The Role of Ependymin in Horseshoe Crab Limb Regeneration

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Abstract

Immunohistology and a partial DNA sequence confirm the presence of the vertebrate neurotrophic factor ependymin in the horseshoe crab. We hypothesized that ependymin concentrates at sites of limb regeneration. We treated regenerating limb stumps prepared for light and electron microscopy with rabbit anti-ependymin (controls: normal rabbit serum) and goat anti-rabbit reporter antibodies. Ependymin is present at wound sites, but also diffusely present in limbs freshly removed from intact controls. Ependymin is likely necessary for normal growth as well as regeneration.

Acknowledgements

We would like to the Biology/Biotechnology department at Worcester Polytechnic Institute for the opportunity to do this project as well as Dr. Daniel Gibson for his continued help, guidance and support throughout the project both as an advisor and personal mentor. It was a pleasure.

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Introduction and Literature Review

Horseshoe Crab (HSC)

The horseshoe crab, or *Limulus polyphemus,* has been the source of legend and lore for centuries, and also has great value in the medical world. It is actually not a crab at all, as its name implies. Rather, it more closely related to the Arachnida class, containing spiders, scorpions, and ticks. In Japan, it was once believed that when a brave warrior died that he would be reborn as a horseshoe crab (Sheekly and Adolfsen 2003). The crab's hard shell resembled the samurai helmets, providing a strong, nearly impenetrable shield from enemies, or predators. Today, horseshoe crabs are just as revered in the medical profession. The horseshoe crab is famous for its blue blood which is copper based and contains cells from which lysate, a substance which is now used to test for purity in medicines, is made. Even the shell is valuable, as certain extracts are used to accelerate blood clotting (www.horsehoecrab.org, Shuster *et al*., 2004).

Anatomy

The anatomy of the horseshoe crab has remained remarkably similar since the Triassic period (Hickman *et al*., 1978). There are 3 sections, the prosoma, or head, opisthosoma, or abdomen, and tail. On the top of the prosoma is a pair of compound eyes, and median eyes which serve as light receptors. The underside of the prosoma is where the chelicerae, or small feeding pincers, 5 pairs of legs, and the mouth are located. On the opisthosoma are the book gills and gill flaps, as well as genital pores. The final section, the telson, or tail, is not used as a weapon, as often thought, but rather it is used

for steering, digging in the sand, and to right the crab if they have capsized. Figure 1 shows a labeled version of the horseshoe crab anatomy.

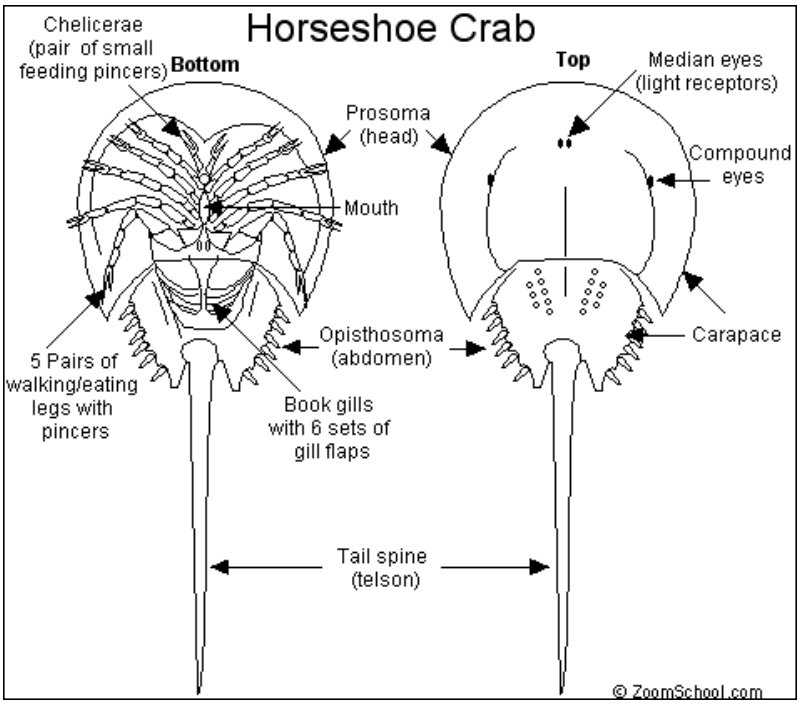

Figure 1: External Anatomy of *Limulus polyphemus* adapted from Delaware Public Schools on the Web (http://www.k12.de.us/warner/structure.html)

