

Ependymin Immunoreactivity in *Limulus polyphemus*

A Major Qualifying Project Report
Submitted to the Faculty
of the
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the
Degree of Bachelor of Science
By

Danielle Dionne
April 24, 2008

And

Stacy Krzyzewski
April 24, 2008

Approved by:

Doctor Daniel Gibson, Advisor

Abstract

Ependymin is a neurotrophic factor that was discovered in memory consolidation in goldfish. Sections of the EPN gene sequences have since been found in many other species, including the horseshoe crab. Previous research suggests that EPN may be instrumental in nerve growth. Using a new antibody against the bioactive 8 aa fragment of EPN, we have shown that Ependymin is present in the central and peripheral nervous system of developing horseshoe crabs. It was evident in the interstices between axons of regenerating limbs. HRP staining and fluorescence microscopy were used to observe Ependymin immunoreactivity. TEM and Immunogold staining were also employed to further support our results from fluorescence and light microscopy techniques and give us a more detailed view on Ependymin's mechanism of action.

Acknowledgements

We would like to thank Dr. Dan Gibson for his all of his help throughout this project. This project would not have been possible without his guidance, support and expertise. In addition we would like to thank Abby White, Mike Buckholt, JoAnn Whitefleet-Smith and Jill Rulfs for taking care of all of the odds and ends including helping us get into the building and our lab.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
Table of Figures.....	v
Introduction	1
Horseshoe Crab Anatomy	2
Ependymin	3
Previous Studies of Ependymin in <i>Limulus polyphemus</i>	4
Project Goals.....	5
Materials and Methods.....	6
<i>Microsurgery and Preparing Specimens</i>	6
<i>Fixation and Embedding</i>	8
<i>Serial Sections on the Microtome</i>	9
<i>Etching</i>	10
<i>Staining for Light and Fluorescent Microscopy</i>	10
<i>Staining for Transmission Electron Microscopy</i>	12
Results.....	13
Discussion.....	21
Ependymin is Present in the Central Nervous System of Juvenile Horseshoe Crabs	21
Ependymin is present in the Peripheral Leg Nerves of Juvenile Horseshoe Crabs.....	22
Future Experiments.....	23
Works Cited.....	25

Table of Figures

Figure 1: Experimental juvenile HRP section, counterstained to show basic structure 13

Figure 2: 1:100 Experimental (A) and control (B) sections of juvenile crabs stained with ABC Peroxidase Kit 14

Figure 3: Close up of Leg Nerves in 1:100 Experimental (A) and control (B) sections of juvenile horseshoe crabs stained with ABC Peroxidase Kit..... 14

Figure 4: Close up of Neuropil in CNS in 1:100 Experimental (A) and control (B) sections of juvenile horseshoe crabs stained with ABC Peroxidase Kit..... 15

Figure 5: 1:100 Alex Flour® experimental (A) and control (B) juvenile horseshoe crab sections..... 16

Figure 6: Close up of leg nerves in the 1:100 Alex Flour® experimental (A) and control (B) juvenile horseshoe crab sections 16

Figure 7: 60-90 nm thick sections of regenerating leg stumps stained with 1% Toluidine Blue, and 1% Sodium Tetra Borate in water, to show structure 17

Figure 8: 1:100 Experimental (A, C) and control (B, D) sections of 27 mm regenerating leg stumps stained with ABC Peroxidase Kit..... 18

Figure 9: 1:100 Alex Flour® experimental (A, C) and control (B, D) sections of regenerating leg stumps of an 18 mm juvenile horseshoe crab..... 19

Figure 10: 1:100 Nickel Variable Grids, 10 nm ImmunoGold experimental ultrathin regenerating leg stump sections 20

Introduction

Horseshoe crabs are often called living fossils because their appearance has changed very little over the past 450 million years. This constant body shape implies that horseshoe crab genes have most likely been conserved. Despite their name, horseshoe crabs are part of the Arthropod family and are more closely related to spiders and scorpions than crabs. Organisms in this family are characterized by brittle outer shells and multiple pairs of jointed legs. Horseshoe crabs and their cousins all have Chelicerae. In ticks and spiders these pinchers act like fangs. Horseshoe crabs use them to push food into their dorsal mouth located in the center of their legs. The ventral neuroesophageal ganglion encircles the mouth. The esophagus enters up through this whole in the nerve cord and loops backwards, and is continuous with the gut tract, which lies ventral to the nerve cord.

Our understanding of the human eye is based on research performed on horseshoe crabs starting over 50 years ago. In 1967, Dr. H. Keffer Hartline received the Nobel Prize for his research on the relatively simple optical organs of *Limulus* compound eye (University of Delaware Graduate College of Marine Studies and Sea Grant, n.d.). The more well known use of horseshoe crabs in the lab however, is for their copper based blue blood. Horseshoe crabs live in shallow water that is high in bacteria so they developed an amebocyte blood cell. If a horseshoe crab is wounded or a limb severed amebocytes will flock to the area and produce a clot to prevent bacteria from getting further into the circulatory system. Horseshoe crabs can be bled and their blood freeze dried to produce *Limulus* amebocyte lysate (LAL). LAL will coagulate in the presence of even small amounts of bacterial endotoxins. LAL is useful in the

medical field to test for and detect gram-negative bacteria in medicines (University of Delaware Graduate College of Marine Studies and Sea Grant, n.d.).

Horseshoe Crab Anatomy

A horseshoe crab's exoskeleton is split into three main parts and made out of a protein called chitin. The anterior, helmet shaped front is the Prosoma. Two compound eyes are located laterally on this segment. The Median eye on the topside senses ultra violet light. At night the decrease in ultra violet light signals the compound eyes to become up to one million times more sensitive to light (Shuster et al., 2003). There are also two eyes on the bottom of a horseshoe crab's shell. Horseshoe crabs don't have ears or balance organs so it is thought that they use these light sensing cells to keep their equilibrium. If the light is stimulating the cells the horseshoe crab will be signaled that it is upside down. The second, more triangular shaped region, is known as the Opisthosoma (Shuster et al., 2003). The five pairs of jointed legs protrude along the central axis underneath this middle section. The first set of uniquely shaped legs is used to dig and push sand aside. The posterior most segment is the tail. There are two hinge joints that separate the three segments of the shell. The heart, open circulatory system, and ventral nerve cord can be easily accessed at the front hinge.

