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The marine gastropod *Nassarius obsoletus* undergoes an irreversible and permanent metamorphosis as it transforms from a veliger larva to a benthic adult. The neurological control for this metamorphic process includes actions mediated by the neurotransmitter γ-aminobutyric acid (GABA), which inhibits metamorphosis and thus preserves the larval state. I used immunocytochemistry (ICC) with antibodies to GABA and the CLARITY procedure to identify GABAergic neurons within *N. obsoletus,* some of which are part of the neuroanatomical framework that controls the initiation of metamorphosis. Results from the ICC experiments documented the presence of GABAergic axons throughout the velar lobes and in the larval foot. Particularly noteworthy is the presence of bilateral posterior pedal nerves that originate in the pedal ganglia and travel to the metapodium (posterior foot). These nerves branch extensively within the foot. The presence of a cluster of putative epithelial glandular cells in the foot was also demonstrated by ICC. An analysis of the adult central ganglia with the CLARITY method was inconclusive. Furthermore, ICC experiments failed to localize cell bodies, hypothesized to be in the apical ganglion, from which velar axons originate. My results support the hypothesis that GABAergic neurons function in the motor system of larval *N. obsoletus,* that GABA or GABA-like compounds are present in mucus-secreting cells of the foot, and that this neurotransmitter is involved in the regulation of the initiation of metamorphosis.

GABA-LIKE IMMUNOREACTIVITY OCCURS IN COMPETENT LARVAE OF THE MARINE GASTROPOD *NASSARIUS OBSOLETUS*

by

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CHAPTER I

INTRODUCTION

Larval metamorphosis is an important, irreversible life history event in which a larva develops into a sexually-immature juvenile that will ultimately mature into an adult capable of reproduction. Metamorphosis is a characteristic shared by many species of marine invertebrates, particularly the molluscs (Fell, 1997). Many commercially relevant species of molluscs undergo metamorphosis. This includes species such as the abalone, *Haliotis iris, H. tuberculata coccinea,* and *H. rufescens;* the mussel, *Mytilus galloprovincialis;* the clams, *Ruditapes philippinarum* and *Venerupis pullastra;* and the oysters, *Ostrea edulis* and *Crassostrea gigas*; to mention a few. Thus, there is an economic reason for understanding the metamorphic process beyond its intrinsic importance to the life histories of most marine invertebrates (Morse et al., 1979; Garcia-Lavandeira et al., 2005; Feng et al., 2006; De Vicose et al., 2010; Roberts et al., 2010). Numerous papers describe the various stages of metamorphosis in multiple species, but further work remains to be done in many species to understand the neuroendocrine circuits that govern this process. I used the marine snail *Nassarius obsoletus* because it is easily reared in the laboratory, is an important member of its ecological community (Kelaher et al., 2003; Cheverie et al., 2014), and has a well-known developmental sequence (Collier, 2002), all of which make it a good model organism for the study of molluscan metamorphosis.

Larvae of marine gastropods vary in size, but are generally microscopic at emergence from the egg capsule, as is the case with *Nassarius obsoletus.* Its larvae are approximately 275µm long at hatching (Scheltema, 1962). These veliger larvae can swim weakly in the water column with the help of larval-specific organs, the velar lobes (Fretter and Graham, 1962). The velar lobes are edged with two bands of cilia that capture particles in an opposed-band fashion while the larvae swim through the water column (Mackie et al., 1976; Strathmann and Leise, 1979). Previous research has shown that these velar lobes, which are shed during metamorphosis, are under neurological control (Mackie et al., 1976).

The central nervous system (CNS) in larval *N. obsoletus* has been described previously (Lin and Leise, 1996a; Lin and Leise, 1996b). At 6 days after hatching, a pair of cerebral ganglia is apparent, as are a pair of pedal ganglia and statocysts, which innervate the foot and control balance, respectively (Lin and Leise, 1996a). An unpaired apical ganglion (AG), a sensorimotor region of the CNS that controls the velar lobes and responds to metamorphic inducers, lies dorsally to the cerebral commissure, which connects the cerebral ganglia (Lin and Leise, 1996a; Marois and Carew, 1997; Mackie et al., 1976; Ruiz-Jones and Hadfield, 2011). By day 8 after hatching, pleural ganglia, which innervate visceral muscles, and the paired buccal ganglia that are associated with the digestive tract have also developed (Lin and Leise, 1996a). Finally, in larvae that are competent for metamorphosis, intestinal and propodial ganglia have formed, as well as a sensory osphradial ganglion (Lin and Leise, 1996a).

Metamorphosis

Larval metamorphosis, a profound physical alteration of the body plan, also alters the nervous system of marine gastropods. For example, the apical ganglion undergoes programmed cell death upon induction of metamorphosis (Barlow and Truman 1992; Marois and Carew, 1997; Gifondorwa and Leise, 2006; Ruiz-Jones and Hadfield, 2011). Recent work with metamorphic induction in *N. obsoletus* has focused on the role of nitric oxide (NO) and its generative enzyme, nitric oxide synthase (NOS). NOS is found in the larval nervous system, especially in the apical ganglion (Lin and Leise, 1996b; Thavaradhara and Leise, 2001). Whether or not levels of NOS are constant during the larval period is unclear, but they decline after metamorphosis is triggered (Lin and Leise, 1996a; Hens et al., 2006). Treatment of larvae with NO donors such as 3-morpholino-sydnonimine (SIN-1) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) can inhibit artificially induced metamorphosis, whereas NOS inhibitors such as *N*-nitro-L-arginine methyl ester (L-NAME) allow its progression, strongly implicating NO in the preservation of the larval state (Froggett and Leise, 1999). Endogenous control of NOS and NO production has yet to be clarified, although experiments in *Lymnaea stagnalis* have shown that expression of a pseudogene for NOS generates an anti-sense RNA transcript that suppresses the production of functional neuronal NOS (Korneev et al., 1999).

Metamorphosis in most marine invertebrates can only proceed when an organism is competent or physiologically able to respond to environmental cues that trigger the transformation. In many of these organisms, this involves a two-step process wherein the larva settles, physically affixing itself to a suitable substrate, and then metamorphoses

(Chia and Koss, 1988; Collier, 1997). Metamorphosis can be induced by compounds from several sources. For example, crustose coralline algae (CCA) seem to be a preferred substrate for several species of *Haliotis*, though the identity of the exact inducing molecule is unclear (Roberts et al., 2010). The natural prey of the nudibranch *Phestilla sibogae*, the finger coral *Porites compressa*, releases a small, diffusible inducer molecule that triggers metamorphosis in its predator (Hadfield and Pennington, 1990; Pires et al., 2000; Koehl and Hadfield, 2004). Signals from conspecific adults and diatoms that are a part of the juvenile diet can also induce settlement and metamorphosis in various species of molluscs (Laimek et al., 2008; Leise et al., 2009).

Settlement and metamorphosis can be artificially induced by bath applications or injections of neuroactive compounds. Dopamine, epinephrine, and norepinephrine, three neurotransmitters with generally excitatory effects, induce settlement and metamorphosis in the tunicate *Styela canopus* (Feng et al., 2006). These catecholamines also promote metamorphosis in *P. sibogae* (Pires et al., 2000). Epinephrine induces settlement and metamorphosis in four species of bivalves: *M. galloprovincialis*, *R. philippinarum*, *V. pullastra*, and *O. edulis* (Garcia-Lavandeira et al., 2005). Serotonin (5-HT) induces metamorphosis in *N. obsoletus* and multiple other larval types, but it is not a universal inducer for all species (Couper and Leise, 1996; Leise et al., 2001; Leise et al., 2004; Leise and Cahoon, 2012). Inhibition of NO production through injection of NOS inhibitors also induces metamorphosis (Froggett and Leise 1999). However, in the sea slug *Alderia willowi*, altering NO levels fails to induce metamorphosis even as larval age increases (Romero et al., 2013). *A. willowi* larvae may transition from a non-selective

state (first 48 hours of larval life), during which NO inhibition induces metamorphosis, to a selective state (around two weeks in duration), during which NO inhibition seems to modulate the larval response to a specific environmental cue (Romero et al, 2013). Inducers generally work, via afferent sensory neurons, on the larval nervous system by eliciting changes in neural activity that leads to metamorphosis (Leise and Hadfield, 2000). However, one neurotransmitter that has widely varying effects between species is γ-aminobutyric acid (GABA).