Life Cycle

The life cycle of *Limulus* consists of four main stages, egg, larval, juvenile, and adult. Adult horseshoe crabs bury the fertilized eggs in the wet sand of the beach where they will begin to develop. They are light green in color and are slightly larger than one and a half millimeters in diameter. Larval horseshoe crabs will remain enclosed in the egg for approximately 14 days, depending on the conditions of their habitat, including temperature. Post-hatching horseshoe crabs have no visible telson at this stage, nor do they have a fully functioning digestive system. Therefore, they will continue to use the nutrients found in yolk droplets stored in the prosoma (www.horsehoecrab.org).

Like many of its relatives, the horseshoe crab must molt in order to grow. After the first post-hatching molt, the horseshoe crab has entered the juvenile stage. Before

becoming sexually mature in about 10 years, the horseshoe crab will undergo 16-17 molts, more heavily weighted in the first years, with 3 or 4 molts the first two or so years. With each molt comes the opportunity to regenerate a limb that has been lost. When the horseshoe crab has reached sexual maturity, it is in the adult stage. They are able to live about 18 years if they are not preyed upon, caught by humans for bait fishing, or perish during a medical bleeding, as are top causes of death (Walls and Berkson 2003).

Ependymin (EPN)

Neurotrophic factors (NTF) are glycoproteins that play a role in the earliest stages of vertebrate sensory neuron development and may be involved in the maintenance of the adult nervous system. Although the precise molecular mechanism by which the brain uses ependymin remains unknown, there are certain functional aspects of EPN that closely resemble the properties of NTFs. Ependymin is a brain glycoprotein that exists in three monomeric forms and is found predominantly in the brain extracellular fluid (ECF) and cerebrospinal fluid (CSF). It is believed that ependymin plays a role in synaptic changes occurring in the consolidation process of long term memory formation like other known neurotrophic factors. For example, EPN was identified as a protein whose turnover rate was enhanced in goldfish brains after various training events (Shashoua, 1976). Ependymin's presence in horseshoe crabs has been confirmed by partial DNA sequence (Cruikshank *et al*., 1993; Brideau, 1993; Selent, 1993; and Barroso, 1999) although EPN was originally discovered in goldfish and appears to have mammalian counterparts. Some mechanisms of ependymin action have been determined in mammalian neuroblastoma cells, and peptide derivatives of EPN have proven useful in therapy for stroke (Arca, 2005).

Methods and Materials

Leg Removal

Eight *Limulus polyphemus* larvae, hatched in the laboratory from collected eggs, were chosen for the experiment. After the larvae molted into juveniles, four of the subjects underwent surgery in 0.2 µl filter-sterilized seawater. The first and second leg on the left side of the body was amputated at the first joint, as indicated in Figure 2.

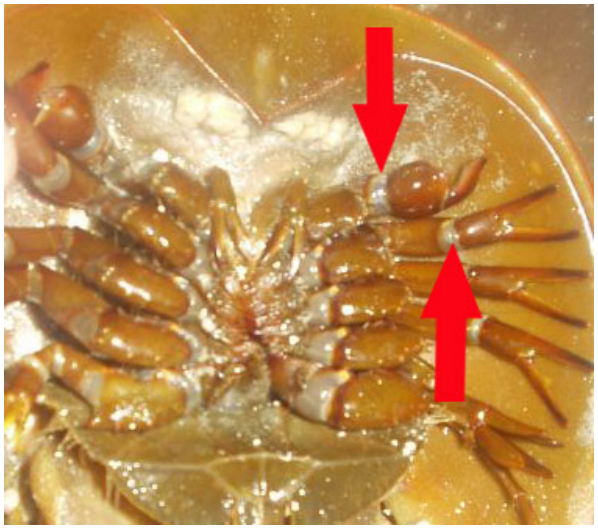

Figure 2: Sites of Amputation adapted from Andrews University Biology Webpage (http://www.biol.andrews.edu/everglades/organisms/Invertebrates/marine_inverts/Arthropoda/Atlantic%20Horseshoe%20Crab/atlantic_horsehoe_index.htm)
Note: The chelate clasper (pedipalp) of the first leg is a feature of mature males only. Larval legs are identical, except for the hindmost one

Injection

The sixteen horseshoe crabs were then divided into four groups. Each group contained two crabs that had undergone an amputation, and two crabs that had not. The "Control group" received no injections. The "Seawater group" received only an injection of 0.2 µl filter-sterilized seawater. The "Rabbit group" was injected with 1:50 normal rabbit serum. The final group, the "Anti-Ependymin group" was injected with 1:50 rabbit anti-ependymin (SHEILA), a polyclonal antibody made against an 18-mer

synthesized peptide that follows the DNA code at the carboxy terminus of the ependymin molecule. Each specimen was given an injection of their respective solution directly into the heart, except the control group. Figure 3 shows the injection location.

