Ependymin Immunoreactivity in *Limulus polyphemus*

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Abstract

Ependymin is a neurotrophic factor found to be present during certain cellular events related to neuronal development, cellular regeneration, and memory consolidation (Adams, D. et. al., 2003). Since its discovery in goldfish in 1976, Ependymin and Ependymin-related proteins have been partially sequenced and show notable conservation through many different species including *Homo sapiens* and *Limulus polyphemus* (the horseshoe crab), showing 87% and 86% homology, respectively (Cruickshank et al., 1993). This amount of conservation, despite the hundreds of millions of years of evolution that separate the horseshoe crab from humans, implies that Ependymin plays a significant role in the development of neuronal tissue in both of these species. It also allowed for the development of an 8 aa antibody against Ependymin which can be used to identify the protein's presence in various tissues. We have attempted here to determine the location of Ependymin in several stages of horseshoe crab development using immunohistological techniques. Our preliminary fluorescent staining of whole-mount crabs for Ependymin indicates a strong presence of Ependymin throughout the central nervous system of horseshoe crabs in the 1st, 3rd and 4th juvenile instar stages. Light and Transmission Electron Microscopy, along with HRP and immunogold staining, respectively, are currently being used to confirm these results in the 3rd, 4th and 5th instars, as well as in the larval stage of development.

Introduction

Purpose

This project will serve to track the presence of the neurotrophic factor Ependymin during the development of *Limulus polyphemus*, the American horseshoe crab, from the larval stage up through the first year juvenile stages. In addition, adult stages may be analyzed for comparison. Several techniques for quantifying levels of the factor and identifying its location will be used. Microscopy, including electron and fluorescence microscopy, will be used in conjunction with newly-developed antibodies and secondary antibodies to determine the quantity and location of Ependymin in the central nervous system of the crabs as they progress through various developmental stages.

Overview of Proposed Methodology and Techniques

A large basis of the research conducted in this project will make use of microscopy. For this reason, the initial stages of this project will focus on proper fixation and sectioning techniques for the various stages of *Limulus* development. The first section of the project will aim to fix the crabs for dissection or microscopy. Following fixation, the first stage of research on fixed crabs will be based on observing microtome-made sections of dissected Central Nervous Systems of crabs (or if necessary, whole crabs) made in the appropriate resin by light microscopy and fluorescence microscopy. Following this sectioning, the sections will be exposed to anti-EPN antibodies followed by HRP or fluorescent secondary antibodies and observed by light or fluorescence microscopy, respectively. Concurrent with this study will be an analysis of the two different types of resins used for sectioning. An epoxy resin, Epon, will be compared to LR white, a newer, water-soluble acrylic resin, which is believed to create more

ragged cuts that provide increased exposure of sections to antibodies. After determining the proper resin to use, additional sections in this resin may be used in similar techniques for electron microscopy with immunogold secondary antibodies. In addition, following successful imaging of sections with fluourescence microscopy and electron microscopy, whole mounts of crabs will be observed with confocal fluourescence microscopy and used to compare the location of Ependymin in the crabs with known areas of the Limulus central nervous system as detailed by Chamberlain and Wyse as well as to determine levels of intracellular and extracellular Ependymin. Confirmation of the presence of Ependymin as well as identifying its location in the CNS in each of five stages of Limulus development (Larvae, and 1st, 2^{nd}, 3^{rd} and 4th Juvenile Instars) will mark the completion of the project and any further study on the cellular location or method of action of Ependymin will be left up to further research.

Ependymin

Ependymin (EPN) is a neurotrophic factor (NTF) found to be present during certain cellular events related to neuronal development, cellular regeneration, and memory consolidation. (Adams, D. et. al., 2003). EPN was originally discovered in goldfish when it was observed that Ependymin-related proteins were increased in extracellular fluid around the brain of goldfish after learning events. (Shashoua, 1976) While the mechanism of action of most neurotrophic factors discovered to date have been identified to be cell-surface receptors that alter signal transduction and gene expression, the mechanism of action of EPN action remains elusive. (Adams, D., et. al., 2003).

Ependymin was first discovered in goldfish using classical conditioning experiments to test learning against different stimuli. Goldfish were submitted to classical conditioning using

light and shock stimulus, and were then dissected at various time intervals, from 0 to 25 hours post-conditioning. Levels of EPN in the brain extracellular fluid were examined by ELISA assay, and found to be significantly lower in trained fish than control (untrained) fish initially. Over time, EPN levels increased in the ECF of trained fish until data showed a return to control levels at 25.5 hours. (Shashoua, 1976). Since its discovery in goldfish, Ependymin and Ependymin-related proteins have been partially sequenced in a number of different organisms including humans, horseshoe crabs (Cruishank, et. al., 1993), sea cucumbers, frogs, and other piscine species. (Suárez-Castillo, E., et. al., 2004). Additional research showed that antibodies to EPN would block the recall of learned conditioned responses (Shashoua 1991). A recent study showed that an EPN-related protein was, in fact, over-expressed during intestinal regeneration in sea cucumbers, Holothuria glaberrima. (Suárez-Castillo, E., et. al., 2004).

Dr. Dave Adams' lab at WPI successfully cloned, sequenced, and characterized a gene for Ependymin. Homology to the gene was demonstrated in ten other species, most notably *Homo sapiens* and *Limulus polyphemus*, (87% and 86% homology respectively) (Cruickshank et al., 1993). The fact that EPN has been highly conserved after approximately 500 million years of evolution that separate Limulus from vertebrates suggests that it plays an important role in the species that produce it. In addition, the discovery of Ependymin in species with regenerative tissue, as well as the evidence that the Ependymin gene has been conserved for centuries, has important applications to other models including that of human development.

Limulus polyphemus

Limulus, otherwise known as the American horseshoe crab, is an arthropod that has evolved very little over the past 450 million years, and is therefore often referred to as a "living

fossil," thought to contain genes that have been conserved in many other organisms (Shuster, 1982). Although *Limulus* is referred to as a crab, it is actually more closely related to spiders and other chelicerates, than it is to crustaceans and true crabs, all of which are classified to the order Xiphosurida (Shuster, 1982). Located primarily along the North American Atlantic coast, *Limulus* has been used extensively in studies of nervous systems, as a model for the nervous systems of higher organisms (Chamberlain and Barlow, 1980; Mittmann and Scholtz, 2003). Although *Limulus* is an invertebrate, its relatively simple nervous system makes it a useful model organism and the identification of many genes and proteins with sequence homology to mammalian genes have provided a basis for using *Limulus* as a foundation for further studies in mammals (Chamberlain and Barlow, 1980; Adams et al., 2003).

Limulus is also of interest to research based on the amoebocytes in its hemolymph (*Limulus* blood), which act to protect the crabs from gram-negative bacteria (Coursey et al., 2004). The amoebocytes, which help the crab form a clot after encountering an antigen, are the basis for the *Limulus* Amoebocyte Lysate assay, used as a field test for gram negative bacteria (Coursey et al., 2004). In addition, Costigan and Gallant examined amoebocytes found in wounded crabs for the presences of ependymin, in hopes of elucidating the regenerative ability of *Limulus* in response to amputated limbs (Costigan and Gallant, 2004).

Limulus Development

Although the full development of *Limulus* from larvae to adulthood can take ten years and consists of approximately eighteen stages and sixteen molts, the first year of development from the embryo to the fifth juvenile instar is of particular importance to development over the next several years (Sekiguchi et al., 1988). Following fertilization, the *Limulus* embryo goes

through twenty-one stages of development, including four molts, that lasts approximately fourteen days before the eggs finally hatch (Shuster, 2003). These newly-hatched larvae are often referred to as the first juvenile instar, but the larval stage is in fact the last embryonic stage as well, having molted in the egg. In addition, it is teleolecithal, does not feed, and its tail is not jointed. Its classification as the first juvenile instar is, as far as we can see, inaccurate. For the purpose of this report, such larvae will be referred to as simply "larvae" (Brown and Barnum, 1983; Shuster, 1990).

With the complex developmental pattern of *Limulus* in mind, it is important to identify the key characteristics of the five stages to be studied. The first stage to be studied, the embryo, is the most difficult to classify due to the rapid and complex changes that the newly-fertilized Limulus egg undergoes through the first two weeks of development, including widespread cleavage and granulation along the surface of the egg followed by the development of embryonic membranes, most notably the chorion and egg capsule, which contain the fluid surrounding the embryo and eventually swell from an influx of seawater prior to hatching (Shuster, 2003). Although the embryo becomes a trilobite larva (a separate stage to be studied) following the rupture of the chorionic membrane, there are several key features of the embryo that occur prior to this event that are useful in characterizing the embryo. From the fifth to eighth days following fertilization, the embryo is approximately 2mm in diameter and begins segmentation and develops appendages along its ventral side (Shuster, 2003). Also, prior to becoming a trilobite larva, the embryo undergoes three molts within the egg, which leave behind thin-walled, transparent molts within the egg capsule (Shuster, 2003).