GABA

GABA is a classical inhibitory neurotransmitter in nervous systems in all bilateria, including mammals (Ko et al., 2015). GABA's involvement has been demonstrated in olfactory responses in invertebrates, memory formation in crabs, as well as the control of swimming in some molluscs (Christensen et al., 1998; Tano et al., 2013; Gunaratne et al., 2014). Further work in molluscs corroborates GABA's involvement in the motor circuitry controlling swimming in abalone, and in the motor control of feeding behavior in the sea hare, *Aplysia californi*a, and the pteropod, *Clione limacina* (Jing et al., 2003; Norekian and Malyshev, 2005; Alfaro et al., 2014).

GABA also induces settlement and metamorphosis in *H. rufescens* (Morse et al., 1979). Subsequent studies demonstrated that molecules homologous to GABA could elicit similar responses, but GABA's effects vary between species of molluscs (Morse et al., 1980). As in *H. rufescens*, GABA induces settlement in *M. galloprovincialis*, *V.*

pullastra, *P. philippinarum*, and *O. edulis* (Garcia-Lavandeira et al., 2005). Baxter and Morse (1992) demonstrated that cilia on cephalic epithelial cells of *H. rufescens* contain G-protein coupled lysine receptors that enhanced the larval response to a GABA-mimetic peptide, possibly through diacylglycerol-dependent activation of protein kinase C. It was unclear whether or not these cilia came from a receptor cell. A GABA receptor in the foot of the abalone *H. asinina* also facilitated settlement and metamorphosis (Stewart et al., 2011). An earlier report provided evidence for the presence of GABA in the pedal mucus glands of several abalone species and other gastropods, as well as in the adult mucus. This could provide a mechanism that allows adults to signal the presence of suitable substrates to conspecific larvae (Laimek et al., 2008). In contrast to these species, in some organisms, such as *S. canopus*, *H. tuberculata coccinea*, and *N. obsoletus*, GABA acts in a more traditional, inhibitory fashion (Feng et al, 2006; de Vocose et al., 2010; Biscocho, 2013). In these species, GABA inhibits settlement and metamorphosis. Decreased levels of larval metamorphosis occur in *N. obsoletus* when GABA is introduced after larvae are given a suboptimal exposure to 5-HT (Leise and Cahoon, 2012; Biscocho, 2013). GABA appears to preserve the larval state, much like NO, and is hypothesized to regulate metamorphosis upstream from NO production (Welch, 2015), as inhibition of NOS appears to directly initiate programmed cell death in the larval apical ganglion (Gifondorwa and Leise, 2006).

Disparities in actions of GABA between induction and inhibition of metamorphosis are the driving forces for this thesis. Species in which GABA induces metamorphosis are largely found in the Pacific rim in rocky habitats where they can

encounter crustose coralline algae. In contrast, the eastern Atlantic coast of North America is comprised of sandy beaches, as well as muddy bays and sounds, where gastropods would not encounter such algae (Ruppert and Fox, 1988). Thus, species of molluscs may have evolved different ways to use GABAergic pathways in metamorphosis as a result of ecosystem distinctiveness (Morse et al., 1979; Garcia-Lavandeira et al., 2005; Leise and Cahoon, 2012). Further studies of GABAergic activity are necessary to fully elucidate the different neural mechanisms that control metamorphosis of Pacific and Atlantic species.

Nassarius obsoletus

The marine gastropod *N. obsoletus* (formerly *Ilyanassa obsoleta*), is commonly known as the eastern mud snail and can be readily found along the east coast of the United States (Fox and Ruppert, 1985). It is a well studied species because adults can be maintained easily in laboratory aquaria, larvae can be easily cultured, and because of its ecological importance. *N. obsoletus* is an important agent of disturbance in its marine ecosystem, shaping benthic biodiversity, altering community structure, and accelerating breakdown of organic matter (Kelaher et al., 2003; Cheverie et al., 2014). Early studies on *N. obsoletus* characterized its veliger larval state and its ability to metamorphose when presented with favorable bottom sediment (Scheltema, 1961; Scheltema, 1962). While the inductive chemical has yet to be identified, experimental evidence indicates that *N. obsoletus* can be induced to metamorphose by diatoms present in its juvenile environment (Leise et al., 2009). Artificially, *N. obsoletus* has been induced to settle and

metamorphose by exposure to 5-HT. This was later discovered to be mimicking endogenous activity, and was not an action of 5-HT at external chemoreceptors (Levantine and Bonar, 1986; Couper and Leise, 1996).

Gangliogenesis is well characterized in *N. obsoletus*. Histological work demonstrated that nitrergic neurons occur in the larval apical ganglion, which ultimately led to the discovery that endogenous NO preserves the larval state, and that its subsequent loss led to metamorphosis (Lin and Leise, 1996a; Lin and Leise, 1996b; Froggett and Leise, 1999, Thavaradhara and Leise, 2001). In *N. obsoletus*, induction of metamorphosis also causes a decrease in NOS gene expression such that the concomitant decrease in NO release leads to programmed cell death in the apical ganglion (Gifondorwa and Leise, 2006; Hens et al., 2006). 5-HT, catecholamines, and neuropeptides can all influence velar activity, altering ciliary beating or muscular contractions, implying that the apical ganglion may be active through a variety of neurotransmitters (Braubach et al., 2006).

Although *Nassarius obsoletus* is a well studied organism, the role of GABAergic neurons in metamorphosis is incompletely characterized. I proposed to use immunocytochemistry (ICC) to localize GABAergic neurons in this organism, to improve our understanding of how GABA exerts its larval and metamorphic effects. That exposure to GABA can inhibit the inductive actions of serotonin implies that the apical ganglion may contain receptors for GABA and, consequently, may be innervated by, or contain, GABAergic neurons. The presence of GABA in the mucus of adult

abalone and the discovery of a GABAergic chemoreceptor in the foot of larval abalone (Laimek et al., 2008; Stewart et al., 2011) suggest that the foot of *N. obsoletus*, a related gastropod, may also harbor GABAergic neurons. Although this finding was in a species of gastropod in which GABA acts as an inducer of metamorphosis (Laimek et al., 2008; Stewart et al., 2011), it would be premature to disregard the relevance of this finding for *N. obsoletus,* where GABA is inhibitory. The foot of most gastropods contains mucous glands and mucus secreting cells (Voltzow, 1994). The foot in *N. obsoletus* is like that of other gastropods and is comprised of an anterior propodium, a connecting mesopodium and a posterior metapodium (Fretter and Graham, 1962). The larval foot contains sensory neurons (Crisp, 1971, Thavaradhara and Leise, 2001) and may be active in detecting metamorphic inducers, as has been postulated for other related species (Arkett and Chia, 1989; Ruiz-Jones and Hadfield, 2011). Thus, the foot could be a site for GABAergic neuronal activity. Finally, the velar lobes show evidence of possible GABAergic control (Biscocho, 2013), as their loss is part of the metamorphic process. Thus, they can be expected to be innervated by GABAergic neurons (Biscocho, 2013). Localization of GABAergic neurons by ICC can provide a framework for understanding how GABA works within *N. obsoletus* to control the larval state prior to, and during metamorphosis.