Figure 3: Location of Solution Injection adapted from http://www.nus.edu.sg/corporate/research/gallery/research2.htm

Embedding Process

After the larvae had been injected, the living larvae sat for 20 minutes to an hour, to allow the antibody time to bind internally. We believed that the antibody would not be degraded by the animal's system because of previous success that Dr. Gibson had with injection of anti-JH, also prepared in rabbits. The hormone apparently persisted long enough to inhibit juvenile hormone and promote early molting (Dr. Dan Gibson, WPI, personal communication). Each larva was then halved along the midsaggital line. One half of each crab was fixed in aldehydes and some post-fixed in osmium tetroxide with seawater prior to embedding in epon-araldite or LR white. All of the eight crab hemisections that had amputated legs were put into 70% ethanol to be prepared for LR White acrylic embedding, along with eight other halves from different crabs that had no appendages amputated. The remaining 16 halves were placed into 1% osmium tetroxide prior to epon-araldite embedding.

Epon-Araldite

The sections of the crabs originally in 1% osmium that were designated to be embedded in epon-araldite went through a dehydration process. This process put the crabs in 70% ethanol for 10 minutes. They were then placed in 90%, 100%, and 100% concentrations of ethanol each for 10 minutes to remove all traces of water. The sections were then placed in propylene oxide for 10 minutes followed by a 1:1 mixture of propylene oxide and epon-araldite overnight. Propylene oxide is a low molecular weight epoxy, capable of scavenging alcohol from the dehydrated specimen and aiding infiltration of the viscous resin. The crab sections were then placed in small polypropylene or polyethylene containers and 100% epon-araldite was poured to fill. These containers were then cured overnight at 60˚ Celsius. For the complete protocol for preparing the Epon-Araldite, see Appendix 1.

LR White

The crab sections that were originally submersed in 70% ethanol were prepared to undergo LR White embedding. The LR White polymerizes around the tissue instead of binding to it like epon-araldite. Therefore, more antigenicity of the proteins is retained. Furthermore, samples were not placed in osmium tetroxide in order to protect the proteins from denaturation. Instead, they were left in a solution of 50% acrylic resin and 50% ethanol at a concentration of 70% overnight. The specimens were then placed in 100% acrylic resin and cured overnight in the oven at 60˚ Celsius.

Sectioning of Crabs

Trimming Samples

Samples in both epon-araldite and LR White were trimmed with a small saw and/or a razor blade prior to machine sectioning. The body was positioned so that the desired body part would be sectioned first. The objective was to trim away as much plastic as possible so that the sections would be mostly composed of body matter.

Machine Sectioning

Glass and diamond knives were used to section the trimmed blocks with a Sorvall MT-2 ultramicrotome. Once the block was "faced off" to the point that tissue was exposed, and full sections 450 nm thick were obtained, one slide was made and fixed by heat. Another slide was fixed and was dyed with a 0.5% Toluidine blue, 0.5% Borax, 1% Ethanol solution. The section thickness was then decreased to 50-90 nm to have sections that diffracted reflected light as a gold color. These sections were then placed on either nickel or copper grids to be observed at a later date under an electron microscope.

Analysis of Samples

Slides of the undyed samples were etched using a dissolving solution of Ethanol, Propylene oxide, and Sodium hydroxide in varying concentrations interrupted with Ethanol and distilled water rinses. Sodium periodate was then used and was followed by a thorough rinse in distilled water. For the complete protocol, see Appendix 2.

The primary antibody was applied at this point, in a 1:100 dilution with phosphate-buffered saline and 6% normal (non-immune) goat serum. To conserve on the amount of rabbit anti-ependymin that was used, grease circles were made on each slide encircling half of the sections on each slide. Only half were treated with the anti-

ependymin primary antibody so that one side of each slide could serve as a control. The control slide received blocking buffer in place of the primary antibody. For the complete protocol, see Appendix 3.