The formation of the initial trilobite larva is marked by the rupture of the original egg casing, the chorion, inside of the egg capsule (Shuster, 2003). Following this rupture, the larva undergoes a third molt within the egg, during which the prosoma and opisthosoma (the top and bottom halves, respectively) are clearly defined and segmented and the crab has the earliest shape consistent with a trilobite larva (Shuster, 2003). Also during this stage, the larva expands rapidly to 4mm in diameter and eventually undergoes its fourth molt, where it is nearly identical to a hatched larva, and awaits a swelling and rupture of the egg membrane in the hatching process (Shuster, 2003). Following hatching, the larvae remain at a 4mm size and can be easily identified by their lack of telsons (tails) (Shuster, 2003).

As soon as 6 days after hatching, the larvae undergo their first post-hatch molt and enter into the first juvenile instar stage (Shuster, 1990). At this point, the crabs resemble adult horseshoe crabs for the first time, most notably due to the development of their telson at the base of their opisthosoma (Shuster, 2003). This stage is often referred to as the "5mm" stage, since the crabs are consistently measured to be approximately 5mm in length (although they can vary from 5 to 6mm) as measured from the top of the prosoma to the bottom of the opisthosoma (not including the length of the telson: Sekiguchi et al., 1988).

Following another molt, the crabs enter into the second instar stage, which has a similar body plan to the first instar, but has a length of approximately 8mm (Sekiguchi et al., 1988). Similarly, a third post-hatch molt leads to the third juvenile instar, which has a length of 11mm and is one of the final stages to be examined in this study.

Although changes in numerous systems, including the cardiovascular and muscular systems, have been studied in *Limulus* during their development in juvenile instars and the

complex differences in these systems between each stage have been studied, little has been done to compare the nervous systems of *Limulus* at each stage of juvenile development (Burggren and Keller, 1997). However, the results of this study hope to elucidate one aspect of this development: the presence or absence of the neurotrophic factor Ependymin at each stage.

Tracking Ependymin in *Limulus*

To determine the presence and location of Ependymin within the CNS of *Limulus*, immunohistological analysis of fixed tissues will be performed using primary and secondary antibodies. The primary antibody to be used in this study is an anti-KKETLQFR antibody which binds to a specific region of the Ependymin glycoprotein which has sequence homology in many organisms, including humans (Adams et al., 2003). The antibody, which contains the eight amino acid fragment KKETLQFR, is a polyclonal antibody produced in rabbits by New England Peptide. To detect the binding of the primary antibody to Ependymin, three secondary antibodies will be used for the various microscopic techniques to be used. For light microscopy, a secondary antibody containing Horseradish Peroxidase will be used in order to stain regions containing Ependymin as dark brown in the presence of DAB (3,3-Diaminobenzidine). For fluorescence microscopy, a fluorescent secondary antibody made by AlexaFluor will be used to detect the primary antibody in both CNS sections and whole mounts. Based on the success of the other immunohistological studies, immunogold secondary antibodies may be used for detection with transmission electron microscopy.

Past Work

Limulus has been studied extensively for nearly a century. However, studies of the *Limulus* central nervous system were common only within the past thirty years. Fahrenbach's study of neuroglia and other CNS structures in *Limulus* increased support for nervous system studies in *Limulus* (Fahrenbach, 1976). However, Chamberlain and Wyse's work to map the entire CNS in *Limulus* is a large foundation of the present work and provides a detailed source for comparing and identifying key locations of neurotrophic factors in development (Chamberlain and Wyse, 1986).

With the work on Ependymin of Adams, Shashoua and others (Discussed in the "Ependymin" section above), recent studies have focused on identifying the action of Ependymin in a wide array of organisms and applying its action to the treatment of neurological disorders and regeneration. The current study will make use of the sequence found for Ependymin and, by analyzing the location of Ependymin within various stages of *Limulus* growth, attempt to reconcile what is already known about its action in one stage of life with several stages of development.

Methodology

Fixation

Prior to CNS removal, the crabs were fixed in a formaldehyde solution to stabilize proteins. The fixative was prepared by mixing 8% Paraformaldehyde (5mL) with 200mM phosphate buffer (2.5mL) and filtered seawater (2.5mL) and a few drops of blue food coloring, yielding a final solution of 4% formaldehyde. The color was to assist in determining the success of perfusion by the fixative. Using insulin syringes, fixative was injected into the heart of the crabs through the membrane that lies within the hinge between the Prosoma and Opisthosoma until their legs puffed out. After injection, crabs were stored in the fixative solution overnight before being transferred to a phosphate buffer rinse solution. Crabs were then dissected within three days of buffer rinse. All fixation stages were refrigerated between manipulations.

CNS Removal

Original Dissection Technique

For the first few rounds of dissection, the following procedure was followed for removal of the CNS from horseshoe crabs. Crabs were first mounted in Petri dishes that had a transparent silicone elastomer (Dow-Corning Silgard) in the bottom to facilitate pinning. The dishes were filled with filtered seawater and micro dissection pins were used to hold the specimen in place. Under a dissecting microscope, scissors were used to remove the midcardiac spine of the exoskeletal system. A flap was then cut around the ocellus revealing the brain and overlying digestive tract. CNSs were carefully removed by separating the gut tissue from the nervous tissue and cutting around the whole of the nervous system. Remains of the crabs were kept in filtered seawater for further use as necessary.

Another technique attempted was to laterally cut around the midcardiac spine and remove the CNS horizontally from the midsection of the crabs. This was done by using sterile razor blades to remove both the left and right sides of the crab followed by a sideways mounting in a Silgard plate. After locating the CNS in the lateral cross-section, forceps and scissors were used to carefully remove the nervous system from the crab.

After dissection of each crab, CNSs were separated by size and stored refrigerated in phosphate buffer until they were ready to be mounted.

Revised Dissection Technique

Since performing the above techniques to remove the CNSs of 5mm and 8mm crabs resulted in only partially intact fragments of CNS, a new technique was attempted which resulted in much cleaner removal of the central nervous system cord. Crabs were mounted dorsal side down on a filtered seawater Silgard dish. Using forceps, a longitudinal separation was made directly in between the ambulatory legs resulting in immediate viewing of the CNS. The cord could then be simply removed by carefully pulling on the posterior side of the cord while being careful to avoid the esophagus which the CNS envelops. This technique was used for the rest of dissections as it resulted in a significant increase in fully intact central nervous systems.

Because larval CNSs could not be removed reliably by either method, entire larvae were embedded and sectioned instead of isolated CNSs.

Resin Mounting

Prior to mounting all of the removed CNSs in resin, each respective size of CNS was divided among each partner to allow for the testing of the two different resins. Just before the

mounting procedure, all CNSs were treated with a 1% osmium tetroxide solution for no more than one hour which allowed for the heavy metal staining of lipid membranes a dark brown color. Following this osmium tetroxide staining, the samples were rinsed with deionized water and allowed to rinse over night.

Epon Araldite

Following OsO_4 staining and a rinse with deionized water, CNSs were then prepared for embedding in Epon Araldite resin, the first several steps being dehydration with increasingly hydrophobic solvents. The samples were rinsed three times in 100% ethanol for 20 minutes each, followed by two rinses in propylene oxide for 5 minutes each. The samples were then soaked for two hours in a 1:2 Epon Araldite: Propylene oxide solution to begin Epon infiltration and then placed in a 2:1 Epon Araldite: Propylene oxide resin overnight. This overnight infiltration was followed by two rinses in pure Epon Araldite for an hour each and then specimens were placed face down (as shown in Figure 1) in the cap of a plastic capsule and filled with Epon Araldite and allowed to cure in the curing oven overnight at 75^{o}C. After the curing step, the plastic capsule was removed with a razor and specimens were prepared for microtome sectioning.