The species *Limulus polyphemus* mate and nest on the shores of America's eastern coast. They are rather easily obtained and easy to take care of. Horseshoe crabs start out their life inside a 2 mm egg with a green chorion that pops off to expose a transparent egg containing a developing embryo. The horseshoe crab will molt its soft shell four times during development, while it's still inside the egg. After the second molt but before the third molt the

embryo has tiny stump legs, and looks kind of like a soft brain or zipper. The egg swells and splits the chorion around the time of the third molt. After the third molt the embryos have longer legs and begin to move around inside their eggs (Gibson and Gibson, 1983). After the fourth molt, the embryos begin to flap their gills and their body plan shows more resemblance to an adult's. That a gene found in modern vertebrates might also exist in a 450 million year old invertebrate type is an amazing possibility which we believe is confirmed by our research.

Ependymin

Ependymin is a neurotrophic factor that was discovered in goldfish memory consolidation. It was discovered due to the observation that certain proteins were increased in the brain cytoplasmic fraction in goldfish after learning events (Shashoua, 1976). Ependymin is a secreted glycoprotein that exists as a dimer and has both a glycosylated β form and a non-glycosylated γ form (Shashoua, 1991). Although the exact mechanism of action is unknown its presence during learning events suggests that it is involved in the synaptic changes that take place during memory formation (Shashoua, 1976).

Since its discovery, Ependymin and similar proteins have been partially sequenced in a number of organisms including humans and horseshoe crabs (Cruikshank et al., 1993). Studies have shown that a human Ependymin analog stimulates the expression of growth related genes in mouse neuroblastoma cells (Saif, 2004) as well as the expression of ribosomal proteins in human neuroblastoma cells (Arca, 2005). There is also evidence that EPN peptides have therapeutic effects in rat stroke models (Shashoua et al., 2003).

Invertebrates such as horseshoe crabs and sea cucumbers are known for their regenerative abilities. A recent study showed that an EPN-like protein in the sea cucumber, *Holothuria glaberrima*, was over-expressed during intestinal regeneration (Suarez-Castillo et al., 2004). This evidence along with the conserved Ependymin gene suggests that it may be important for neuronal growth and regeneration throughout many different species.

Previous Studies of Ependymin in *Limulus polyphemus*

Previous experiments attempting to locate Ependymin in horseshoe crabs used an anti-Ependymin antibody called SHEILA which was made in rabbits against an 18-mer peptide at the carboxy terminus of the protein. This antibody did show evidence of Ependymin expression in the ganglion and neuropils of juvenile horseshoe crab (Barroso, 1999) as well as in the blood of a wounded adult horseshoe crab (Costigan and Gallant, 2004). However, another study showed no immunoreactivity to SHEILA in regenerating limbs of juvenile horseshoe crabs (Castillo and Garabedian, 2007).

A recent study has shown that an 8 amino acid fragment, CMX-8933 with sequence KKETLQFR, has the same neurotrophic effects as the full protein (Adams et al., 2003). A new antibody to this fragment was ordered from New England Peptide in Gardner, MA. It was produced in the hope that it would be a better reporter of Ependymin than SHEILA and that it may be used to experimentally inhibit regeneration (Castillo & Garabedian, 2007).

Project Goals

The purpose of this project was to use the new anti-Ependymin (Anti-KKETLQFR) in order to determine if Ependymin is present in *Limulus polyphemus*. This project focused on finding Ependymin expression in two locations: the central nervous system of small developing juvenile horseshoe crabs and the peripheral nerves of regenerating legs in larger juvenile horseshoe crabs. The presence of Ependymin in horseshoe crabs would make them a good model for research on Ependymin's role in regeneration, neuronal development, and therapeutic actions.

Materials and Methods

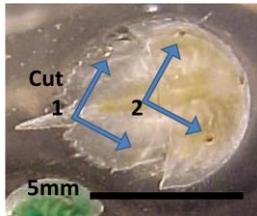
To begin studying the effects of Ependymin in horseshoe crabs some basic skills were first acquired. Sodium azide was added to the stock anti-EPN antibody, normal goat serum (NGS) and normal rabbit serum (NRS) to help prevent bacterial growth in the experimental samples. The stock solutions were aliquotted into 2ml Eppendorf tubes and frozen in the -80°C freezer for later use. Glass pipette pulling was one of the first basic skills practiced. These tiny glass pipettes were used with plastic tubing for microscopic horseshoe crab injections. Injection was practiced with horseshoe crab larvae. The glass pipettes were used to puncture larvae in the center of their hinge and inject them with anit-EPN containing blue dye or normal rabbit serum containing red dye. Microsurgery was another skill practiced before the onset of the actual experiment. Legs were removed from horseshoe crab larvae with Germany Stainless FST Microscissors under a dissecting scope.

Horseshoe crab eggs were fertilized *in vitro* by extracting the tiny green eggs from the female and placing in a petri dish with filtered sea water. Sperm was then extracted from the male and released over the eggs with a transfer pipette. The water was changed to fresh, filtered sea water after the eggs were left at room temperature for three hours. They were allowed about 6 weeks to develop into larva.

Microsurgery and Preparing Specimens

The first experiment was performed to provide immunohistochemical evidence of the Ependymin molecule in the nerve cord of developing juvenile horseshoe crabs. After the first

juvenile molt, nervous systems were isolated from 5mm *Limulus* to provide maximum access and binding for the antibody. The crabs were placed in petri dishes containing 1:1 8% paraformaldehyde in filtered seawater -Triton-X (0.3%) to make a 4% buffered formaldehyde solution. Injection with the same solution started the fixation process. The hinge of the crab was punctured with a 1/2cc Insulin Syringe and pointed towards the anterior end of the crab. Injection by this method allows for a quick and direct delivery of the “poison” because the nervous system is continuous with the heart membrane, also minimizing the amount of pain, if any is felt. Next, sections of the outer shell were removed with Vannas- style microscissors



under the dissecting scope at 10X magnification. This was done in two steps. While holding the crab by the side of the shell with tweezers, the center ridge was removed by cutting from the tail towards the hinge (posterior and anterior). Next, the center ridge was cut from the prosoma by starting at the hinge and cutting towards the eyes. After the portions of shell were removed the green gut tract was visible, still containing some yolk, and was removed by cutting where it connects to the esophagus, and pulling back with tweezers. Resulting was an exposed nerve cord with a portion of the esophagus protruding through the center of the nerve cord. Juvenile crabs were then used for whole mounts or preserved and embedded for serial sections.