CHAPTER II

HYPOTHESIS AND SPECIFIC AIMS

I tested the following hypothesis in my study: That GABAergic neurons occur in larval *Nassarius obsoletus* and that they are present in the apical ganglion, velar lobes, and the foot of *Nassarius obsoletus*, where they may play roles in pre-metamorphic or metamorphic control of the organism. Specifically, I tested these hypotheses by: Identifying and locating GABAergic neurons through immunocytochemistry and the CLARITY procedure and by localizing their somata to known ganglia of the CNS; describing their axonal branching patterns; and identifying target structures in competent larvae of *Nassarius obsoletus*.

A full accomplishment of these aims would characterize the GABAergic cellular populations and systems that occur in larval *Nassarius obsoletus*. These neuronal circuits appear to be required for metamorphosis and important in larval functions, thus providing impetus for further study of the relationship between GABA and metamorphosis.

CHAPTER III

MATERIALS AND METHODS

Larval Cultures

Adult *Nassarius obsoletus* were retrieved from the north side of the dock at the Center for Marine Science CREST Research Park at UNCW in Wilmington, NC and kept in laboratory aquaria from which egg capsules were collected. Larvae that developed from these egg capsules were placed in 1L beakers with 650 ml of Larval Seawater (1:1 natural sea water and 0.2µm filtered Instant Ocean) with an aeration device (Miller and Hadfield, 1986; Gharbiah et al, 2009). Each beaker contained approximately 750 larvae. Beakers were placed in a Precision Scientific Incubator (Model 818) at 26.0 ºC with a 12/12-hour light/dark period. Larval age for all experiments was determined as days after establishment of the culture. All larvae used for experiments were competent at approximately 7 days old.

Immunocytochemistry (ICC)

The basic ICC procedure is as follows: Competent larvae were decalcified overnight in low pH (6.0) MBL Artificial Seawater (Cavanaugh, 1956) to remove their shells and were then anesthetized by exposure to 7.5% $MgCl_2$ for 30 minutes. In experiments in which decapitations were conducted, a maximum of 150 larvae were

selected and the head and foot regions were cut away from the visceral mass. This step increased penetration of antibodies into key anterior ganglia in preliminary experiments. In whole-organism experiments, with approximately 600 larvae per experiment, this step was omitted. Specimens were then fixed in 100% methanol at room temperature for four hours, the fixation interval that best preserved tissue antigenicity in initial tests. The specimens were then washed four times, for 30 minutes each, in a solution of 24% sucrose in 80mM phosphate buffered saline (PBS). Specimens were transferred to small snap-cap vials and underwent four, 30-minute washes in 100mM PTA (0.1M PBS, 2% Triton-X, 0.1% Sodium Azide, all in dH_2O) to disrupt membranes and allow antibodies to penetrate into cells (Diaz-Rios et al., 1999; Dr. Shaun Cain, personal correspondence). Following this step, specimens were left overnight at 4ºC on a shaker in 100mM PTA.

After the overnight incubation in 100mM PTA, specimens in some experiments (Figures 10-13) were run through an ascending ethanol series (15%, 40%, 70%, 100%) for 10 minutes at each step, followed by 2-4 rinses in an organic solvent (chloroform, hexanes, or xylene) for 10 minutes at each rinse and then a descending ethanol series (100%, 70%, 40%, 15%) into 100mM PTA overnight to remove lipids and improve antibody penetration (Markham et al., 2006) before continuing to the next step. Specimens that did not undergo this dehydration process remained in 100mM PTA overnight, after which this solution was removed and specimens were immediately immersed in a blocking medium with 5 or 10% normal goat serum (NGS) in 100mM PTA for two hours at room temperature to reduce background immunoreactivity (IR). The primary antibody, an anti-GABA antibody raised in rabbits (Sigma A2052), was

diluted 1:100 in blocking medium. Specimens were incubated with the primary antibody for 48 hours. Excess primary antibodies were washed away by four, 30 minute washes in 100mM PTA. The secondary antibody, an AlexaFluor 555 goat anti-rabbit IgG (Invitrogen Cat. #A-21248), was diluted 1:1000 in the blocking medium and incubated with the specimens for 64.5 hours. After four, 30-minute washes in 100mM PTA to remove excess secondary antibody and four, 30-minute washes in 100mM PBS to remove the PTA, specimens were cleared in either 16.7% glycerol in 100mM PBS or in 100% methyl salicylate directly following an ascending ethanol series (15%, 40%, 70%, 100%) for 10 minutes per step (Diaz-Rios, et al., 1999).

Specimens were then placed on microscope slides coated with Sylgard (Dow Corning Corporation) in various orientations prior to confocal microscopy. Only a subset of specimens in each experiment underwent microscopy to determine representative results. Exemplary images are displayed in the figures (Figures 1-13) and estimates of the number of specimens viewed are provided in the figure legends as n=x.

Two control conditions were used to ensure relevant IR of GABAergic neurons: pre-absorbed primary antibody and primary antibody omission. To test for the specificity of the primary antibody, the pre-absorption control involved exposing the primary antibody, a rabbit anti-GABA, diluted 1:1000 in blocking medium to 1.26M GABA (a saturated solution) overnight. The solution was then divided into six Beckman-Coulter Thinwall UltraClear tubes (Item No. 344718) and the balanced tubes were placed in a Beckman-Coulter A-100/18 rotor (Item No. 347593) for centrifugation in a Beckman-Coulter Airfuge at 30 psi, 122,000xg, for 30 minutes. The supernatant was then added to

the specimens and ICC continued as previously described. Whole-mount specimens of this control were uniquely imaged using an OLYMPUS IX81 mounted camera and Microsuite Five software.

To test for the specificity of the secondary antibody to the primary antibody, specimens in this control group were exposed to the same quantity of the blocking medium vehicle, without the addition of the primary antibody. Specimens were incubated for 48 hours before undergoing four, 30 minute washes in 100mM PTA and subsequent exposure to the secondary antibody.

CLARITY

The CLARITY method was originally developed to readily identify structures in mammalian brains by polymerizing proteins to acrylamide, thereby allowing lipids to be removed from the intact brain. The organ is then sectioned and green fluorescent protein is usually expressed to visualize whole fibers and brain regions containing molecules of interest (Chung et al., 2013). I attempted to adapt this procedure for use with *N. obsoletus* and ICC to obtain more robust results with greater antibody penetration.

I initially used the CLARITY method on ganglia of the CNS dissected from adult *N. obsoletus*. In each of four experiments, 2 - 4 snail shells were cracked with a hammer and the snails removed from the shell remnants. The animals were then anesthetized in 7.5% $MgCl_2$ for 30 minutes. Afterwards, ganglia were removed under an Olympus SZ-40 dissecting microscope with dissecting scissors and watchmaker's forceps. The ganglia

were fixed, at first overnight, and in further experiments, for 3 days, at 4ºC in a mixture of 4% paraformaldehyde, 4% acrylamide, 0.05% bis-acrylamide, and 0.25% VA044 in PBS (Chung et al., 2013). The paraformaldehyde acted as the primary fixative agent, while the acrylamide cross-linked with cellular proteins. VA044 was the polymerization agent.