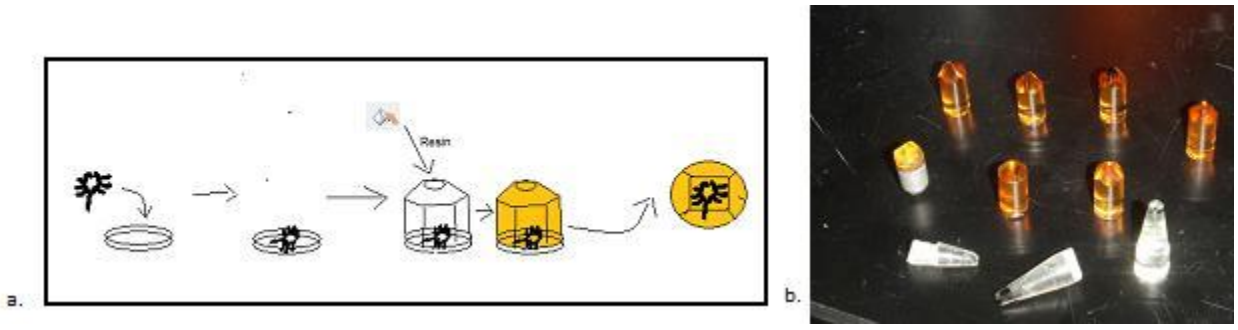


Figure 1: a. Schematic Representation of Embedding CNS b. Flat Embedded Samples

LR White

LR White is a polyhydroxy-aromatic acrylic resin with low toxicity and ultra-low viscosity. LR White does not actually polymerize to the CNS and should therefore be gentler on the tissue in question. Additionally LR White does not require etching prior to TEM staining. The procedure for mounting tissue in LR White is as follows:

After the OsO_4 rinse, solution was removed using heat elongated transfer pipettes. This solution was replaced with 100% ethanol for 20 minutes. Again, the solution was removed and replaced with a 1:1 EtOH : LR White solution over night. When the 24 hours was up, this solution was removed and replaced with 100% LR White for two rinses and then left for at least an hour before curing. Curing was done in an eppendorf (microcentrifuge) tube in an oven at 75^{o}C for no less than one hour. After the curing step, LR White mounts were removed from the eppendorf tubes by cutting of the tip with a sterile razor blade and pushing it out with a wooden dowel.

Whole Mounts

Isolated CNS

Following removal of the CNS from crabs, the entire paraformaldehyde-fixed CNS was allowed to sit in 100mM Phosphate buffer prior to treatments with fluorescent staining. Following staining (described below), the whole central nervous system cords of 5, 11 and 15mm crabs were analyzed for the presence of Ependymin.

Thicker Cutting

In contrast to isolated CNS whole mounts, a different procedure was used for whole mounts, in which the CNS was allowed to remain inside the crabs. The crabs were first fixed with 4% paraformaldehyde as before, but instead of removing the CNS, the crabs were

sectioned into thick sections using a sterile razor blade to make coronal sections to crabs laid on their dorsal sides on Silgard dishes (as shown in Figure 2). These sections were then stained according the normal whole mount procedure.

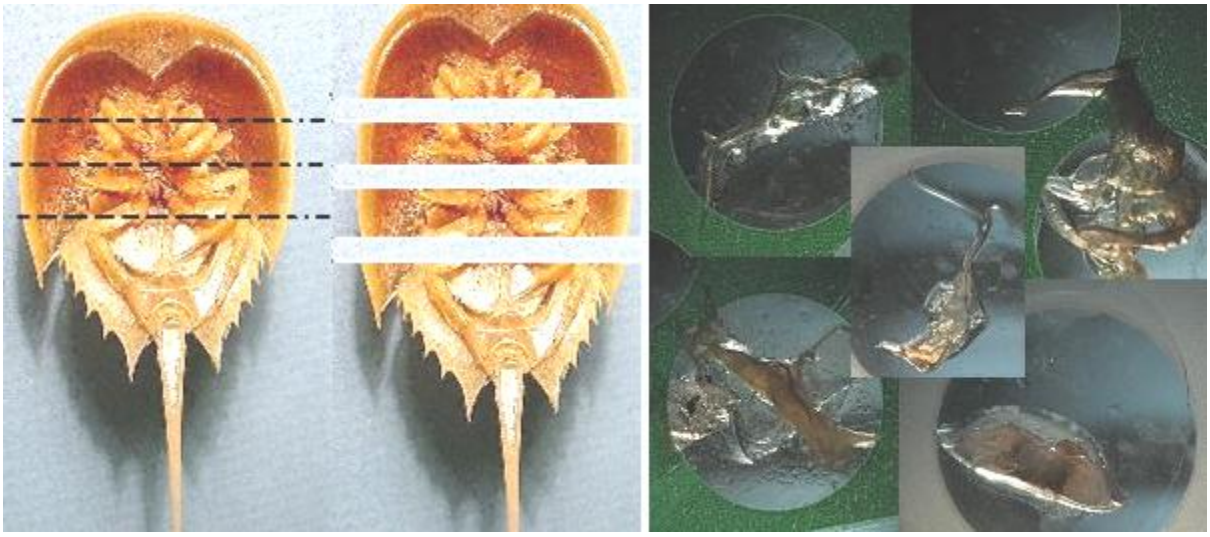


Figure 2: (Left) Schematic of thick section cutting. (Right) Examples of thick cut whole mount sections.

Microtome Sectioning

Epon Araldite

Transverse Sections

Following embedding in Epon Araldite, two sectioning angles were used. The first technique, which was the standard technique used for most of the initial sectioning, used the standard embedding technique for Epon, as described above, with the isolated central nervous system cut in a transverse section, as shown in Figure 3.a., with the orientation of the CNS shown as it would be within a whole crab. This type of sectioning was done on 5, 8, 11 and 15mm crabs with varying levels of success.

Coronal Sections

An alternative angle was used for larvae, 11 and 15mm crabs where the CNS was sectioned along a coronal plane (as shown in Figure 3.b). This angle was accomplished by

removing these specimens from their original Epon-Araldite embeddings and re-embedding them in small plastic capsules in this alternate orientation.

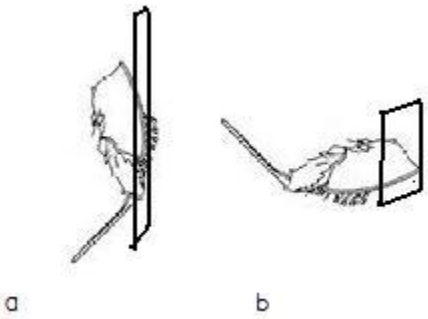


Figure 3: a. Transverse sectioning of CNSs at the orientation they would have inside the whole crab. b. Coronal sectioning.

LR White

Transverse

Initially, fixed crabs were mounted in LR White such that sections of CNS would come off as transverse cross-sections. Sections were collected from 5mm, 11mm, and 15mm crabs and used for light, fluorescence, and electron microscopy. Light and fluourescence microscopy sections were cut with homemade glass knives while sections for TEM were cut with a diamond knife.

Cryosectioning

Tissue Preparation and Whole Mount Sectioning

Cryosectioning was an added attempt to get cleaner, more antigenic sections from a frozen sample. All sectioning was done in a cryotome, a larger microtome that is inside a -20^{o}C freezer unit. Crabs used as cryosectioning whole mounts had to be fixed and prepared in a slightly different manner than other crab specimens. In addition to the typical formaldehyde fixation, crabs that were to be cryosectioned were also treated with 30 % sucrose and kept in a

refrigerator as a preparative step before freezing. Prior to sectioning the crabs, a 100% ethanol solution was cooled down with dry ice, in which the whole crab was then submerged. After about 5 minutes, the frozen crab was mounted in the cryotome by placing the sample on the cryotome slide and freezing it in place with a cryosectioning glycerol solution. The freezing process was sped up by applying the glycerol on the slide after cooling it with dry ice as well. Sections were collected on slides for use with light and fluorescence microscopy.

Slide Preparation

In working with whole mounts sections with liquid glycerol already on them, it was necessary to rinse the thick mounting solution off with buffer prior to staining with antibodies or colorimetric stains. However, in doing so, much of the crab tissue was lost in the process, so various techniques were attempted to try to alleviate this issue. In addition to trying multiple varieties of store bought slides, a few slides were prepared with egg white coating - which were made simply by "painting" the white of an egg onto a yellow frost plus slide. It was these egg white coated slides that resulted in the least amount of tissue lost compared to un-coated gold plus slides, which lost nearly all of the tissue by the time fluorescent staining was done.

Antibody Staining

Etching

Prior to any microscopy staining, sections first had to be etched to allow for greater antibody penetration. First, a dissolving solution was prepared by adding 1.0 g NaOH pellets to 10 mL 100% EtOH mixed with 10 mL propylene oxide. Next, a 1% sodium periodate solution was prepared by dissolving 0.1 g $NaIO_4$ in 10 mL dH_2O. Sections were incubated for 3 minutes in dissolving solution then rinsed three times in 95% EtOH for 2 minutes each, followed by a 3

minutes rinse in 50% EtOH, and then a 5 minute rinse in dH_2O. The 1% $NaIO_4$ was then applied to the section and allowed to incubate for 7 minutes, followed again by two 3 minute rinses in dH_2O. Slides were then allowed to equilibrate in 0.1M PBS for at least 5 minutes prior to further staining. While dissolving solution could be stored in freezer for next use, sodium periodate was made fresh each time. This protocol was used for all stages of horseshoe crabs embedded in Epon and each microscopy staining technique.