A second experiment was performed to test for the presence of EPN outside of the CNS, during regeneration in the peripheral nerves of the legs. Legs were ablated from 11, 18, 27, 53,



and 76mm juvenile crabs at the tibia or patella. The sections removed were immediately placed in 4% formaldehyde in filtered seawater to preserve for later use. After 4-6 weeks of regeneration, the legs were



ablated again on the other side of the next joint, in either the patella or femur, respectively. The leg stumps were also placed in the 4% formaldehyde to begin the preservation process.

They were allowed to set in formaldehyde. Leg stumps and claws were prepared for serial sectioning in the same manner as the 5mm juvenile horseshoe crabs mentioned in the first experiment.

Fixation and Embedding

Tissue proteins were fixed with 4% paraformaldehyde in 100 mM cacodylate buffer, pH 7.6, adjusted to 900 mOs/kg with sucrose for one hour at room temperature, then rinsed twice in identical buffer. Specimens were then treated with 1% osmium tetroxide in cacodylate buffer for an hour and briefly rinsed twice in distilled water. To dehydrate the specimens they were soaked three times in ethanol for ten minutes each. Then, crabs were placed in propylene oxide which was allowed to penetrate for a few hours. This was followed by three soaks, of at least one hour, in 2:1, 1:1, and 1:2 propylene oxide to Spurr's Resin.



Another one hour soak in 100% Spurr's resin followed. Then, the Spurr's resin was decanted and the specimens were transferred to small plastic film caps. A new change of Spurr's resin was added to the film caps, allowed to soak for 2-6 hours, and they were then placed in the curing oven overnight at 65°C. The resulting specimens were 5mm crabs embedded in blocks of

hard plastic. Ablated leg stumps were fixed and embedded by the same procedure but cured in smaller centrifuge caps.

Serial Sections on the Microtome

The plastic was trimmed off the outer edges of the blocks with jewelers' saws until tissue was exposed, and shaved smooth with razor blades. The specimens were then glued to the mounting rod of the Sorvall Porter-Blum Ultra-microtome or placed in a clamp. Various sized sections were cut according to the Leica color scale. 0.5 μm thick clear sections of 5mm juveniles and regenerating stumps were prepared on Fisher Frost glass slides by removing from the boat with a wire loop and hair and placing down the centerline of the slides. The slides were placed on an 80° hot plate and allowed to dry for 30 minutes, as to not disturb the integrity of the proteins. Some unetched sections were stained with 1% Toluidine Blue and 1% Sodium Tetra Borate in distilled water to observe different tissue structures. Other slides were ready to be etched and stained for light and fluorescent microscopy.

Ultra thin 60-90nm gold and silver sections of regenerating leg stumps and juvenile crabs were placed on nickel grids with variable mesh sizes. The grids were held with jeweler's tweezers, submerged in the water and brought up underneath the sections, while guiding the sections on to the grid with a hair. Unetched grids were contrast-enhanced with uranyl acetate and lead citrate viewed with the Electron Microscope to observe basic tissue structure. Other grids were etched and treated with immunogold antibody and heavy metal staining.

Etching

Next, the plastic was removed from the sections on slides. First, Dissolving solution and Sodium periodate were prepared. Dissolving solution was prepared by adding 1.0g of NaOH pellets to 10ml of EtOH mixed with 10ml of propylene oxide, and placed on the stir plate for five minutes. 1% Sodium periodate was made by dissolving 0.1g of NaIO₄ in 10ml of dH₂O. Dissolving solution could be kept in the freezer but 1% NaIO₄ was prepared fresh each time. Sections were incubated for 3 minutes in Dissolving solution then rinsed three times, for 2 minutes each, in 95% EtOH. A 2-3 minute rinse in 50% EtOH and a 5 minute rinse in dH₂O followed. Then, the 1% NaIO₄ was applied to the sections and allowed to incubate for 5-7 minutes. Two more 3 minute rinses in dH₂O followed. The slides were then allowed to equilibrate in 0.1M PBS for at least 5 minutes. Both 5mm crab and leg stump sections were etched according to this protocol.

Staining for Light and Fluorescent Microscopy

Experimental sections were stained with 1:10 or 1:100 dilutions of anti-EPN antibody while control slides received the same dilutions of normal rabbit serum (NRS). Both antibodies were prepared in PBS and 1% normal goat serum (NGS). Secondary antibodies were used from the Invitrogen Alexa Fluor488[®] and Pierce ABC Peroxidase Staining Kits. 1:100 dilution of primary antibody was found to show sufficient immunoreactivity in juvenile crabs so to preserve our antibody only a 1:100 dilution was used in staining regenerating leg stumps. HRP slides were blocked with 6% NGS for 20-30 minutes, then rinsed in PBS. Incubation in primary

antibody followed and was allowed to set overnight at room temperature. Next, slides were soaked in PBS three times for ten minutes each. A 30 minute incubation in the goat anti-rabbit biotinylated secondary antibody from the ABC Kit followed. The slides were rinsed in twice in PBS for 10 minutes and then soaked in the ABC reagent for 30 minutes. The ABC reagent is avidin linked to horseradish peroxidase via a biotin link. The biotin on the secondary antibody also binds to this complex, forming a bridge that binds the horseradish peroxidase. Two 10 minute rinses in PBS followed and then a 2-7 minute incubation in 1:1 1X nickel enhanced Diamino-benzidine in hydrogen peroxide (DAB substrate Kit). Then two 10 minute rinses in PBS were performed and lastly a 2-3 minute rinse in distilled water. Fluorescent slides followed the same protocol up to the secondary antibody. Instead of the biotinylated secondary antibody, slides were treated with the Alexa Fluor® 488 goat anti-rabbit conjugated secondary antibody. Then the slides were rinsed twice in PBS for 10 minutes each and once in distilled water. The slides were allowed to dry, glycerol was applied as a clearing agent, and a glass coverslip was set on top. . HRP and Alexa Fluor® slides were observed under light and fluorescent microscopy at 4X, 10X, and 100X oil emersion magnifications. Some changes were made to this initial protocol after the first set of etching on the regenerating leg stumps. The 1X Nickel enhanced DAB was diluted more, at 1:20 DAB to hydrogen peroxide because dark staining developed almost instantaneously with a 1:10 dilution. Also, FITC (Fluorescein Isothiocyanate) goat anti rabbit were used as a secondary antibody and enhancer on the second set of regenerating limbs because the Alexa Fluor secondary antibody was used up.