Specimens were heated at 37ºC for three hours for polymerization, then passively cleared for approximately 12 days in 4% sodium dodecyl sulfate in 200mM sodium borate buffer (pH 8.5), at 50ºC, and the clearing solution was changed daily (Chung et al., 2013). Remaining procedures were modified from Chung et al. (2013) as follows: Specimens were rinsed with 100mM PTA at 37ºC for 48-72 hours, followed by incubation in the blocking medium for 2 hours at room temperature as described above. The rest of the protocol followed the antibody incubation methods as described above, including relevant controls.

Confocal Microscopy

All experimental specimens and the control lacking the primary antibody were imaged under a confocal microscope. Confocal microscopy was conducted on an inverted Olympus IX81 laser scanning confocal microscope equipped with FLUOVIEW 500 software for imaging. A helium-neon (HeNe) laser excited the Alexa Fluor 555 tag at 555nm (Green) wavelength, which emits at a peak of 565nm (Orange-Red), detected by a photomultiplier tube (PMT). The Alexa Fluor 555 dye was selected for specimen

dyeing, and images were captured with a slow scan speed, an X4 Focus Mode, with a Line Kalman Filter. PMT settings were set between 370nm and 694nm, depending on the specimen. Gain was kept at 1x with Offset between 3-4% as necessary to reduce background fluorescence. Images were displayed as z-stacks with 5 to 20 μm slices to ensure that the stack contained approximately 20 images, or as selected slices from these stacks. Images were viewed in Adobe Photoshop CS4 and contrast was enhanced to ensure visualization of printed images.

CHAPTER IV

RESULTS

Immunocytochemistry (ICC)

Results of the pre-absorption control yielded specimens with minimal background IR after introduction of the secondary antibody. IR of axonal networks in the velar lobes and the foot was extinguished, although auto-fluorescent algae in the gut were visible (Figure 1A). Specificity of the AlexaFluor 555 goat anti-rabbit secondary antibody was also confirmed using a treatment without the primary antibody, in which specimens were exposed to the blocking medium alone (Figure 1B). These specimens also displayed little to no background IR at maximal PMT (=694nm) levels on the confocal microscope.

Representative results from three experiments that utilized whole organisms demonstrated GABA-like immunoreactivity (GABA-LIR) in axonal projections in the velar lobes and particularly in the posterior part of the foot (metapodium, Figure 2). In multiple specimens, nerves in the metapodium displayed consistent GABA-LIR (Figure 3). Furthermore, experiments with whole organisms demonstrated radiating axonal fibers in the velar lobes (Figure 3).

Larval decapitation was integrated into the experimental method to improve antibody penetration into the CNS of the head. IR of cell bodies was evident in the foot of such specimens (Figure 4). Further procedures with decapitated heads also revealed the existence of IR cell bodies throughout the foot, along with the previously described

nerves in the metapodium (Figure 5). More results from the same experiment indicated that GABA-LIR occurring throughout the foot could be segregated into a population of cells in the pedal ganglia in the propodium and a putative glandular population in the metapodium (Figure 6). Putative glandular cells may not be restricted to the metapodium; they may also occur in the propodium (Figure 7). Another experiment showed a vast network of GABA-LIR fibers occurring throughout the velar lobes and the head, reaching into the pedal ganglia (Figure 8). A series of images from a z-stack of a single specimen demonstrated the variability of GABA-LIR with depth within the specimen, supporting the idea that the metapodium contains GABAergic nerves that are surrounded on the z-axis with glandular cells (Figure 9). There is also IR in the pedal ganglia and a nerve arising from these ganglia.

To improve antibody penetration into the CNS, I utilized different organic solvents in an attempt to dissolve lipids from cellular membranes. The first process involved an alcohol dehydration to chloroform at room temperature (Figure 10). The results of this experiment included velar IR, but a loss of IR in the foot and no IR in the CNS. A second experiment utilized hexanes, instead of chloroform, at room temperature, which yielded no CNS or peripheral IR (Figure 11). A third experiment used hexanes, again instead of chloroform, heated to 60ºC. This yielded more promising results, with preserved velar GABA-LIR, GABA-LIR in the foot, as well as outlines of CNS ganglia, although with what appears to be leakage of IR from the cells (Figure 12). The final organic solvent used in a dehydration series was xylene. Results from this experiment

corroborated the presence of axons in the foot and the velar lobes, as well as cell bodies in the propodium (Figure 13). There was, however, no IR in the CNS (Figure 13).

CLARITY

The CLARITY procedure was conducted on 14 adult CNSs to develop a useful protocol in larger, more easily handled specimens. An example of the results from these experiments contains high background IR, probably arising from the presence of polymerized acrylamide gel and insufficient clearing (Figure 14). Though some cell bodies are present, the tissue is insufficiently transparent for good visualization of GABA-LIR. Furthermore, internal ganglionic tissue appeared to be insufficiently well fixed, leading to large areas devoid of IR where it was expected (Figure 14). Further experimentation with the CLARITY protocol was abandoned.

CHAPTER V

DISCUSSION

Immunocytochemistry (ICC)

Neuroanatomical data from the experiments described herein demonstrated the presence of GABA-LIR in the velar lobes, the foot, and possibly the apical ganglion of competent larvae of *N. obsoletus* (Figures 2-9). In the foot, IR appeared consistently in cell bodies of the pedal ganglia in the propodium, in a cluster of putative gland cells in the metapodium, and in bilateral nerves in the metapodium. IR in the apical ganglion occurred inconsistently. I conducted two controls which are typical for ICC, protocols conducted without the primary antibody and with the primary antibody preabsorbed to GABA (Figure 1). The results demonstrated little to no background IR, which indicated specificity for authentic GABA.

Multiple experiments demonstrated the presence of a network of GABAergic axons in the velar lobes (Figures 2, 3, and 8). Axons radiating throughout the velar lobes were consistently observed in multiple specimens. Previous work in the Leise laboratory suggests that bath application of GABA slows ciliary beat frequency in the velar lobes (Leise and Cahoon, 2012; Biscocho, 2013). The GABA-LIR in velar axons supports the suggestion that GABA may play a role in the motor control of the velum.

IR in the foot occurred consistently (Figures 2-7, 9, 10, 12, and 13) and appeared in cell bodies of the pedal ganglia and in epithelial cells of the foot. The GABA-LIR

occurring in neuronal somata of the pedal ganglia supports previous work that demonstrated the projection of the paired pedal ganglia into the propodium (Lin and Leise, 1996a) and provides evidence for a potential role for GABA in motor control of the foot.

The epithelium of the foot of most gastropods contains numerous mucus-secreting gland cells (Voltzow, 1994). GABA-LIR in these epithelial cells suggests that this neurotransmitter is secreted in the mucus along with the usual mucopolysaccharides. Some epitopes of these mucopolysaccharides may have been bound by my primary antibody, but previous research on the haliotids has demonstrated the presence of GABA in their mucous secretion (Laimek et al., 2008; Stewart et al., 2011). GABA in adult mucus secretion has been implicated in the recruitment of conspecific, competent larvae (Laimek et al., 2008, Rivera-Ingraham et al., 2015). Although GABA-LIR occurs in similar mucus gland cells in competent larvae of *N. obsoletus*, these animals are preparing for an adult benthic lifestyle. The foot is retained during metamorphosis and the presence of GABA in these putative gland cells may be part of the preparation for adult secretory activities.

The GABA-LIR in the metapodium also demonstrated the existence of branching nerves arising from the pedal ganglia (Figures 2, 3, 5, and 9). These nerves could innervate pedal musculature, and thus may play a role in larval settling behavior (Morse, 1979). Braubach et al. (2006) demonstrated that motor control in *N. obsoletus* is governed by several different neurotransmitters, including 5-HT, dopamine, and various neuropeptides. Bilateral serotonin-like immunoreactive fibers have been demonstrated in

the foot of the nudibranch *P. sibogae* as well (Croll, 2006). The nerve in the *N. obsoletus* metapodium may provide similar evidence for GABA's involvement in crawling behavior.