Fluorescent - Sections

Experimental

After etching slides for fluorescence microscopy, experimental sections were prepared by blocking with 6% NGS for 30 minutes and then rinsed in PBS. The blocking step prevents the rabbit primary antiserum from non-specifically binding to the tissue. After blocking, a 1:100 dilution of anti-EPN was applied and allowed to incubate overnight at room temperature. The next day, slides were rinsed three times for 10 minutes each in PBS. A 1:100 dilution of AlexaFluor 488 goat anti-rabbit conjugated secondary antibody was then applied and allowed to incubate overnight at room temperature. After the incubation, slides were rinsed twice in PBS for 10 minutes each and once in dH_2O. After allowing the slides to dry, glycerol was applied as a clearing agent and slides were observed in the light/fluorescence microscope. All dilutions were made in PBS and 1% normal goat serum (NGS).

Control

Controls for fluorescence staining followed the same procedure up to the primary antibody. Instead of anti-EPN, control slides were incubated with 1:100 normal rabbit serum (NRS) overnight and rinsed in PBS three times for 10 minutes. The secondary antibody and

rinsing techniques followed as above as well as glycerol application and observations under the fluorescence microscope.

Horseradish Peroxidase - Sections

Experimental

Experimental HRP sections were first washed in dH_2O for 5 minutes before a 20-minute PBS rinse. Slides were then blocked as with fluorescent sections with 6% NGS for 20 minutes and then rinsed with PBS. Following blocking, sections were incubated for 30 minutes at room temperature in 1:100 anti-EPN and then washed in PBS for 10 minutes. Biotinylated Secondary Antibody was then prepared from the Pierce ImmunoPure Ultra-Sensitive ABC Peroxidase Staining Kit, applied to tissue sections, and allowed to incubate for 30 minutes. "ABC" of the kit stands for Avidin-Biotin Complex, because the secondary antibody is biotinylated and so is the 'reporting' enzyme, HRP. The two are bound together by the natural adhesion of the biotin on both to avidin (egg white protein). Following another 10 minute rinse with PBS, the Pierce ABC reagent was applied to sections and allowed to incubate for 30 minutes followed by another 10 minute wash in PBS. Finally the metal-enhanced DAB substrate was added and allowed to incubate until color appeared. The 1X DAB/Metal Concentrate was prepared by diluting 10X DAB/Metal Concentrate with Stable Peroxide Buffer, which caused the HRP enzyme to degrade the peroxide, release oxygen, and oxidize the DAB from clear to dark brown. .HRP slides were visualized under light microscopes and were used to confirm fluorescence results.

Control

HRP control slides were prepared following the same procedure as above up to the primary antibody. Instead of anti-EPN, control sections were incubated with 1:100 NRS

overnight and rinsed in PBS three times for 10 minutes. The rest of the procedure followed as above.

Fluorescent - Whole Mounts (Isolated CNS and Thick Crab Sections)

Experimental

Whole mount tissue (CNS and thick-cut crab sections) were prepared by rinsing in 0.1M PB/ 0.3 %Triton X /NGS(1%) 6 times in three hours. Triton-X (Tx) is a detergent useful in making tissues more permeable. After rinsing, tissue was incubated in eppendorf tubes with anti-EPN 1:100 overnight at 4^{o}C. Another 0.1M PB/Tx(0.3%)/NGS(1%) rinse was done followed by overnight incubation in AlexaFluor 488 at 4^{o}C in the dark. Stained whole mounts were then rinsed one last time in 0.1M PB/Tx(0.3%)/NGS(1%) and mounted on slides using glycerol as a clearing/mounting agent. The phosphate buffer was made by mixing 20mL 0.1M phosphate buffer, 60µL Triton-X, and 500µL NGS.

Control

Again, control whole mounts were prepared exactly as experimental except for the primary antibody. The primary antibody step was substituted with 1:100 NRS for an overnight incubation at 4^{o}C. The rest of the protocol follows as above. Control and experimentally stained whole mounts were visualized under fluorescence microscopes.

Immunogold

For TEM immunogold staining, ultra-thin gold sections from Larvae, 11mm and 15mm crabs were collected on multi-mesh and 300-hex nickel grids and etched with 10% hydrogen peroxide. Grids were placed face-down on a drop of peroxide and incubated for 15 minutes at room temperature followed by three distilled water rinses for 5 minutes each. Grids were then

incubated for two hours in a 1:100 solution of primary antibody in 0.1M PBS/Tween-80(0.05%)/NGS(0.25%), pH 7.2.

Experimental

The primary antibody for experimental grids was anti-EPN. After the two hour incubation, grids were rinsed in PBS-Tween-80/NGS three times for 5 minutes each and then rinsed in PBS for 5 minutes. The grids were then incubated for 1 hour in goat anti-rabbit secondary antibody conjugated to 25nm gold balls diluted 1:50 in PBS-Tween-NGS. The sections were then rinsed three times in PBS-Tween-80-NGS for 5 minutes each, then twice in PBS for 5 minutes, then twice in distilled water for 5 minutes. The grids were then placed on drops of 2% glutaraldehyde in PBS for 10 minutes to stabilize the bound gold and then rinsed three times for 5 minutes each with deionized water. The sections were stained for 5 minutes in Uranyl Acetate and rinsed for 5 minutes in deionized water. Grids were then stained with newly-centrifuged Lead Citrate for 1 minute and then washed for 5 minutes in deionized water.

In addition to these grids, another set was stained as above, but with an additional staining step in Phosphotungstic Acid (PTA). Following the Lead Citrate staining step from above, experimental and control grids were placed on drops of PTA for 5 minutes and then rinsed with dH_20 for 5 minutes.

All sections were examined with a JEOL CX-100 transmission electron microscope at 80 kV.

Control

The primary antibody step for control grids used PBS-Tween-NGS instead of anti-EPN. All steps following this incubation were the same for both control and experimental sections.

An additional control was done to see the effects of Uranyl Acetate and Lead Citrate on untreated tissue. Following, grids that weren't treated with any antibodies were stained with Uranyl Acetate and Lead Citrate as above and viewed in the same way as treated grids.

Microscopy

Light Microscopy Staining

Toluidine Blue and Basic Fuchsine

While sectioning *Limulus* CNSs with the microtome, the occasional section was removed and stained initially with just Toluidine Blue and visualized with light microscopy in order to determine if there was actually tissue being sectioned, and about how much there was in each section. Later, the Toluidine stain was coupled with a diluted Basic Fuchsine stain to help improve the contrast on tissue sections.

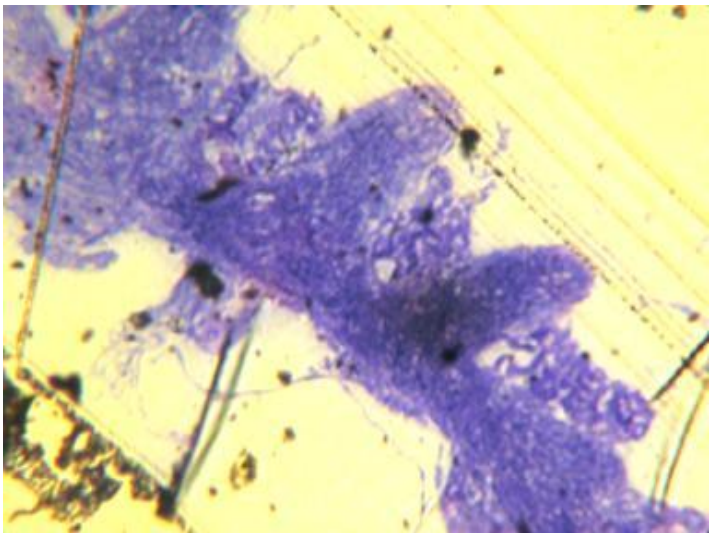


Figure 4: Basic Fuchsine/Toluidine Blue Staining of a 5mm CNS Section. The prominent bumps are nerves to the legs.

Methylene Blue

Methylene Blue stain was used on living tissue with light microscopy to visualize nervous tissue. When Methylene Blue was applied to living tissue, it turns nerves blue allowing for

visualization of CNS, peripheral nervous system, and nerve tracts. We also used it together with cryosectioning as a quick verification technique before moving on to fluorescence staining.

Fluorescence Microscopy

AlexaFluor-488

After overnight incubation and PBS rinsing, slides were mounted in glycerol and visualized with fluourescence microscopy. AlexaFluor 488 absorbs light in the blue wavelength and emits a brilliant green color. Tissue was visualized in the blue fluorescent wavelength at 10X, 25X, and 40X and then compared with other wavelengths to identify "artifacts", something that auto-fluoresces under every wavelength. Ependymin was identified as fluorescing at 488 (blue), but disappearing under other wavelengths.