Staining for Transmission Electron Microscopy

Ultra thin gold sections on nickel grids were etched in 10% Hydrogen Peroxide by soaking shiny side down for 5 minutes then rinsed several times in distilled water. They were then incubated for two hours in a 1:100 dilution of primary antibody with 0.1M PBS-Tween-80 (0.05%)-NGS(0.25%), pH 7.2. Experimental grids received anti-EPN as the primary antibody while control grids received NRS. After two hours, the sections were rinsed in PBS-Tween-80 (0.05%)-NGS(0.25%) three times and allowed to soak in the last rinse for 5 minutes. An overnight incubation in goat anti-rabbit secondary antibody conjugated to 10nm gold balls followed (10nm ImmunoGold, Sigma). The sections were rinsed again in PBS-Tween-80 (0.05%)-NGS(0.25%), once in PBS, and once in distilled water. The next step performed was heavy metal staining. The sections were stained for 1 minute, face down, in uranyl acetate, rinsed in distilled water, and lastly stained briefly in lead citrate.

Results

Figure 1 shows an experimental section of juvenile horseshoe crab stained with the Pierce Immunopure ABC Peroxidase Kit. This section has also been counterstained with 1% Fuchsin in 50% EtOH and 50% water, in order to show the basic structure of the horseshoe crab sections. The ganglion and neuropil of the central nervous system and the peripheral leg nerves can be seen as well as the Ependymin staining that is present within these structures. In addition the Esophagus and muscle are visible.

Figure 1: Experimental juvenile HRP section, counterstained to show basic structure



Figures 2-4 show an experimental and control section of juvenile horseshoe crab stained with the Pierce Immunopure ABC Peroxidase Kit. Experimental section was stained with 1:100 dilution of anti-EPN primary antibody. Control section was stained with 1:100 dilution of NRS as the primary antibody. Dark lines in the nerve cord and axons entering the legs show

Ependymin immunoreactivity and its presumptive tracts in the CNS. Sections were observed by light microscopy.

Figure 2: 1:100 Experimental (A) and control (B) sections of juvenile crabs stained with ABC Peroxidase Kit

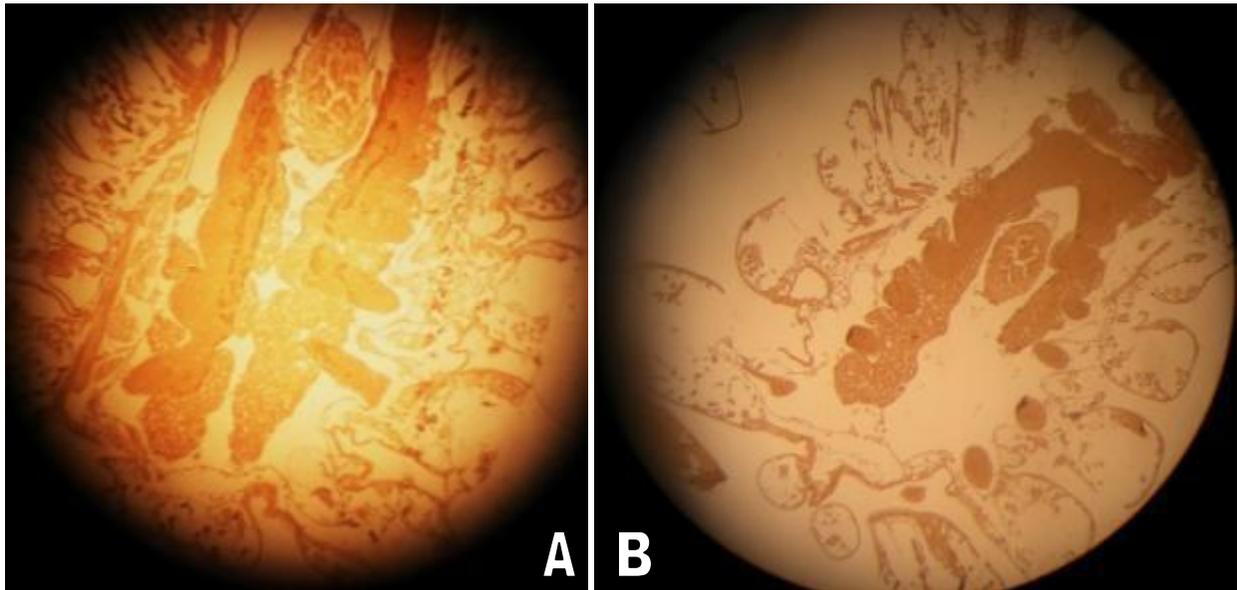


Figure 3: Close up of Leg Nerves in 1:100 Experimental (A) and control (B) sections of juvenile horseshoe crabs stained with ABC Peroxidase Kit