After the slides were properly etched and treated, they were stained using the ABC Immunopure Peroxidase Staining Kit. The tissue was first treated with the Biotinylated Secondary Antibody conjugated to Horseradish Peroxidase (HRP) with several rinses in PBS followed by the DAB Substrate Working Solution. Development of the brown color characteristic of oxidized DAB occurred if horseradish peroxidase was present to release Oxygen from the peroxide buffer, and HRP was only present if there had been binding of the secondary antibody/ABC complex. When the slides were properly developed, they were left to air dry. When this was completed, they were then covered in Brite brand floor wax to protect the slides and allow for the samples to be seen with less internal refraction. The wax has a refractive index very close to that of the resin, and therefore fills in cracks and knife marks and "clears" the specimen.

Results

Table 1 is a representation of the HSC groups that were used in the main experiment.

Table 1: Representation of Group Categorization. Group numbers in the same color are halves of the same HSC.

	Injection Received			
	No Injection	Sterile Seawater	Rabbit Serum	Rabbit Anti-Ependymin
Amputee HSC in Epon-Araldite	Group 1	Group 2	Group 3	Group 4
Amputee HSC in LR White	Group 5	Group 6	Group 7	Group 8
Intact HSC in Epon-Araldite	Group 9	Group 10	Group 11	Group 12
Intact HSC in LR White	Group 13	Group 14	Group 15	Group 16

Figure 4 shows a slide of Group 12 with two enlarged sections. There are two distinct cell clusters that have stained dark brown in the red enlarged photograph, which are likely amebocytes. Amebocytes from wounded animals have been shown to bind anti-EPN, so this finding is not surprising; however, the discoverers of EPN-like immunoreactivity in amebocytes doubted that the cells were a probable location for ependymin production. The enlarged light blue section shows the noticeable difference in staining between a section of muscle and part of the gastrointestinal tract against the other body tissues. Because muscle is growing and gut is remodeling, it is reasonable to assume that these are two sites of (1)ependymin action ***or*** (2)ependymin secretion.

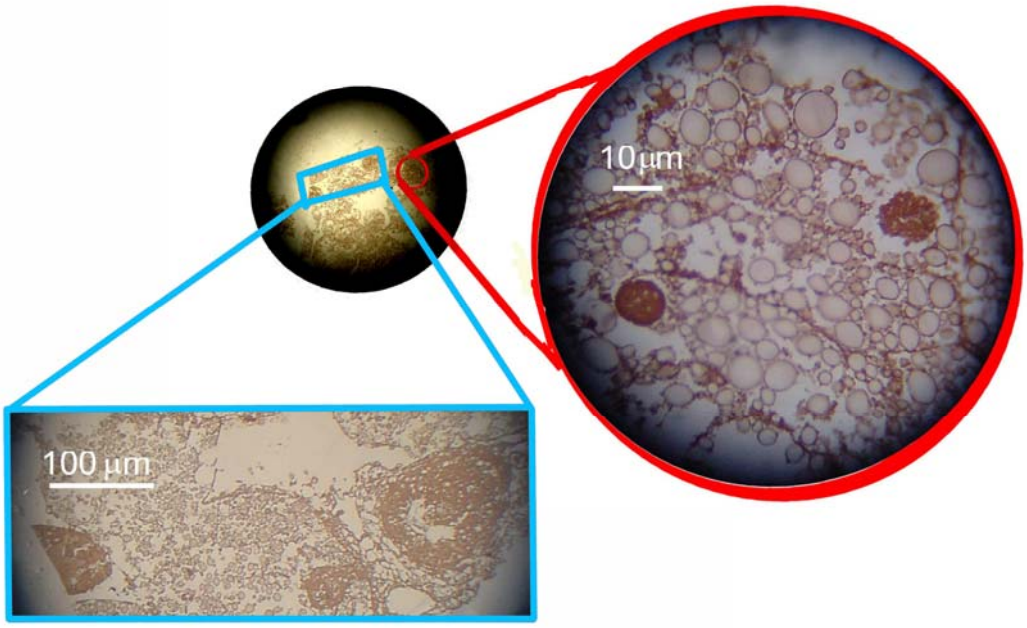

Figure 4: Group 12 with Enlarged Darker Staining Regions

Furthermore, Figure 5 shows a section from Group 9 that served as a control, and did not receive an injection of anti-ependymin antibody. This slide was developed for the same amount of time as that in Figure 4. The distinct difference in color between the control and experimental sections of the slides indicate a binding of anti-EPN by the experimental sections that clearly exceeds background.