4', 6-diamidino-2-phenylindole Staining

After identifying what was believed to be Ependymin in tissue sections or whole mounts and photographing them, a DAPI stain was applied to identify cell nuclei near sites of Ependymin aggregation. DAPI binds strongly to DNA and fluoresces under near-UV light, emitting a cyan blue color. The drawback to DAPI is that AlexaFluor-488 is essentially negated by adding DAPI, and therefore doesn't allow for follow up screening of Ependymin under the blue fluorescence.

Transmission Electron Microscopy

Transmission Electron Microscopy Grids

After collecting and staining ultra-thin crab sections for TEM, grids were carefully placed in a TEM grid carrier with forceps and passed through an air and into the high vacuum chamber of the TEM (10^{-7} Torr) of a JEOL 100CX TEM at 80 kV. Grids were first visualized at low mag to identify tissue location and then magnified up to 40,000x.

Results

Larvae

Results from Larval-stage crabs showed a large presence of Ependymin in pockets around esophageal tissue. Figure 5 shows two close-ups of Ependymin pockets (the right and bottom-right pictures). The figure also shows an excellent example of auto fluorescence in horseshoe crab shells, which sometimes is so bright that weaker fluorescence is eclipsed by it.

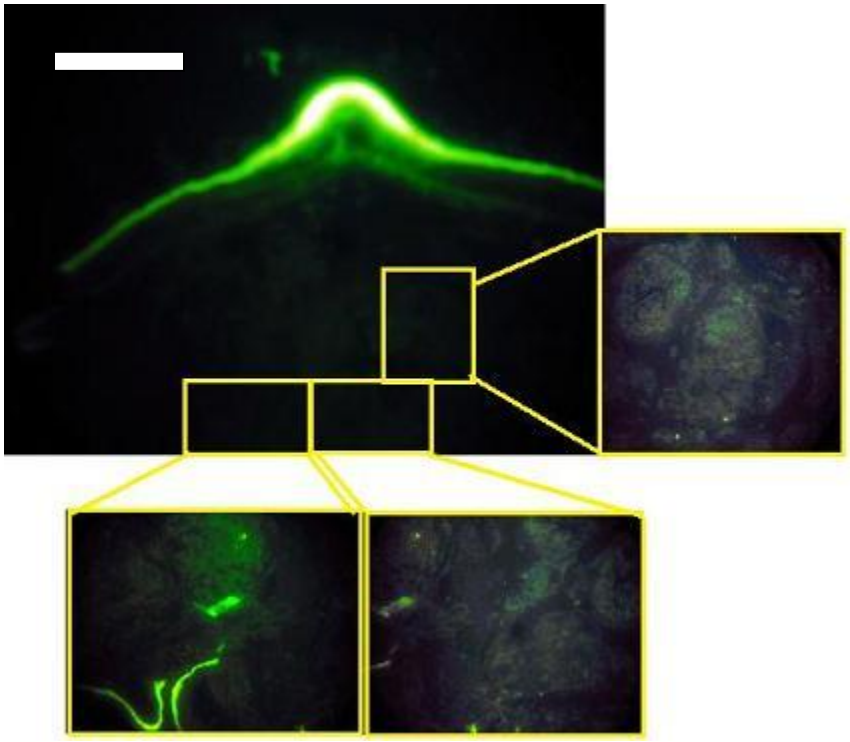


Figure 5: Larval fluorescent stain composite showing ependymin pockets around circum-esophageal tissue. Scale bar is 1mm

HRP-stained sections of larval tissue confirm that anti-EPN binds to nerve tissue in the larvae. In the experimental vs. control sections below, the cell bodies of the anti-EPN sections stain darker than those of the control. Neuropil (axon mass) also stains darker but specific tracts as seen in sagittal sections (Dionne and Krzyzewski, 2008) are not discernable

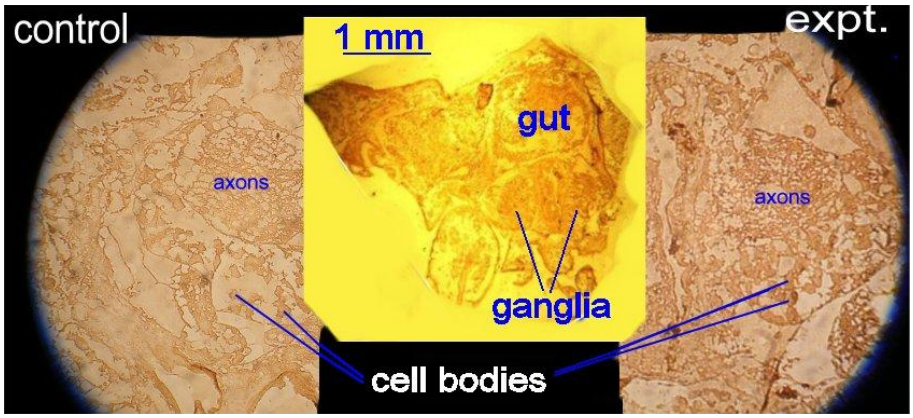


Figure 6: Larval HRP stained tissue.

5mm -Whole mounts

Due to the few 5mm crabs available, the only 5mm results obtained were from fluorescent staining. Figure 7 shows a blurry, yet revealing picture of a 5mm microtome section showing Ependymin around the CNS.

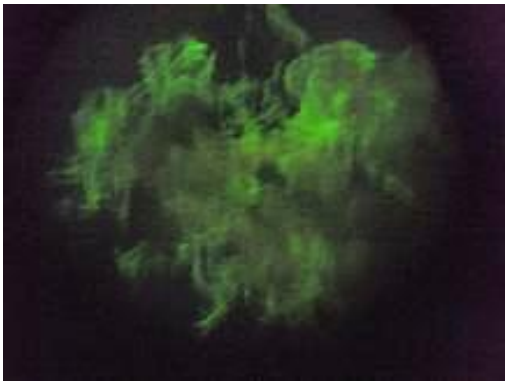


Figure 7: 5mm Isolated CNS whole mount showing possible Ependymin tracts within the CNS.

11mm

Results from 11mm sections and whole mounts showed a presence of Ependymin-like immunorreactivity located around the main CNS and around the peripheral area nerves as well. Figure 8 shows an 11mm isolated CNS whole mount which has a nerve fiber coming off the main CNS going out to one of the crab's legs. The brilliant green color is Ependymin staining while the dull green area to the bottom right is just background fluorescence.

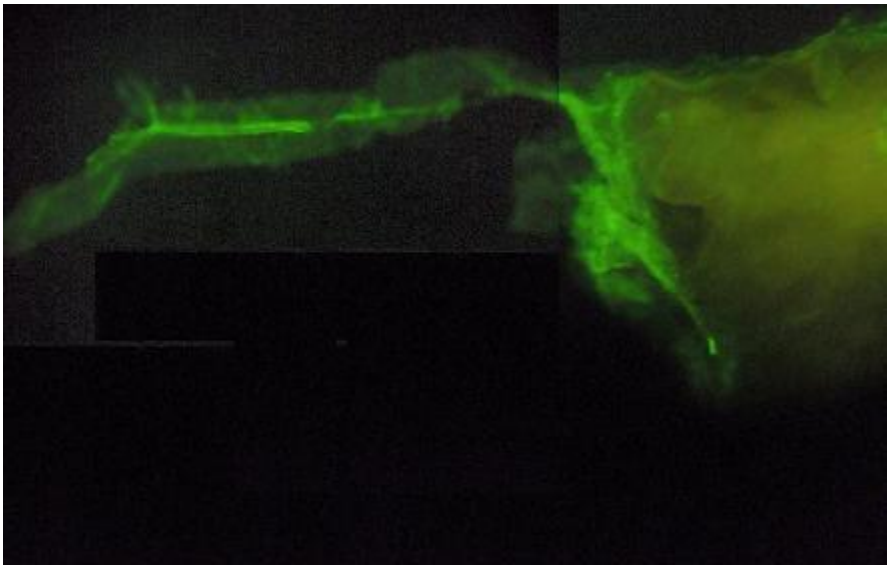


Figure 8: 11mm fluorescent stain showing a nerve fiber coming off of a leg sprout.

HRP staining of 11mm microtome sections show that Ependymin is, indeed, present around the CNS as shown in the dark tract in Figure 9. This figure shows a coronal section in which the CNS spans the width of the section.

Figure 9: 11mm coronal-sectioned HRP stained tissue showing intact CNS tract.

15mm

Results from 15mm horseshoe crabs were obtained from cryosection, HRP sections, isolated CNS whole mount and fluorescently stained microtome sections. Below, in Figure 10, a fluorescently stained cryosection shows 15mm shell above the CNS which extends down and right due to the friction caused by the blade on the softer tissue in the cryotome.