The unpaired apical ganglion, present only in the larval CNS, is thought to function in the sensorimotor control of the larval-specific velar lobes (Lin and Leise, 1996a; Marois and Carew, 1997). Whole organism neuroanatomical investigations of competent larvae of *N. obsoletus* failed to yield reproducible GABA-LIR in neuronal somata of the CNS, with the exception of the pedal ganglia. Axons of neurites in the velar lobes were unconnected to any somata in the CNS. This seeming discrepancy could be explained by at least two rationales. GABAergic vesicles may be rapidly transported out of the soma into distal neurites or the antibodies used may have been unable to penetrate the other non-pedal ganglia of the CNS. One way to locate GABAergic somata would be to conduct a similar ICC study but with antibodies to glutamic acid decarboxylase (GAD), GABA's generative enzyme (Ribak, et al., 1978). Decapitation of specimens and trials with four organic solvents elicited no additional results (Figures 4-13). Previous work with nitric oxide synthase (NOS) in molluscs has demonstrated that endogenous mechanisms exist for downregulation of neurotransmitter expression (Korneev et al., 1999). So a third possible explanation for the lack of GABA-LIR in somata of much of the CNS is that GABA production may have been downregulated as the animal becomes competent, which would allow the CNS to respond to metamorphic cues.

CLARITY

I utilized a slightly modified CLARITY procedure in an attempt to improve antibody penetrance into the larval CNS. CLARITY was developed to study neuroanatomy in mammalian brains through protein polymerization and lipid removal from an intact specimen (Chung et al., 2013). This was accomplished by incubating the neural tissue with a mixture of acrylamides, paraformaldehyde, and a polymerization agent, VA044 (Chung et al., 2013). Free radicals generated by VA044 promote covalent binding of amine groups in neural proteins and peptides to the acrylamide and paraformaldehyde (Chung et al., 2013). Thus, removal of the lipids leaves behind a transparent brain that could be subjected to ICC.

In conducting this procedure on *N. obsoletus*, I passively extracted the lipids by incubating the samples in a clearing buffer of 4% sodium dodecyl sulfate in 200mM sodium borate buffer (pH = 8.5), refreshing the solution daily for 12 days (Murray et al., 2014). This decreased the amount of unwanted debris relative to samples subjected to electrophoretic clearing. Perfusion of my samples with the gel solution was also omitted.

Results of my CLARITY experiments on adult CNSs demonstrated some IR within ganglionic cell bodies. However, this is largely lost in the background IR caused by incompletely cleared gel. The CLARITY procedure presents several problems for use with larval *N. obsoletus*. CLARITY works best on samples prepared with minimal excess gel around the tissue ("Hydrogel Embedding", no date), which is difficult to accomplish on adult ganglia and especially on small larvae. The gel auto-fluoresces and

disguises meaningful data (Figure 14). Furthermore, the time course for the CLARITY procedure is much longer than a traditional ICC protocol, requiring over one month versus 8 days for ICC (Katie Kaugars, personal communication). My 12-day clearing procedure was probably insufficiently long. The fixative may have been unable to penetrate deep into the adult ganglia (Figure 14B), or GABA may not have been cross-linked along with the larger proteins. No experiments were conducted on larvae as a result of these issues.

Conclusion

My experiments demonstrate the existence of GABA-LIR in the velar lobes and the foot of competent, larval *Nassarius obsoletus*. I also described the existence of a pair of posterior pedal nerves, originating in the pedal ganglia and possibly innervating the foot as a motor nerve. Putative glandular cells, larger than the ganglionic soma, contain GABA-LIR throughout the foot of competent larvae and may deposit GABA in their mucosal secretions. This set of cells is distinct from the population of cell bodies in the pedal ganglia. The velar lobes displayed radiating fibers that could also be involved in motor control, though this description is incomplete, as no corresponding cell bodies were located. This could be due to an actual lack of GABA in the neuronal somata, or lack of penetrance of antibodies into the CNS. But taken as a whole, my work provides evidence supporting the existence of endogenous GABA in the larvae of *Nassarius obsoletus* and provides ample avenues for future study that could further characterize the role of GABA in the biology and metamorphosis of marine gastropods.

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APPENDIX A

FIGURES

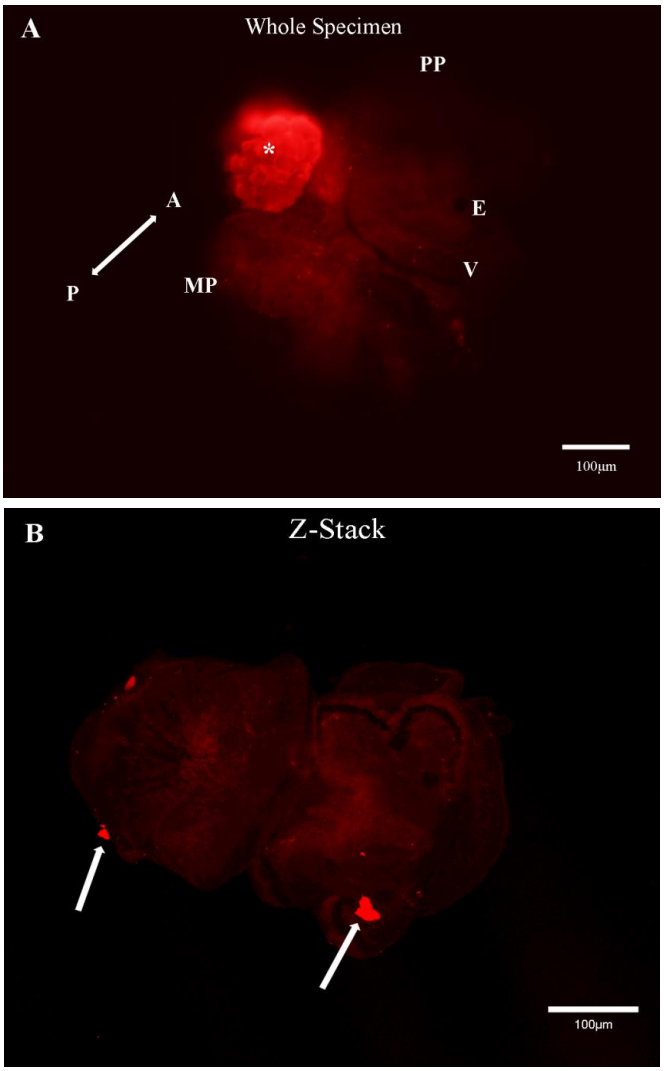

Figure 1. Whole Competent Larva Treated with Pre-Absorbed or No Primary Antibody Show Specificity of Antibodies. (A) A whole-mount specimen, representative of primary pre-absorption controls, shows lack of GABA-like immunoreactivity (GABA-LIR), providing evidence for specificity of the primary antibody for authentic GABA. Autofluorescent algae are visible in the gut (*). 27 specimens were viewed (n=27). (B) A z-stack of a specimen lacking exposure to the primary antibody shows no significant immunoreactivity (IR), displaying the specificity of the goat anti-rabbit secondary antibody used to visualize primary anti-GABA-LIR. Autofluorescent debris (arrows) is present; n=9. Double headed arrow in (A) indicates anterior to posterior axis. PP, propodium; MP, metapodium; E, eyespots; V, velar lobes. Images have been artificially brightened in Adobe Photoshop CS4 to better visualize larva in print. x150.