Figure 5: Group 9 at 40x

Figure 6 shows the amputated limbs from a unique juvenile in the correct orientation. This HSC in particular, was in the process of regenerating two chelate limbs and a "snowshoe"-like leg, but in the wrong location (last leg is usually the only snowshoe; this regenerating limb was leg 4). When these limbs were tested for ependymin, they were also positive.

Figure 6: Amputated Limbs from Juvenile HSC in Correct Order: R2, (R3 normal for comparison), L2, and L4. Scale in centimeters.

An adult crab with the clasper joint removed from its body was also studied in a whole mount. Figure 7 displays the differences between the intact limb (left) and the regenerating limb (right). By concentrating on the enlarged photographs, it is possible to see the new tissue as it is growing within the limb. It is clear in the photographs in the third row that there is a larger amount of new tissue extending towards the tip of the limb. The staining in the top right and left photographs clearly shows the difference in ependymin concentration between the amputated and intact limbs. The amputated limb has stained much darker, indicating a higher concentration of ependymin in this area.

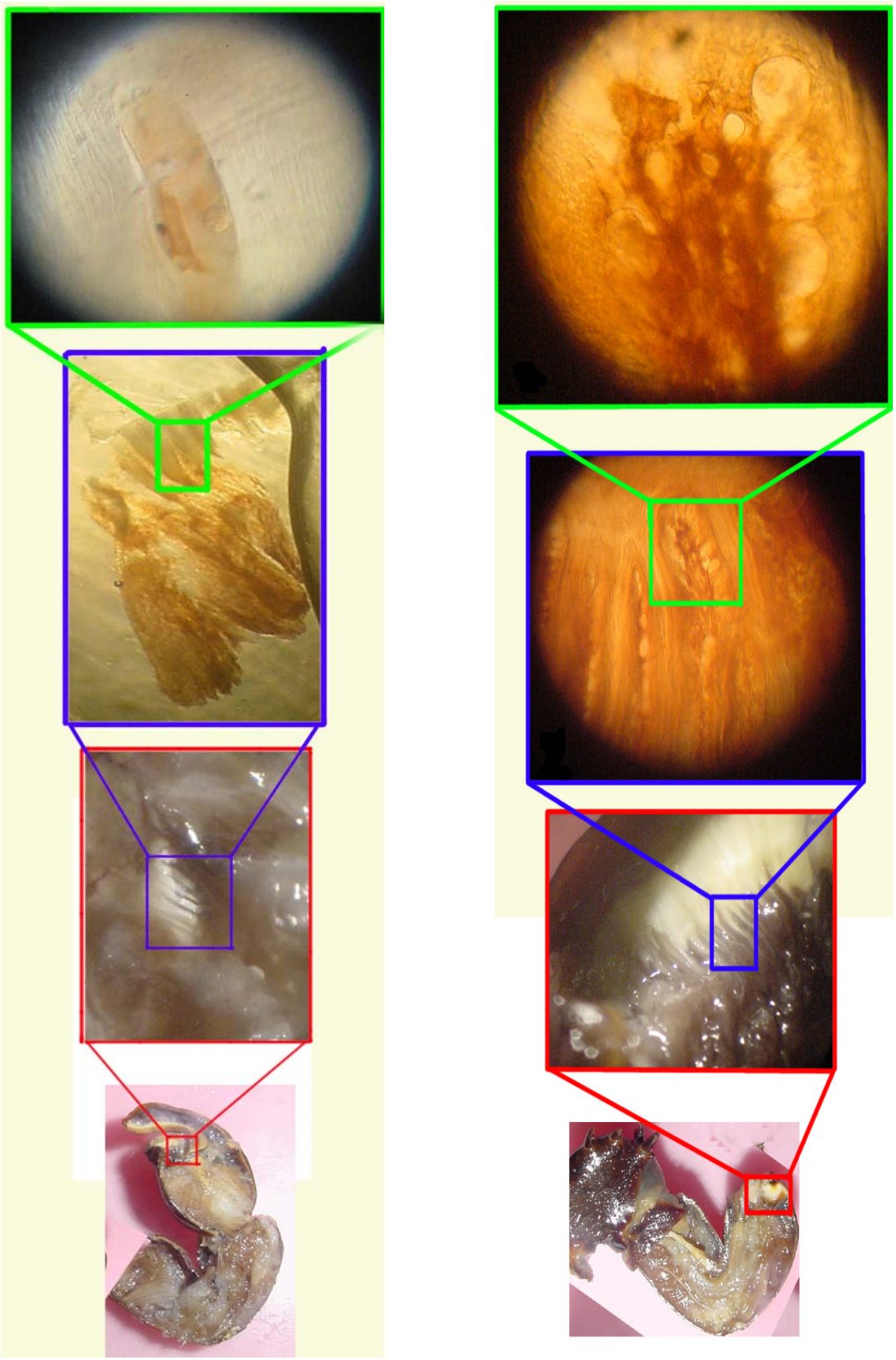

Figure 7: Tissue Growth Compared at Increasing Magnifications in an Intact Limb (Left) and an Amputated Limb (Right)

Discussion and Conclusions

The original hope that injected anti-EPN would bind in the living animal and allow us to fix, embed, section, and then stain with secondary antibody was not realized. There were no differences unless the sectioned material was etched to expose the antigens and then treated conventionally, starting with primary antibody (anti EPN) and normal goat serum (NGS) for a control. Success with the first scheme would mean that only extracellular ependymin, or cell-surface ependymin, would likely be bound in the living animal, but it appears to be well-represented intracellularly on the etched and stained sections. A much higher concentration of anti-EPN staining was seen in the sections of gastrointestinal and muscle areas of the HSC incubated with anti-epenydmin antibody at a 1:100 dilution (see Figure 4) than in that of the HSC that received no primary antibody (see Figure 5). The lack of staining of the tissue (See Figure 5) indicates that there was no non-specific binding of the secondary antibody. If brown staining could be seen in Figure 5 it would have be indicative of either non-specific binding or binding to anything other than the primary antibody. This is because the binding of the secondary antibody allows the attachment of peroxidase to the specimen and results in the subsequent brown staining. Therefore, we can conclude that the brown staining in Figure 4 is resultant of the binding of primary antibody to sites of ependymin, or at least an ependymin-like peptide.