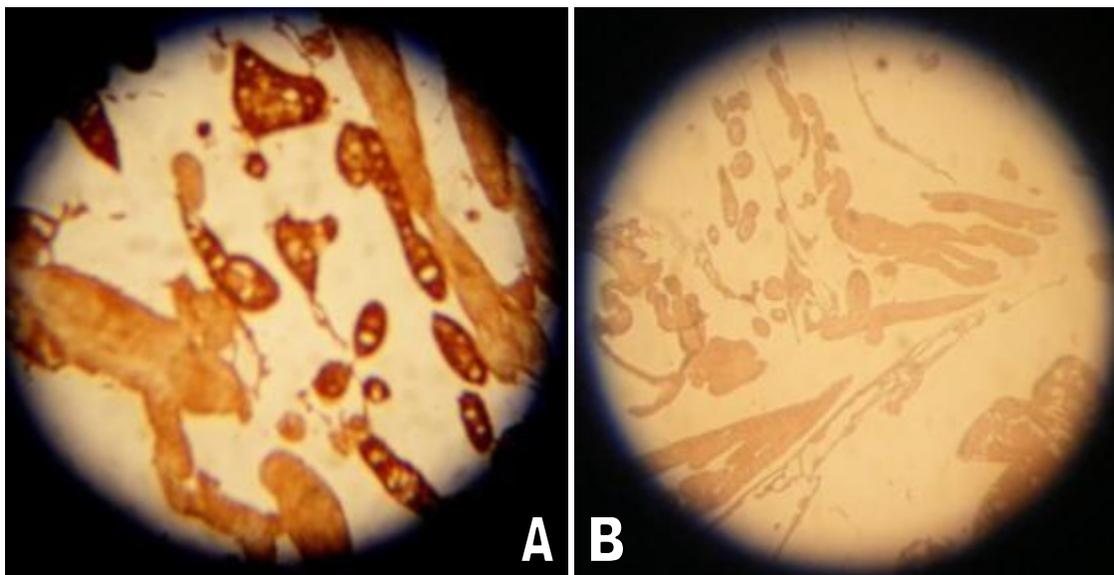
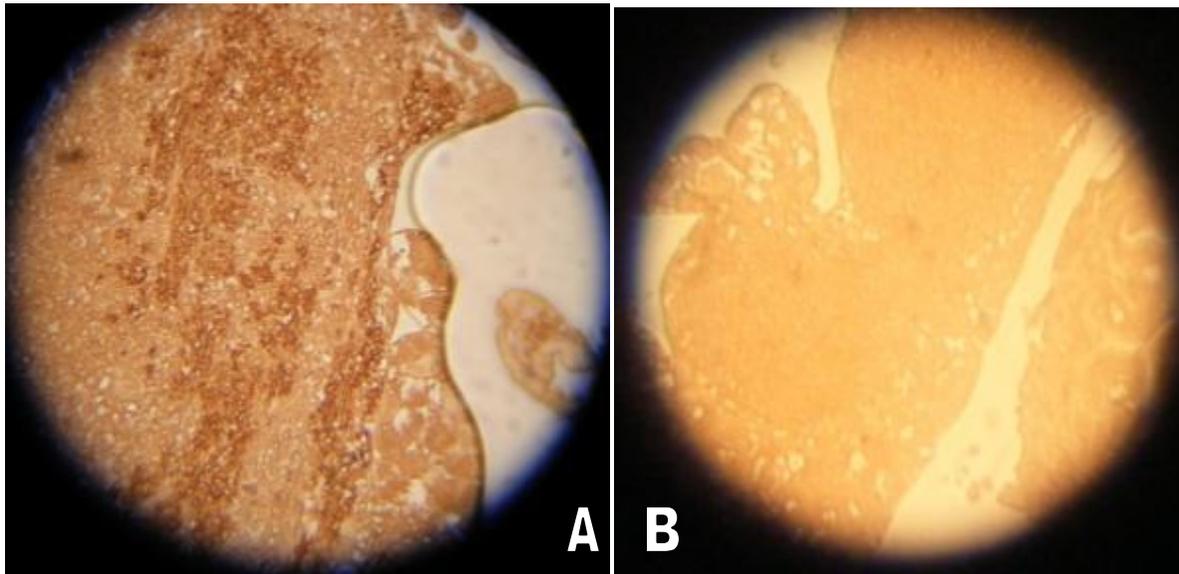


Figure 4: Close up of Neuropil in CNS in 1:100 Experimental (A) and control (B) sections of juvenile horseshoe crabs stained with ABC Peroxidase Kit



Figures 5 and 6 show the immunoreactivity of Ependymin in the experimental sections of juvenile horseshoe crabs stained with the Alexa Flour® kit as compared to the control sections. Experimental section stained with 1:100 dilution of anti-EPN primary antibody. Control section stained with 1:100 dilution of NRS as primary antibody. Bright green fluorescence in the nerve cord and leg joints of experimental sections show areas of Ependymin immunoreactivity.

Figure 5: 1:100 Alex Flour® experimental (A) and control (B) juvenile horseshoe crab sections

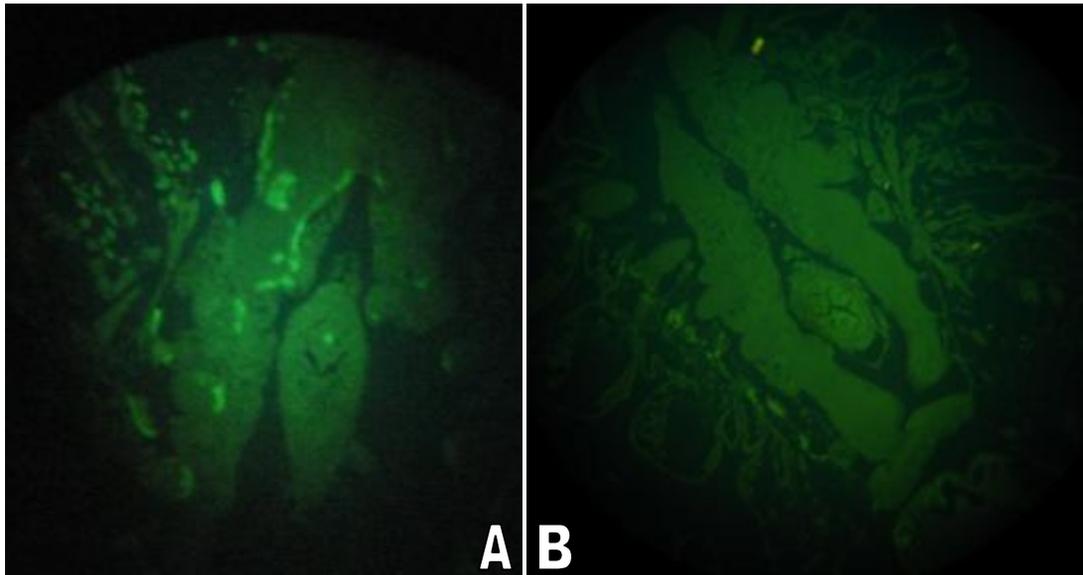


Figure 6: Close up of leg nerves in the 1:100 Alex Flour® experimental (A) and control (B) juvenile horseshoe crab sections