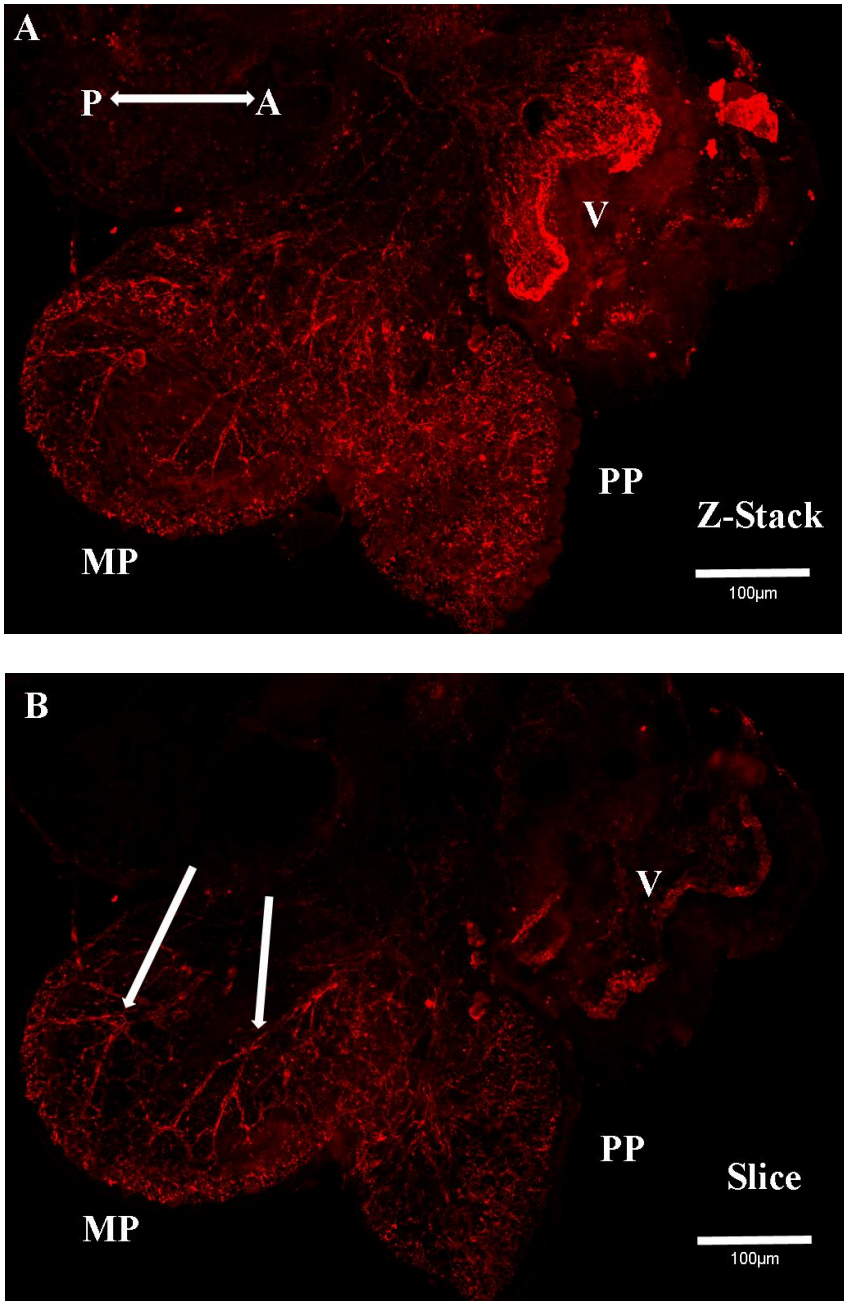

Figure 2. Whole Organism Displays GABA-LIR in Axonal Projections. (A) A z-stack composite image of a whole larva shows IR throughout the foot (MP, metapodium; PP, propodium) and velar lobes (V). (B) A single section from this z-stack shows commonly occurring nerves in the metapodium of the specimen (arrows) with conserved branching patterns. Double headed arrow in (A) indicates anterior to posterior axis. PP, propodium; n=18. x160.

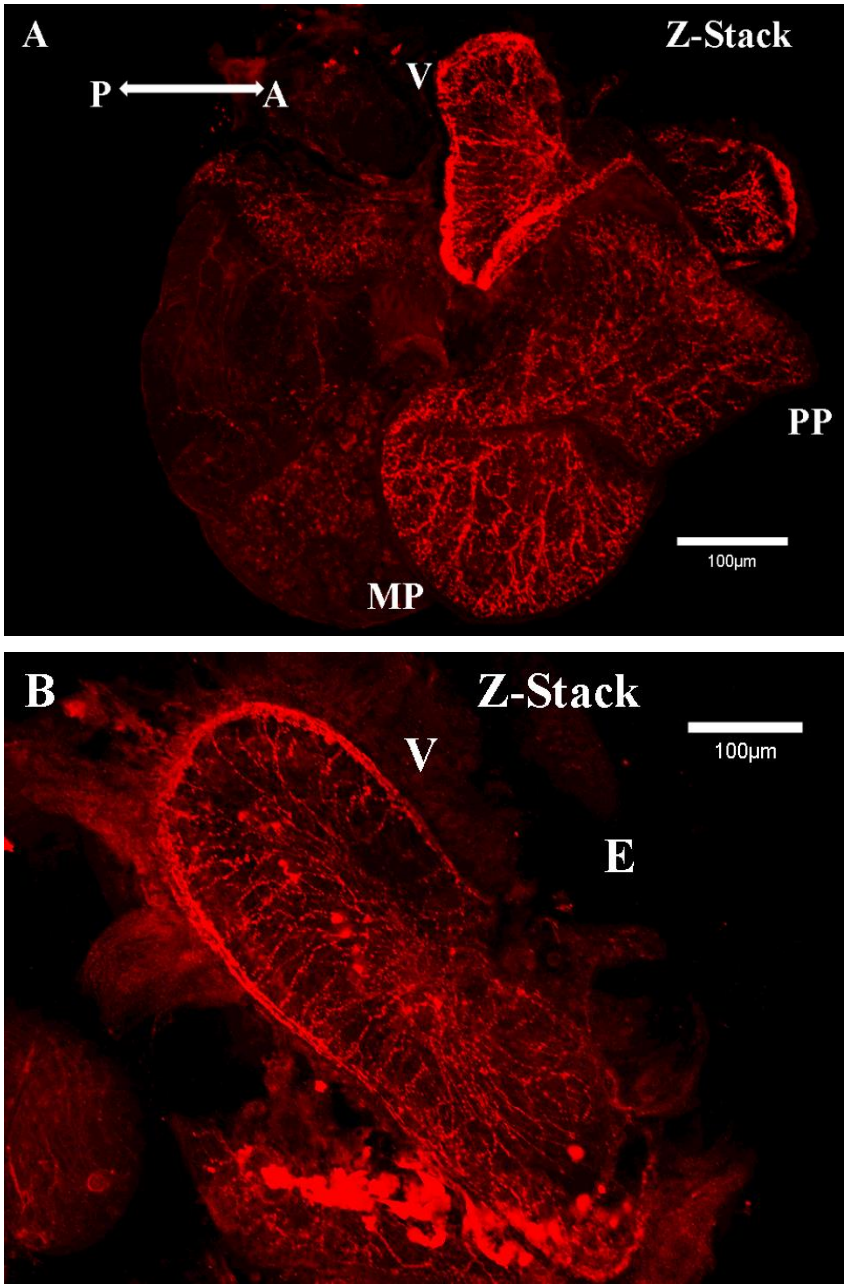

Figure 3. GABA-LIR Axons Radiate Throughout the Velum and Foot. (A) A z-stack composite image of a whole larva revealed GABA-LIR occurring throughout the foot and velar lobes (V) with branching in the metapodium (MP). (B) A velar lobe contains axons with GABA-LIR that radiate towards the ciliated bands on outer edge. Double headed arrow in (A) indicates anterior to posterior axis. E, eyespot; PP, propodium; n=18. x160.