The presence of brown staining around the yolk cells in the enlarged portions of Figure 4 indicates that the primary antibody is specifically binding to ependymin at the edges of the yolk cells. It is interesting, however, that there is no binding within the yolk cell, showing that no ependymin is present within the cell. When sectioned, the cytoplasm of the yolk cells becomes exposed and one would expect binding of the anti-

ependymin antibody if there were ependymin present inside the cell (Barroso, 1999). Furthermore, if the anti-ependymin antibody were binding non-specifically, one would expect staining within the yolk cells or random staining throughout the specimen rather than the clustered staining seen in the figures.

We did section tissue for immunoelectron microscopy but have only begun to look at this material, for which the secondary antibody is tagged with colloidal gold (Brumwell and Martin, 1996). This will be a natural extension of the project for future MQPs to work on, and may reveal just where within the stained tissues (i.e., which organelles) the ependymin is located. It is a thrilling prospect to consider that a gene for a neurotrophic factor is so essential and widespread in the animal kingdom that we can find it in ourselves and in animals that branched off from us 600 million years ago.

Appendices

Appendix 1

Epon Araldite Preparation Procedure

Procedure taken from Anderson and Ellis 1965: J. Protozool 12:483

Araldite 502…….11g
Epon (or EmBed) 812……...15.3g
DER 732 or 736……. 5.2g
DDSA ……..35g
DMP-30 …….1.5g

The only critical measurement is the DMP-30 accelerator, which can cause premature curing or incomplete curing if the dose isn't right. This mix is very viscous and has to be infiltrated in stages with dilutions of propylene oxide. After 3x 100% ethanol and 2x propylene oxide, infiltrate with 1:2 EA:po, then 2:1 EA:po, then straight EA, at least 1 hour each. Change into fresh EA before curing at 60˚C for 24h.

Appendix 2

Slide Etching Procedure

Procedure taken from Barroso, (1999) pg. 18

Procedure

1. Incubate slides for 3 minutes in Dissolving Solution (50 ml EtOH, 50 ml propylene oxide, 5.0 g NaOH pellets, stirred vigorously for 5 minutes until precipitate forms).
2. Perform three 2 minutes rinses in 95% EtOH.
3. Perform a 2-3 minutes rinse in 50% EtOH followed by a 5 minute rinse in dH2O.
4. The specimens were then treated for 5-7 minutes with 1% NaIO4, made fresh, in dH2O.
5. Perform three more 3 minutes rinses in dH2O. Then proceed to the ABC Peroxidase Staining Kit procedure (See Appendix 3).