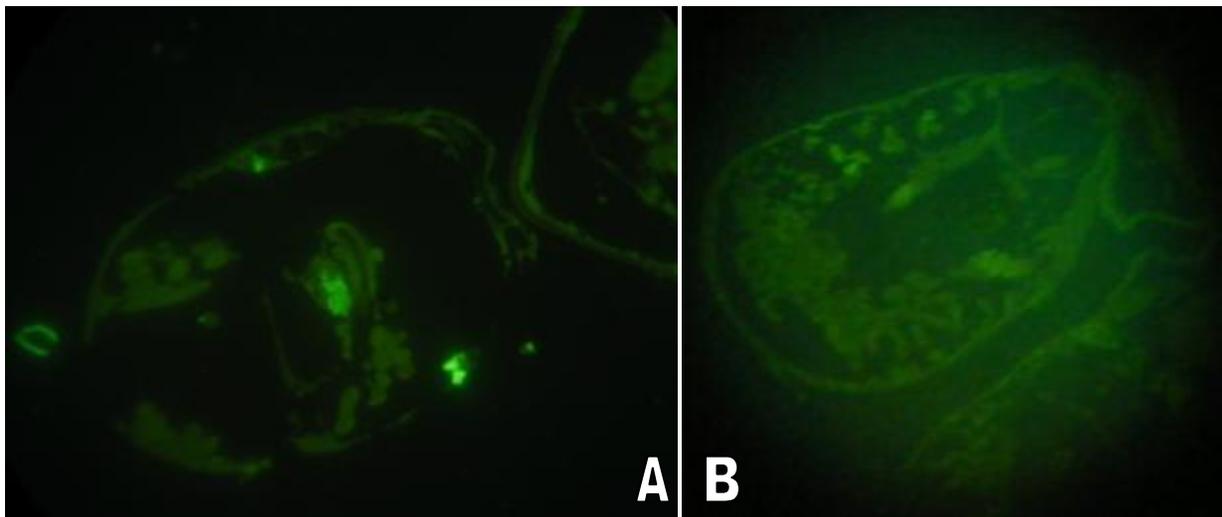


Figure 7 shows a semi-thin section of regenerating leg stump from an 18 mm horseshoe crab stained with Toluidine Blue in order to show its structure. Where the leg stump comes to an end in the upper left hand corner of the micrograph, a callus is present that has formed over

the cut end of the leg. Below that, nerve and muscle can be seen which get more organized as you look farther away from the regenerating end.

Figure 7: 60-90 nm thick sections of regenerating leg stumps stained with 1% Toluidine Blue, and 1% Sodium Tetra Borate in water, to show structure



Figure 8 shows the experimental (A, B) and control (C,D) regenerating leg stumps from a 27 mm horseshoe crab stained with the Pierce Immunopure ABC kit. Each of these 4 micrographs shows dark staining throughout the entire structure of the leg. This does not show clear results of Ependymin presence in the regenerating leg stumps and will be discussed further in the next chapter.

Figure 8: 1:100 Experimental (A, C) and control (B, D) sections of 27 mm regenerating leg stumps stained with ABC Peroxidase Kit

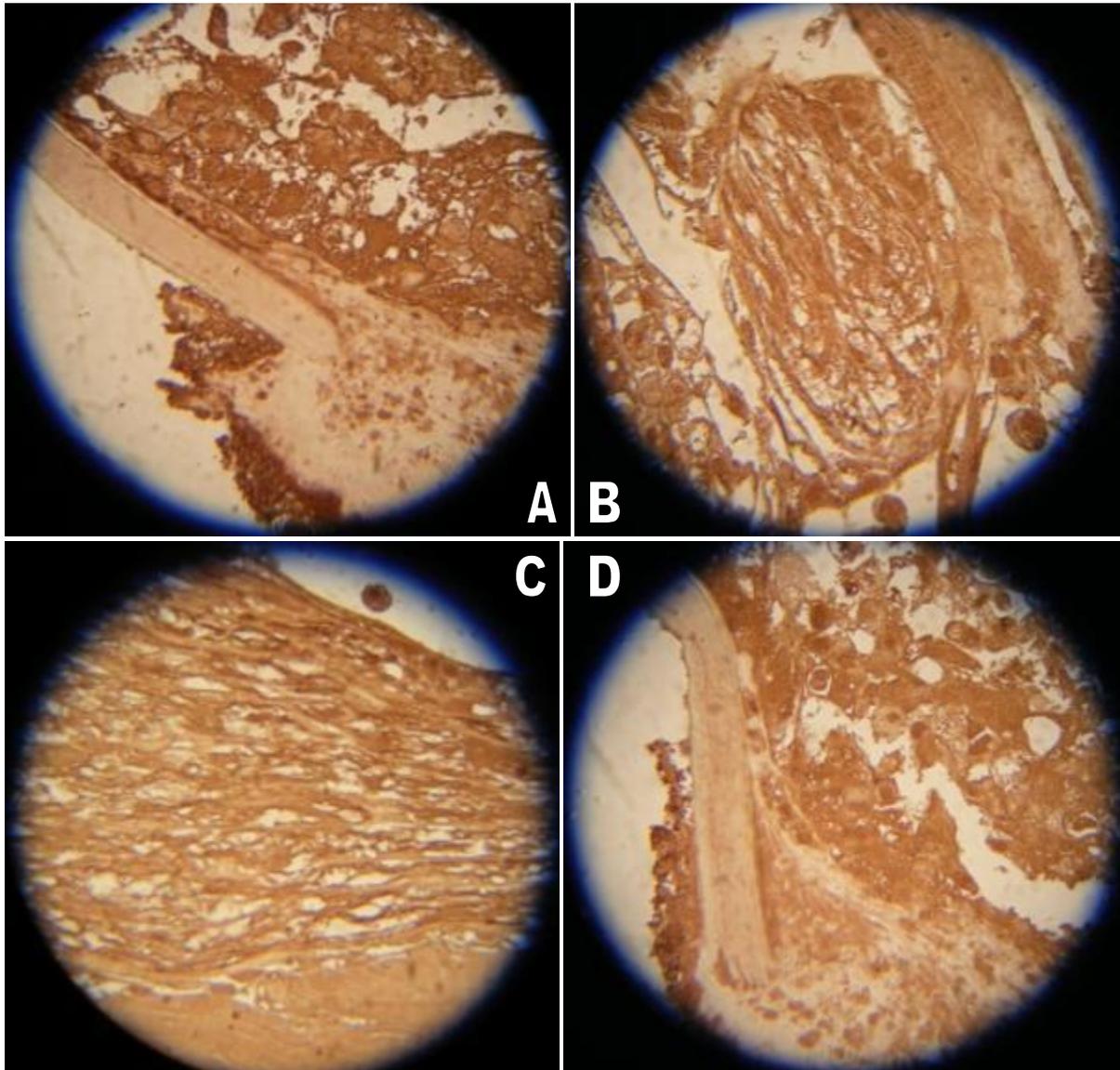


Figure 9 shows the micrographs of experimental (A and C) and control (B and D) regenerating leg stumps from an 18 mm juvenile horseshoe crab. In the controls (B and D), the nerve cannot be distinguished from the muscle from its staining because the nerve and muscle have the same level of brightness throughout the entire leg. However, in the experimental sections, the nerve tissue is stained brighter than the muscle which shows that Ependymin is

present in the nerve tissue of these regenerating juvenile horseshoe crab legs. The white arrows in pictures A and C point to the brightly stained nerves while the red arrow points to the muscle which is not brightly stained.