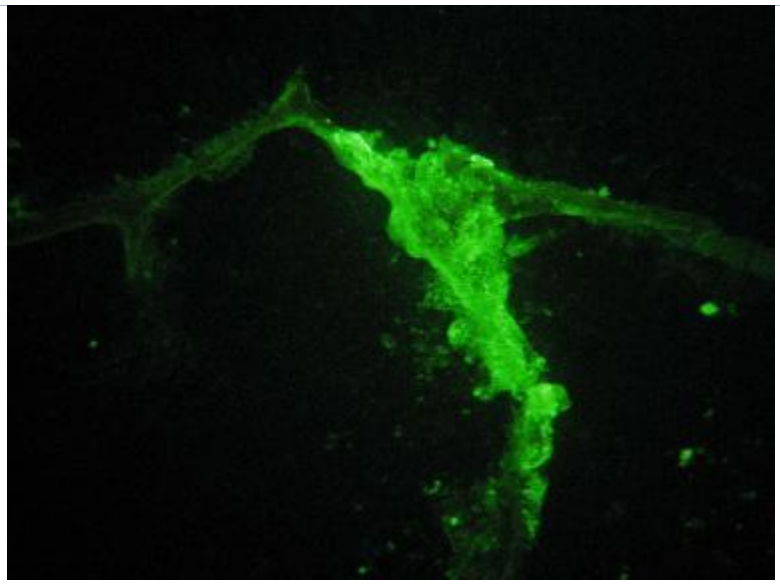


Figure 10: 15mm Crysection after Fluorescent Staining.

Illumination with other wavelengths did not produce fluorescence, arguing against autofluorescence. Figure 11 is the same as figure 10 except that the excitation wavelength is long UV (as for DAPI stain) and not the 488 nm that would excite AlexaFluor. The intense fluorescence is therefore unlikely to be an artifact, but the tissue that it is staining is not neuronal, judging from its location.

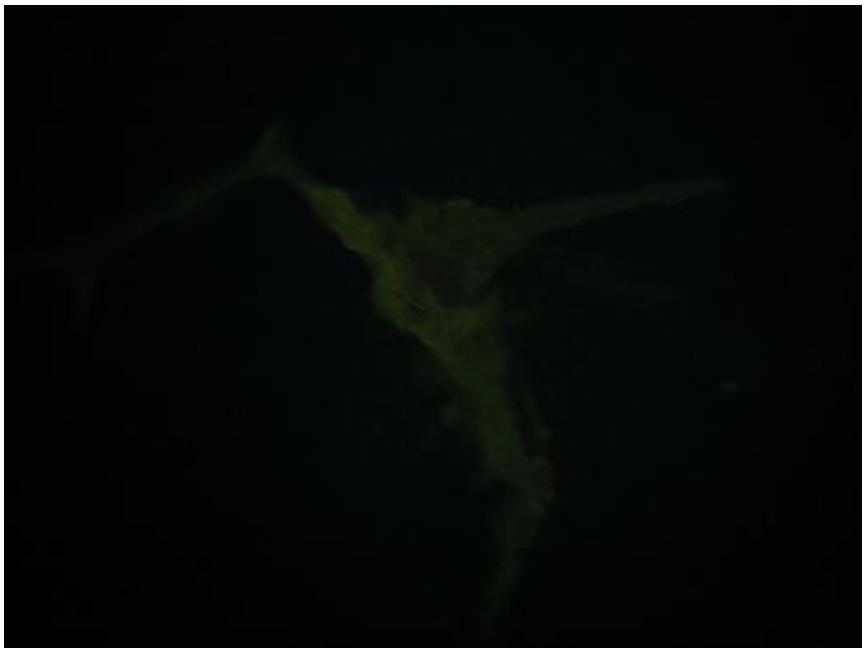


Figure 11: The same 15mm shot after DAPI staining under blue light.

Figure 12, below, confirms that the fluorescing area is not shell, because nucleated cells can be seen within it when stained with the nuclear dye DAPI and illuminated at near-ultraviolet wavelengths.

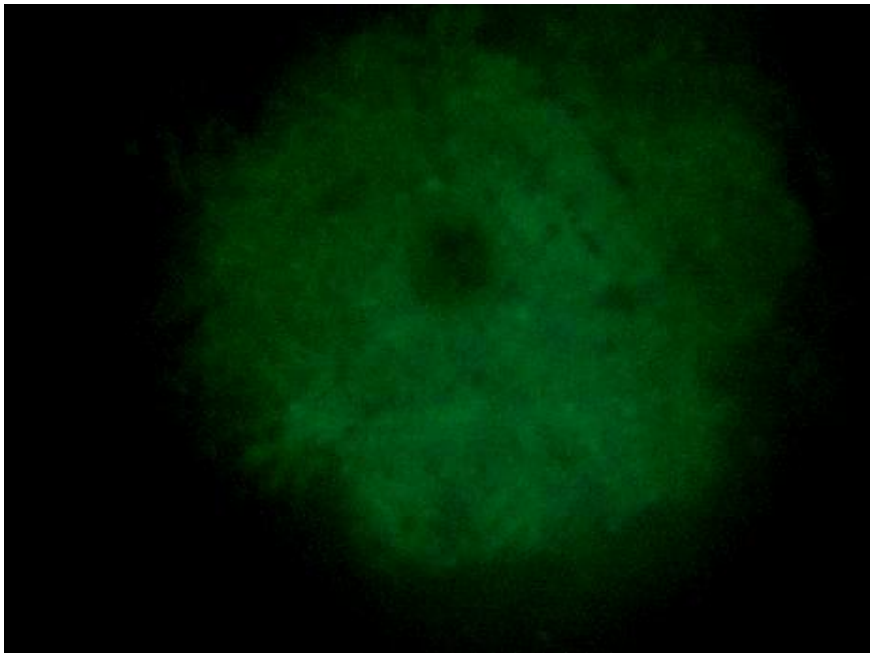


Figure 12: A crysection after DAPI staining under UV light.

Figures 13 and 14 are HRP and fluorescently stained 15mm microtome sections, respectively, and both show a presence of Ependymin around nerve fibers which extend from the CNS. The HRP section shows darker areas where primary antibody bound to EPN. Figure 14 is a correlating section with fluorescence in tracts showing EPN presence.

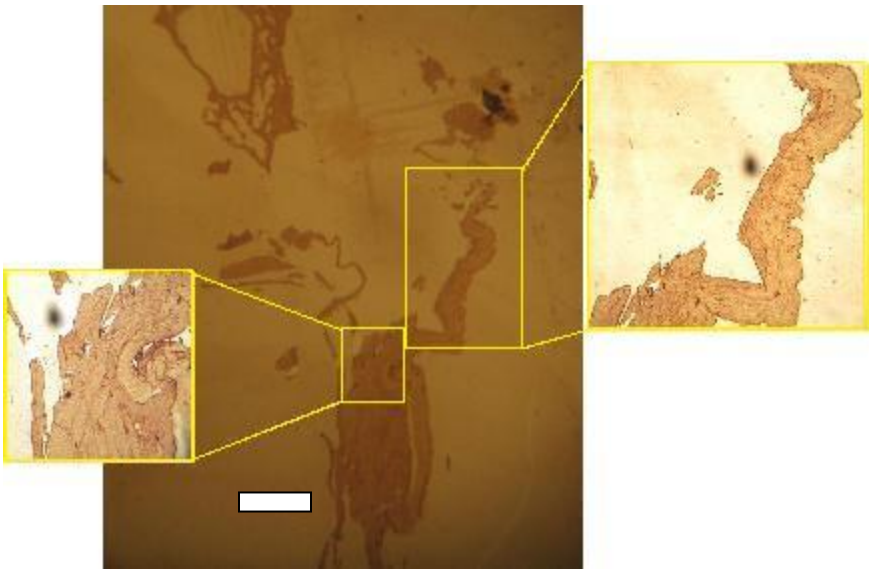


Figure 13: 15mm HRP staining showing ependymin tracts in a nerve fiber extending from the CNS. Scale bar is 250 um

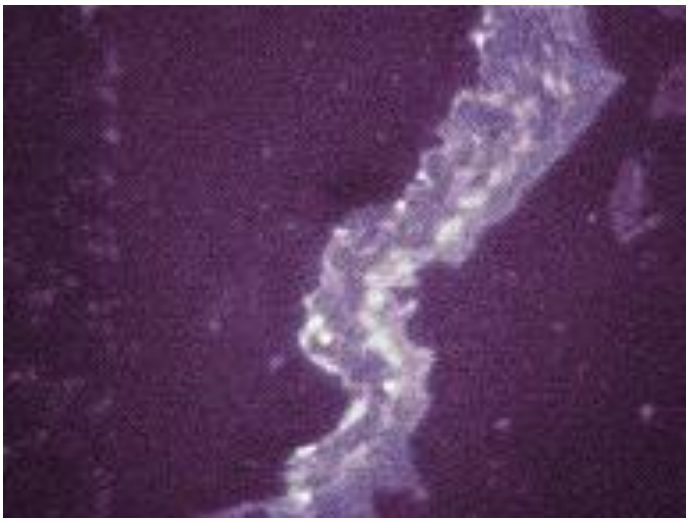


Figure 14: 15mm Fluorescent-stained section correlating to HRP stained nerve fiber in Figure 13

Figure 15 and 16 are isolated CNS whole mounts stained with fluorescent antibody showing Ependymin tracts on the CNS and coming off the CNS, respectively.