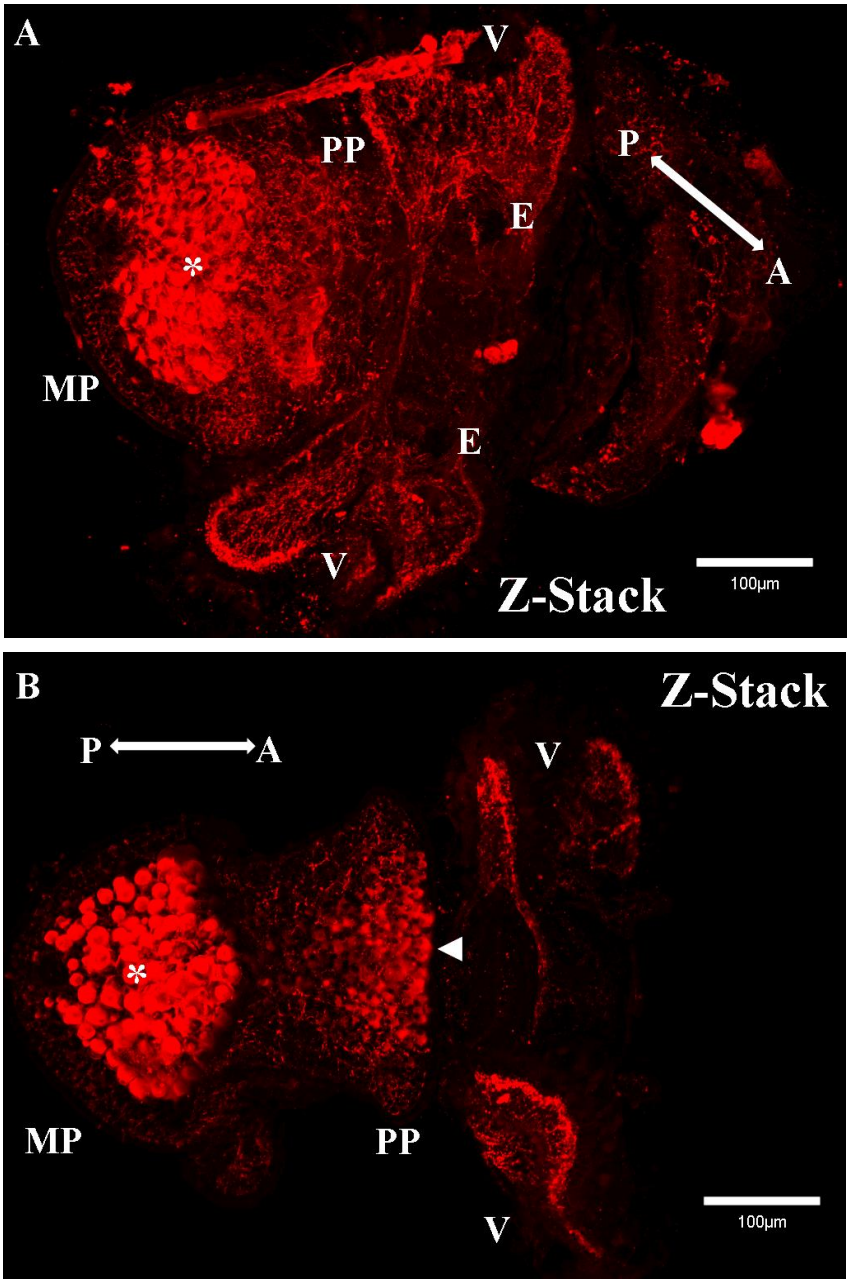

Figure 4. GABA-LIR in Cell Bodies of the Foot in Two Specimens. (A) A z-stack composite image of a decapitated larva shows IR in cell bodies (*) in the metapodium (MP). IR in velar lobe (V) axons is also visible. (B) Two seemingly distinct populations of GABA-LIR cells occur in the propodium (PP) (arrowhead) and metapodium (*). Cells in the metapodium are larger than those in the propodium. Double headed arrows indicates anterior to posterior axis. E, eyespot; n=30. x160.

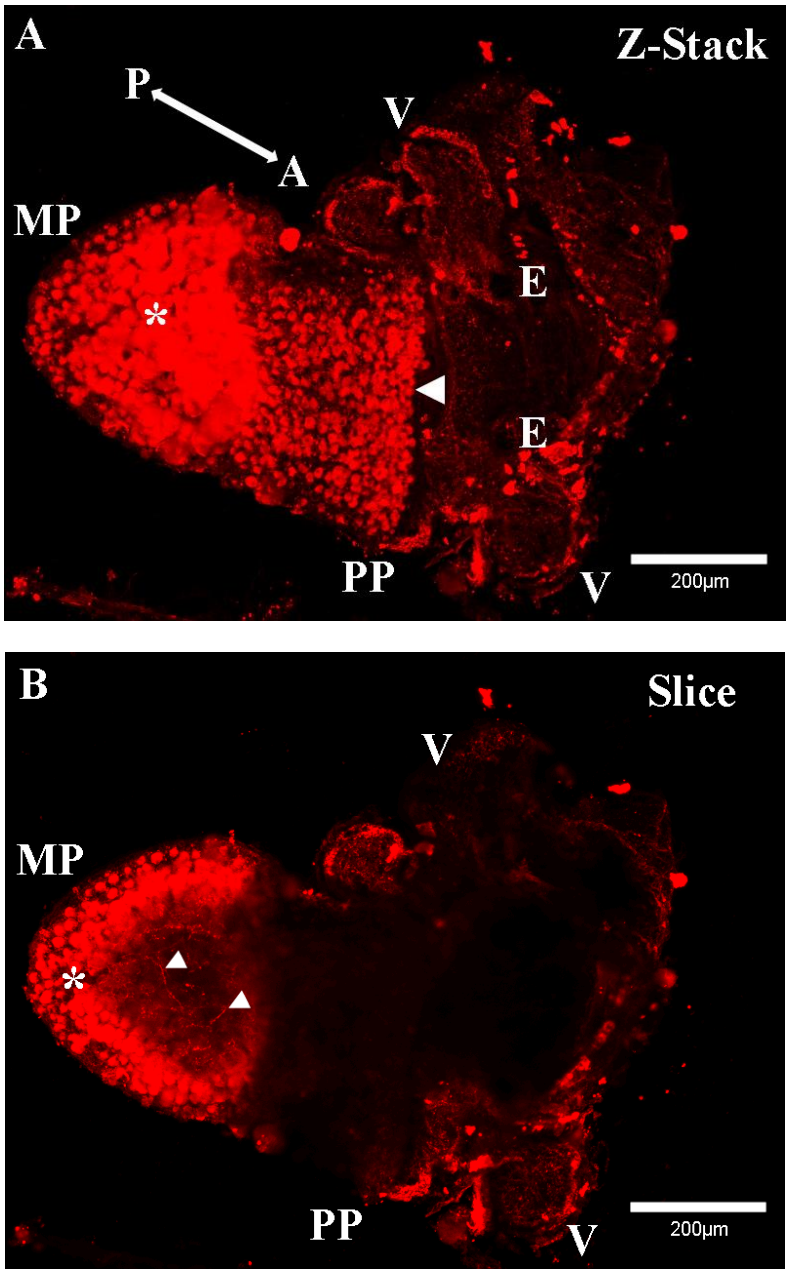

Figure 5. Pedal Nerves Visible in a Slice from a Z-Stack. (A) Larger cells occur throughout the metapodium (MP,*) than in the propodium (PP, arrowhead) of the specimen. Pale IR in velar lobe (V) axons is visible. (B) A slice from the z-stack (in A) of a competent larva shows GABA-LIR of bilateral pedal nerves (arrowheads) in the metapodium, surrounded by GABA-LIR cells (*). Double headed arrow in (A) indicates anterior to posterior axis. E, eyespot; n=30. x97.5.