Figure 9: 1:100 Alex Flour® experimental (A, C) and control (B, D) sections of regenerating leg stumps of an 18 mm juvenile horseshoe crab

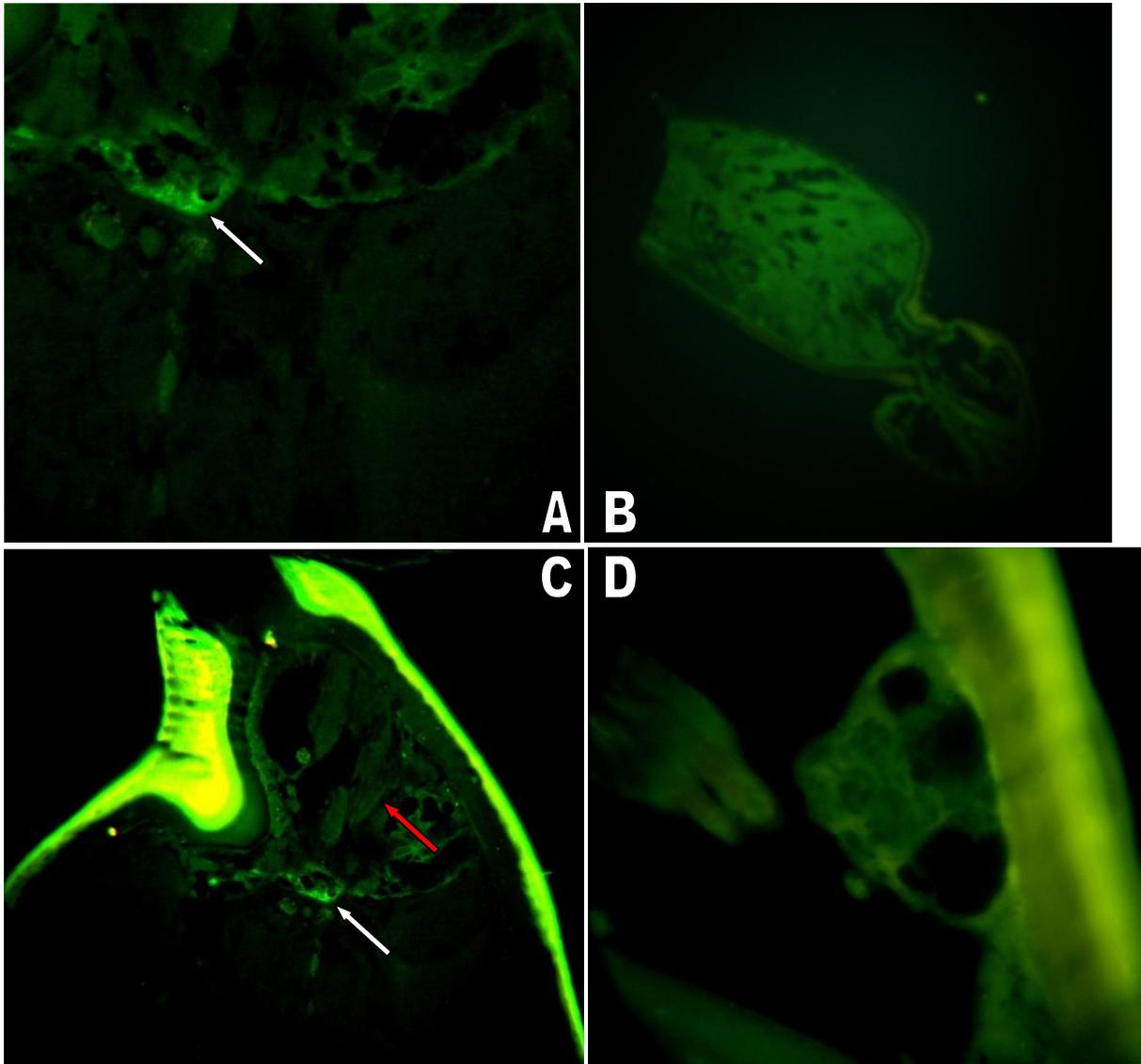
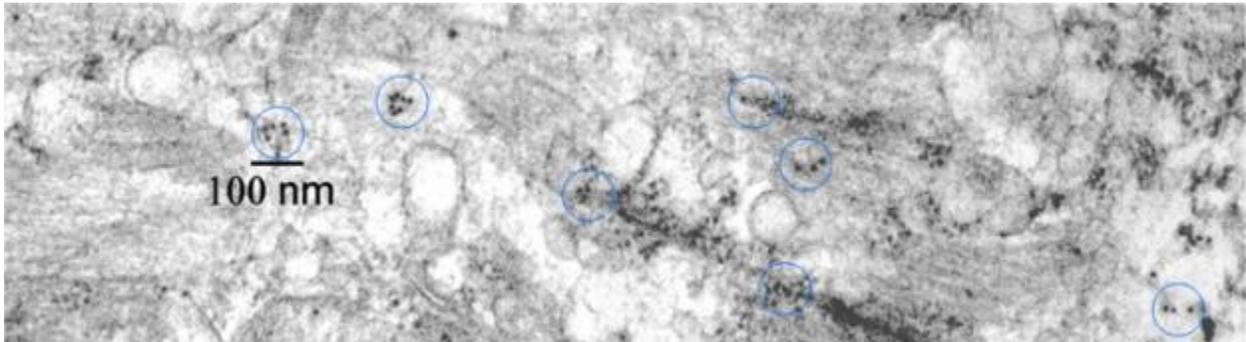


Figure 10 shows a transmission electron micrograph of an experimental regenerating leg stump that has been stained with 1:100 dilution of anti-EPN as the primary antibody and 10 nm

gold balls attached to goat anti-rabbit Immunoglobulin as the secondary antibody. The presence of 10 nm uniform black balls in the interstitial spaces between leg nerve axons shows the presence of Ependymin immunoreactivity.