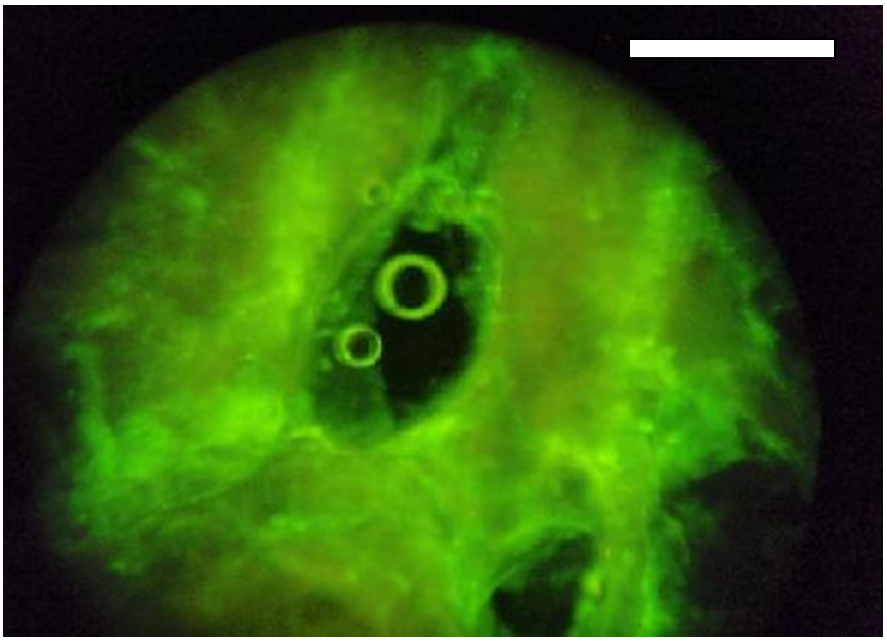
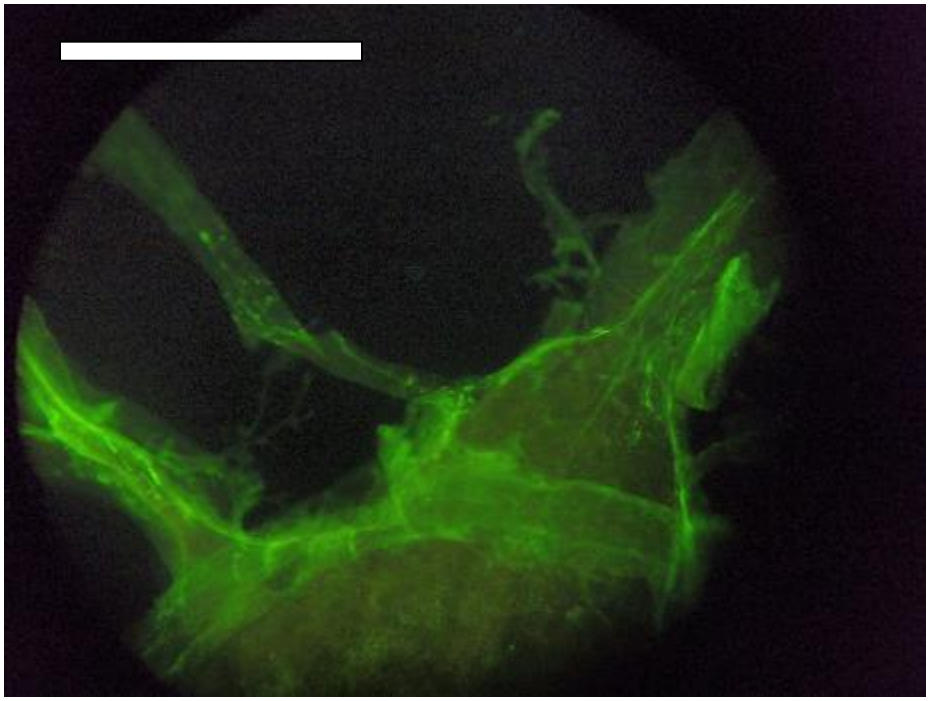


Figure 15: 15mm isolated CNS whole mount showing tracts of EPN encircling the esophagus scale bar is 1mm

Figure 16: 15mm Isolated CNS whole mount showing tracts of Ependymin exiting the CNS Scale Bar is 1 mm



Discussion

In the present study, we have confirmed the presence of the neurotrophic factor Ependymin in the central nervous system of Horseshoe crabs in the larval stage as well as in the 3rd and 4th juvenile instar stages. In the 3rd and 4th instars, Ependymin was found to be aggregated in the interstices of axons in nerves exiting the central nervous system. In larvae, Ependymin was found there also, but also appeared to be found in the area surrounding yolk droplets.

There were, however, several problems encountered during this study that need to be addressed to clarify the findings made thus far. Although LR White was believed to allow better antibody infiltration for tissues, no viable sections were produced in this resin. Only Epon Araldite allowed for proper sectioning since LR White proved too brittle. Therefore all results shown were either embedded in Epon or in cryosectioning medium.

Also, due to a lack of crabs in the 1st, 2nd and 3rd instars stage, we ran out of tissue to analyze for these stages. So, data for these stages was lacking. Furthermore, several problems in the microtome and knives used for sectioning tissue resulted in a lack of resolution and clarity in several of the fluorescent and HRP-stained sections. Also, adjustments in the primary and secondary antibody concentrations for larvae sections may be needed for increased antibody infiltration and clarity.

Larvae

Ependymin has been shown to be present in the larval stage running through the CNS as well as surrounding yolk droplets. Fluorescent staining indicated its presence in discrete areas surrounding the esophagus and moving more laterally towards its nerve cords. This was

confirmed by HRP staining. The most universal problems with analyzing larvae for Ependymin were their small size, the large amount of interfering yolk and an inability to isolate the CNS from the remainder of the crab. These might have hindered antibody infiltration and analysis.

HRP

Experimental sections of larval tissue stained much darker than corresponding control sections and had staining in much more discrete locations rather than the generalized brown haze on control sections. As can be seen in Figure 6, very dark HRP staining can be seen in pockets surrounding the esophagus as well as moving more laterally along the shell and towards the legs, indicating the presence of Ependymin in nerves in these locations.

Although this data seems to strongly indicate the presence of Ependymin in larvae, even the darkest stained sections were still relatively faint compared to HRP staining of other stages. This was most likely due to the small size of larval tissues relative to the other stages. Furthermore, antibodies may not have had complete access to the central nervous systems of the larvae, since sections of larvae were done on entire larvae instead of isolated CNSs (unlike 11mm and 15mm crabs which were isolated CNSs). To counteract this limited access, a longer incubation in primary antibody or a higher concentration of antibody might be beneficial.

Fluorescence

HRP was used to confirm the initial results of fluorescent staining on larval sections. Similar to HRP results, fluorescent staining indicated discrete locations of fluorescence in larval tissue, indicating the presence of Ependymin around the esophagus and areas leading into the legs.

Slides

Sections of embedded larvae proved to show some fluorescence (as shown in Figure 5). This fluorescence was aggregated in similar regions as those shown in HRP staining. However,

photography of these regions was difficult due to the overwhelming brightness of the shell which seemed to prevent capturing the fluorescence of the underlying nervous tissue. Furthermore, contamination of the lenses of the fluourescence microscope used caused blurry pictures and a decrease ability to focus on specific regions of Ependymin fluorescence.

Whole Mounts

Whole mounts of larvae could not be done due to an inability to remove the CNS from the crab. Furthermore the large amount of yolk present made it difficult to create thick sections, cryo-section, or even an attempted "squishing" technique to isolate the CNS. For this reason, the only viable way to obtain larval specimens was to embed the entire crab and section it on a microtome. Therefore, the only fluorescence images are of thin sections that provide less distinct and concrete evidence of the magnitude of Ependymin's presence in the larval stage.