Appendix 3

ImmunoPure ABC Peroxidase Staining Kit Procedure

Blocking Buffer: 3 drops of normal goat serum (provided in kit) in 10 ml of PBS.
Primary Antibody: Dilute anti-epndymin antibody to 1:100 with blocking buffer.
Biotinylated Secondary Antibody: 3 drops of normal goat serum and 1 drop of Biotinylated Secondary Antibody (provided in kit) in 10 ml PBS.
ABC Reagent: (Must be prepared 30 minutes prior to use). 2 drops of Reagent A (provided in kit) in 10 ml of PBS, followed by 2 drops of Reagent B (provided in kit). Immediately mix the solution and allow 30 minutes for complex to form.

Preparation of DAB Substrate Working Solution

1. Determine amount of substrate required.
2. Remove the DAB/Metal Concentrate (10X) from -20°C storage. Mix well by inversion. (Do NOT bring to room temperature.)
3. Dilute the DAB/Metal Concentrate (10X) with the Stable Peroxide Buffer to a 1X working solution. For example, if 5 ml of substrate is required, dilute 500 µl of the DAB/Metal Concentrate with 4.5 ml of the Stable Peroxide Buffer.
4. Mix well. Note: Do not add hydrogen peroxide. Stable peroxide is already present at an optimal concentration. Adding any additional hydrogen peroxide will increase background.
5. Add the 1X working solution to the tissue until desired substrate development.

Procedure:

1. Wash slides for 20 minutes in PBS.
2. Block the slide for 20 minutes with the prepared Blocking Buffer.
3. Blot excess buffer from tissue sections. Apply the diluted Primary Antibody and incubate tissue for 30 minutes.
4. Wash slides for 10 minutes in PBS.
5. Apply the prepared Biotinylated Secondary Antibody and incubate tissue for 30 minutes.
6. Wash slides for 10 minutes in PBS.
7. Incubate tissue for 30 minutes in the prepared ABC Reagent.
8. Wash slides for 10 minutes in PBS.
9. Add the Metal Enhanced DAB Substrate working solution (1X) and incubate tissue until desired staining has been achieved. Typical incubations are from 5 to 15 minutes.

Literature Cited

Adams, D.S.M. Kiyokawa, M.E. Getman and V.E. Shashoua. 1996. Genes encoding giant danio and golden shiner ependymin. *Neurochemical Research.* 21: 377-384.

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

Barroso, G. 1999. "Immunohistochemical evidence for the expression of ependymin in *Limulus polyphemus*." Master's Thesis, Worcester Polytechnic Institute.

Brideau, A. 1993. "Evidence for ependymin outside the teleost fish." Master's Thesis, Worcester Polytechnic Institute.

Brumwell, G. and Martin, V. 1996. Ultrastructural localization of Rfamide-like peptides in neuronal dense-cored vesicles of a cnidarian planula larva. *Invertebrate Biology*. 115 (1):13-19.

Cruikshank, A., N. Faulkner, J. Gosling, K. Ingemi, and J. Vescio. 1993. Cloning and sequencing of ependymin. Major Qualifying Project, Worcester Polytechnic Institute

Hickman, Cleveland., Hickman, Cleaveland, Jr.., Hickman, Frances. *Biology of Animals*. Saint Louis: The C.V. Mosby Company, 1978. second edition

Selent, C. 1993. "The search for ependymin ouside the cyprinidae family." Master's Thesis, Worcester Polytechnic Institute.

Shashoua, V.E. 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.

Shuster, C., R. Barlow, and H.J. Brockman. 2003. The American Horseshoe Crab. ISBN 0-674-01159-7. Harvard University Press, Cambridge, MA. 427p.

Sheekey, Megan., Adolfsen, Eric. (2003). Retrieved April 1, 2005, from http://www.nycgovparks.org/sub_newsroom/media_advisories/press_releases.php?id=16774

Vernberg, F.J., W.B. Vernberg. 1983. *The Biology of Crustacea. Vol. 7: Behavior and Ecology*. Academic Press, New York.

Wallas, Elizabeth A.., Berkson, Jim. (2003). Effects of blood extraction on horseshoe crabs. *Fishery Bulletin* 101: 457-459.

www.horseshoecrab.org (2003) Retrieved April 1-26, 2005.