Figure 10: 1:100 Nickel Variable Grids, 10 nm ImmunoGold experimental ultrathin regenerating leg stump sections



Discussion

The purpose of this project was to determine if Ependymin is present in the developing central nervous system of juvenile horseshoe crabs as well as in the regenerating leg stumps of injured horseshoe crabs. This was done through immunostaining with an anti-Ependymin antibody (anti-KKETLQFR) and three different types of secondary staining: the Pierce Immunopure ABC Peroxidase and Alexa Flour staining kits for light microscopy and 10 nm Immunogold for transmission Electron Microscopy. These stains were used on fixed 5 mm juvenile horseshoe crabs as well as on the regenerating leg stumps of 18 and 27 mm juvenile horseshoe crabs.

Ependymin is Present in the Central Nervous System of Juvenile Horseshoe Crabs

Figures 1, 2 and 4 show dark staining in the ABC stained juvenile crab central nervous systems. This represents the expression and presence of Ependymin in these areas. The dark staining is concentrated in the neuropil, or axons, of the central nervous system. This supports the theory that Ependymin is involved in axonal growth. In addition figure 5 shows the same results of bright staining in the central nervous system of experimental horseshoe crabs using the Alexa Flour® system.

Ependymin is present in the Peripheral Leg Nerves of Juvenile Horseshoe Crabs

Figures 3 and 6 show staining in the peripheral leg nerve of experimental juvenile horseshoe crabs stained with the Pierce Immunopure ABC kit and the Alexa Flour kit respectively. In addition, figure 10 shows staining with 10 nm gold balls in the regenerating leg stump of an 18 mm juvenile horseshoe crab. This staining is present in the interstitial spaces between axons. These results support the involvement of Ependymin in synaptic changes that occur during nerve growth.

Figure 8 shows 4 micrographs of regenerating leg stumps that have been stained with the Pierce Immunopure ABC kit, two with anti-EPN and two with NRS as the primary antibody. The overall dark staining that can be seen throughout each of these pictures does not represent any proof of Ependymin presence. This dark staining is likely due to an overexposure or too high a concentration of antibody at some point during the staining process. Evidence for the existence of Ependymin in the regenerating leg stumps however is present in figure 9 which shows the experimental and control regenerating leg stumps that have been stained with the Alexa Flour kit. The distribution of EPN in the CNS and leg nerves suggests that it is necessary for neuronal development

This project attempted to demonstrate the presence of Ependymin in regeneration, however that was not achieved. Although the fluorescent micrograph in figure 9 and the transmission electron micrograph in figure 10 demonstrates the existence of Ependymin in this regenerating leg stump it is unclear whether it is present due to regeneration alone. The

presence of Ependymin in the peripheral leg nerves of uninjured juvenile horseshoe crabs shows that Ependymin is present in the legs already, likely due to development and growth. Therefore although it has been shown that the Ependymin is present, further studies will have to be conducted in order to determine if it is for the purpose of regeneration.

Future Experiments

The anti-KKETLQFR antibody that was used in this experiment has been shown to be effective in immunostaining uses. This project will hopefully be extended in future years in order to further understand Ependymin. For example, a current MQP proposal for next year is to study Ependymin in *C. elegans* using this antibody.

In order to study Ependymin in relation to regeneration in horseshoe crabs it is likely that these studies will have to be conducted on fully grown adult horseshoe crabs. This should help to determine if Ependymin is present because of regeneration or if it is there due to normal development. A similar experiment to the one conducted on juvenile horseshoe crabs could be done on adult crabs in order to see if there is any Ependymin present in regenerating legs as compared to non-regenerating legs. In addition experiments involving the injection of anti-EPN into injured leg stumps could be interesting in determining if it can prevent regeneration.

The expression of Ependymin in horseshoe crabs, along with the fact that they are common laboratory animals and easy to care for, makes them a good model for the study of

Ependymin. Studies in the horseshoe crab will likely help to learn more about Ependymin's mechanism of action, location within cells, and its purpose.

Works Cited

- Adams D., Hasson B., Boyer-Boiteau, A., El-Khishin A., Shashoua V. (2003). A peptide fragment of ependymin neurotrophic factor uses protein kinase C and the mitogen activated protein kinase pathway to activate c-Jun N-terminal kinase and a functional AP-1 containing c-Jun and c-Fos proteins in mouse NB2a cells. *Journal of Neuroscience Research*. 72:405-416.
- Arca, T. (2005). Attempts to clone the *Limulus* ependymin gene, and the effects of a human ependymin peptide on human shsy neuroblastoma cells. MS Thesis, Worcester Polytechnic Institute.
- Castillo, Y. & Garabedian, L. (2007). Limb Regeneration in Horseshoe Crabs. Major Qualifying Project, Worcester Polytechnic Institute.
- Costigan, J. & Gallant J. (2004). Horseshoe Crab Growth Factors: Immunohistology. Major Qualifying Project, Worcester Polytechnic Institute.
- Cruikshank, A., N. Faulkner, J. Gosling, K. Ingemi, and J. Vescio. (1993). Cloning and sequencing of ependymin. Major Qualifying Project, Worcester Polytechnic Institute.
- Gibson, D. & Gibson V. (1983). The Living Fossil's Habits. *The Cape Naturalist*. 12: 18-22.
- Shashoua, V. (1976). Brain metabolism and the acquisition of new behaviors. I. Evidence for specific changes in the pattern of protein synthesis. *Brain Research*. 111: 347-364.
- Shashoua V., Adams D., Boyer-Boiteau A. (2003) Neuroprotective effects of a new synthetic peptide, CMX-9236, in *in vitro* and *in vivo* models of cerebral ischemia. *Brain Research* 936: 214-223.
- Shuster, C., Barlow, R., Brockmann, H.J., eds. (2003). *The American Horseshoe Crab*. Cambridge: Harvard University Press.
- Suarez-Castillo E., Medina-Ortiz W., Roig-Lopez J., Garcia-Arraras J. (2004). Ependymin, a gene involved in regeneration and neuroplasticity in vertebrates, is overexpressed during regeneration in the echinoderm *Holothuria glaberrima*. *Gene* 334:133-43.
- University of Delaware Graduate College of Marine Studies and Sea Grant (n.d). Retrieved March 20, 2008 from the Horseshoe Crab website:
<http://www.ocean.udel.edu/horseshoecrab/index.html>.