TEM

Sections of immunogold stained larval sections are still in the process of being studied by electron microscopy, but as of yet, have yielded insubstantial results. Tissue has been found to have scattered spots of Ependymin between neurons, but not in amounts expected. Images are currently being developed and will be included in a later appendix.

5mm (1st Instar)

Although past work has indicated the presence of Ependymin in 5mm crabs, the current study had little success in confirming this. Unfortunately, the largest reason for this was a lack of 5mm crabs or their nervous systems to analyze. The only data achieved for 5mm crabs was a slight indication of tracts of Ependymin through the central nervous system in a whole mount fluorescence sample.

Fluorescence

Sections

Unfortunately, in the process of creating sections of 5mm crabs, a lot of sections were not usable because of imperfections in the knives used to cut them. Furthermore, by the time problems with the cutting process had been fixed and viable sections could have been produced, the supply of 5mm specimens ran out, leaving only a few sections to use. Unfortunately, problems with the mounting medium for these sections as well as a contaminant in the DAPI used to counter-stain the sections prevented finding any useful results on fluorescent sections (data not shown).

Whole Mounts

The one remaining isolated 5mm CNS used for whole mount fluorescence microscopy did yield a somewhat useful image of fluorescence marking Ependymin (as shown in Figure 7). However, there were several contaminating substances surrounding the CNS that hindered determining whether the fluorescence observed was actually indicating tracts of Ependymin or just background fluorescence. An orange-colored artifact was seen surrounding the CNS, which was probably chitin or connective tissue, which blocked much of the CNS (data not shown). Furthermore, the lenses used on the fluorescence microscope were contaminated with some unknown substance that blurred any distinct photography at the time the specimen was observed. To overcome this, not only should the microscope lenses be thoroughly cleaned or replaced, but also confocal fluourescence microscopy would be beneficial to possibly circumvent the contaminating orange substance surrounding the CNS.

HRP & TEM

Unfortunately, the supply of 5mm crabs was depleted before any slides could be prepared for HRP staining or TEM analysis. For this reason, no results are available for these analytical techniques on this stage.

8mm (2nd Instar)

Although a few CNSs were removed from 8mm crabs and sectioning was started on these specimens, problems with the knives used to cut these specimens prevented the use of any 8mm sections produced. Also, the supply of 8mm crabs ran out before any CNSs could be isolated for whole mount analysis either. For this reason, detection of Ependymin in 8mm crabs could not be done as was originally proposed.

11mm (3rd Instar)

11mm crabs, in addition to the 15mm crabs, were among the most abundant stages available for experimentation. Because of this, many more results were obtained with 11mm crabs than with the 4, 5, and 8mm stages. Fluorescent staining showed Ependymin's presence in varying locations including extracellular CNS tissue as well as more peripheral nerves. In addition, using HRP staining allowed for the confirmation of Ependymin's presence in the 11mm stage and TEM analysis is still underway.

HRP

Experimental sections of 11mm tissue stained much more profoundly than corresponding control sections. As can be seen in Figure 9, a very dark HRP streak can be seen stretching along the width of the coronal section of an 11mm crab, which implies that Ependymin is located around this region near the CNS.

Fluorescence

Fluorescence staining of 11mm crabs indicated a large presence of Ependymin along the peripheral area of the nervous system and outer nerve tissue. As shown in Figure 8, Ependymin seems

to be present along nerve tissue that extends from the CNS toward the legs and outer nervous tissue, as compared to control slides which show only autofluorescence.

Sections

Fluorescence of 11mm microtome sections typically showed so much fluorescence that obtaining any useful pictures from them became an issue. Any pictures that were obtained came out blurry and inconclusive. This could be attributed to any number of issues including underblocking or overstaining, but in general it seemed that glycerol was the biggest issue where rather than clearing things up, it tended to make sections blurrier.

Whole Mounts

Whole mounts of the 11mm isolated CNS showed much more conclusive results, like that of Figure 8, which, again, shows a nerve fiber stretching into the peripheral nervous system of an isolated CNS whole mount of an 11mm crab.

TEM

While TEM results are still in the process of being collected, the general impression is that no conclusive evidence will be found using the electron microscope with the current grids. In general, the three grids with 11mm tissue on them showed either overstaining (immunogold everywhere) or no significant presence of Ependymin where it should otherwise be expected. As of right now, control and experimental grids look almost identical; that is, heavy metal staining which imparts some contrast but either too many or too few gold balls. Some of issues concerning this problem are the etching process, which sometimes causes loss of tissue, and the use of Epon as the mounting resin, as LR White is said to provide better TEM results.

15mm (4th Instar)

As with 11mm crabs, results from 15mm crabs show a large presence of Ependymin throughout the main CNS as well as peripheral regions of the nervous system. 15mm crabs are also the only

developmental stage to have been sectioned with the cryotome, which resulted in some issues of its own but unique results nonetheless. Fluorescent staining of 15mm crab sections and whole mounts showed a number of positive results showing tracts of Ependymin around the esophageal CNS and HRP staining confirmed these results.

HRP

HRP staining of 15mm crabs showed a presence of Ependymin along the main CNS as well as nerve tissue extending from the CNS, as in Figure 13. Looking closely at this picture, it can be seen that the main CNS (circumesophageal) is pointing vertically from the bottom of the photograph. Extending from this is a peripheral extending nerve pointing off in the direction of a leg to the right of the picture.

Fluorescence

Fluorescent staining of 15 mm crabs resulted in some confirming evidence that Ependymin is present in 15mm crabs, in addition to all previous stages that were tested. Of all the fluorescence images taken, 15mm crabs resulted in the clearest results of Ependymin immunoreactivity and location.

Sections

15mm fluorescent sections, like that of Figure 14 which shows a fluorescently stained section that was an adjacent section to that of Figure 13. This combination is interesting because it shows the clearest proof that HRP and the Alexafluor secondary antibodies very clearly bound to the same protein, that is, Ependymin. On top of that, the fact that Ependymin appears to be expressed up through the 4th instar stage of development implies that the neurotrophic factor has serious implications in growth and nerve development during the lifespan of horseshoe crabs.

Whole Mount

In addition to the fluorescent sections, isolated CNS whole mounts of 15mm crabs resulted in more straightforward results showing that Ependymin is present around the CNS and peripheral areas of the nervous system. Figure 15 shows the presence of Ependymin towards the top part of the CNS (close

to the head) around the esophageal duct which separates the two halves of the CNS. Figure 16 shows the peripheral nerve tracts around the CNS comparable to that of the 11mm crab in Figure 8. On top of that, the cryosection in Figure 10 very clearly shows an outline of shell, complete with a disfigured CNS that's been dragged by the rough blade of the cryotome.

TEM

As with 11mm crabs, TEM results are still in the process of being collected for 15 mm crabs. However, while one of the grids examined has shown a relatively clear tissue, the other two grids examined so far have shown to be over-stained with background stain or no clear presence of immunogold. As of right now, control and experimental grids look almost identical, with only a few gold balls being found in the experimental tissues. Similar issues to those of the 11mm sections are possible, including etching and resin choices. However, it is possible that a higher concentration of primary or secondary antibody may be needed or there may need to be a change in rinsing or glutaraldehyde protocols.

Conclusion and Future Work

From the present study, we conclude that Ependymin is found in the central nervous system of developing horseshoe crabs, specifically in 3 stages of development (larva and 3rd and 4th instars). The presence of Ependymin in these stages, which come before and after the stage analyzed previously, indicates ependymin's importance as a neutrophic factor in developing organisms. The fact that Ependymin is found in different anatomical locations throughout the crab's development indicates its versatility and importance in the development or survival of nervous tissue.

Although TEM is still underway to confirm the presence of Ependymin between neurons (which indicates it acts as a secreted glycoprotein), the presence of Ependymin around yolk droplets in the larval stages is particularly interesting and calls for further study of how Ependymin is propagated throughout the organism. However, despite this strange finding, the nearly standard presence of Ependymin in the central nervous system of all three stages of developments indicates is importance and activity as a neurotrophic factor.

Further research must be done to confirm these findings as well as to further determine the mechanism and cellular location of Ependymin's activity. The use of confocal fluorescent microscopy of whole mount specimens is expected to yield further evidence for exact locations of Ependymin throughout the crab's entire nervous system. Furthermore, continued electron microscopy must be done to characterize the nature of Ependymin's cellular activity and location. Also, further work must be done on later stages of horseshoe crab development to determine when or if Ependymin expression ever ceases to help shed light on its role as a regenerative factor as well as developmental factor.

With further understanding of Ependymin's role in horseshoe crabs, eventually further work involving bioinformatics and immunohistological techniques should be done to characterize the presence of homologous neurotrophic factors in mammals, in hopes of furthering understanding of neurotrophic factor applications in mammals.

Acknowledgements

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