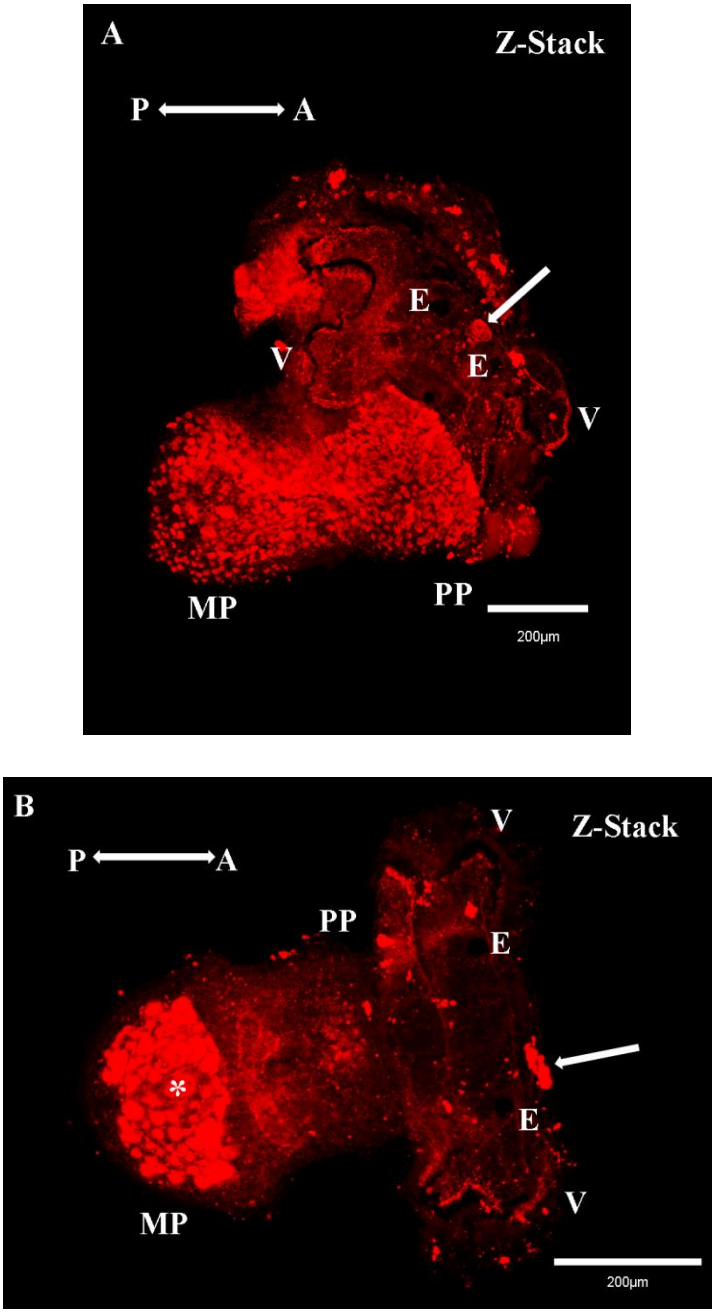

Figure 6. Distinct Populations of Pedal Cells. (A) Cells occur throughout the foot of the specimen and lack clearly defined populations. IR in putative cells of the apical ganglion (arrow) is present between the eyespots (E). x70. (B) A second specimen from the same experiment shows IR putatively in the apical ganglion (arrow) along with GABA-LIR in the propodium (PP) and putative glandular cells in the metapodium (MP, *). IR in the pedal ganglia occurs anterior to the metapodium. Double headed arrow indicates anterior to posterior axis. V, velar lobes; n=30. x90.

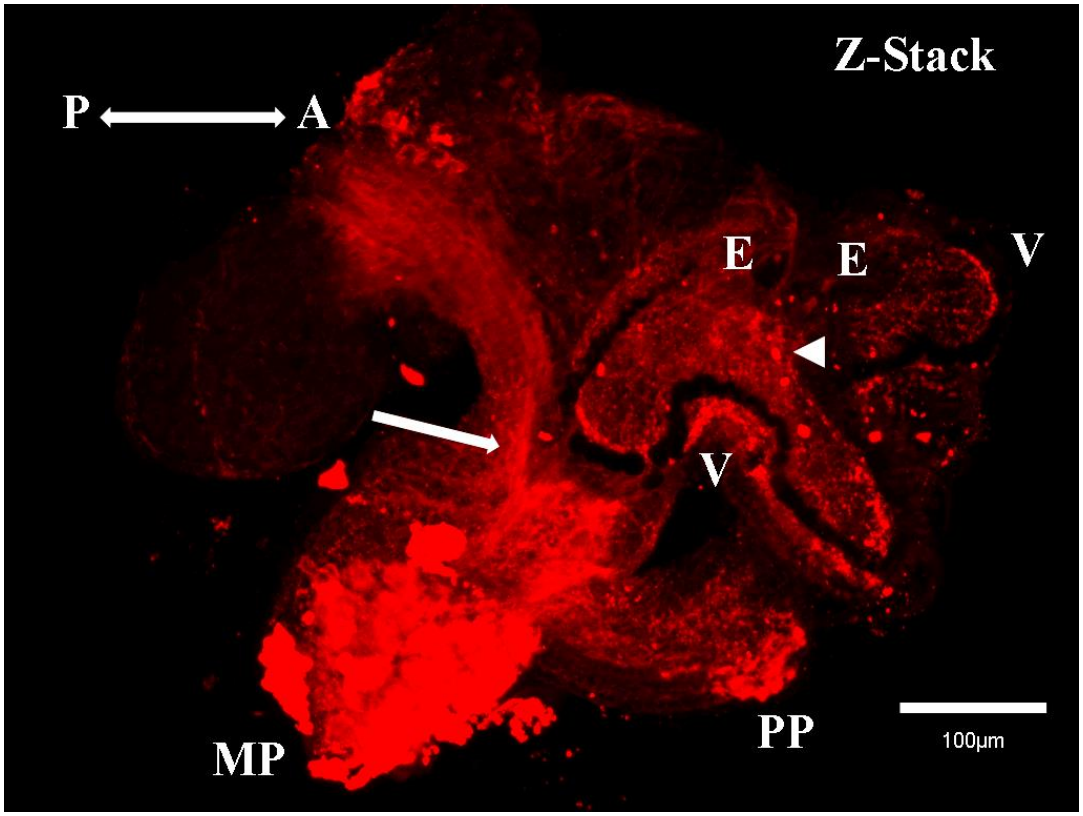

Figure 7. Distinct Populations of GABA-LIR Cells in the Foot. GABA-LIR occurs in the foot in distinct propodial (PP) and metapodial (MP) populations. Velar lobes (V) show radiating axons and possible CNS IR (arrowhead) below the eyespots (E). IR of the remaining visceral mass of this head-foot preparation can be seen extending from the middle of the foot towards the top of the image (arrow). Double headed arrow indicates anterior to posterior axis. n=30. x200.

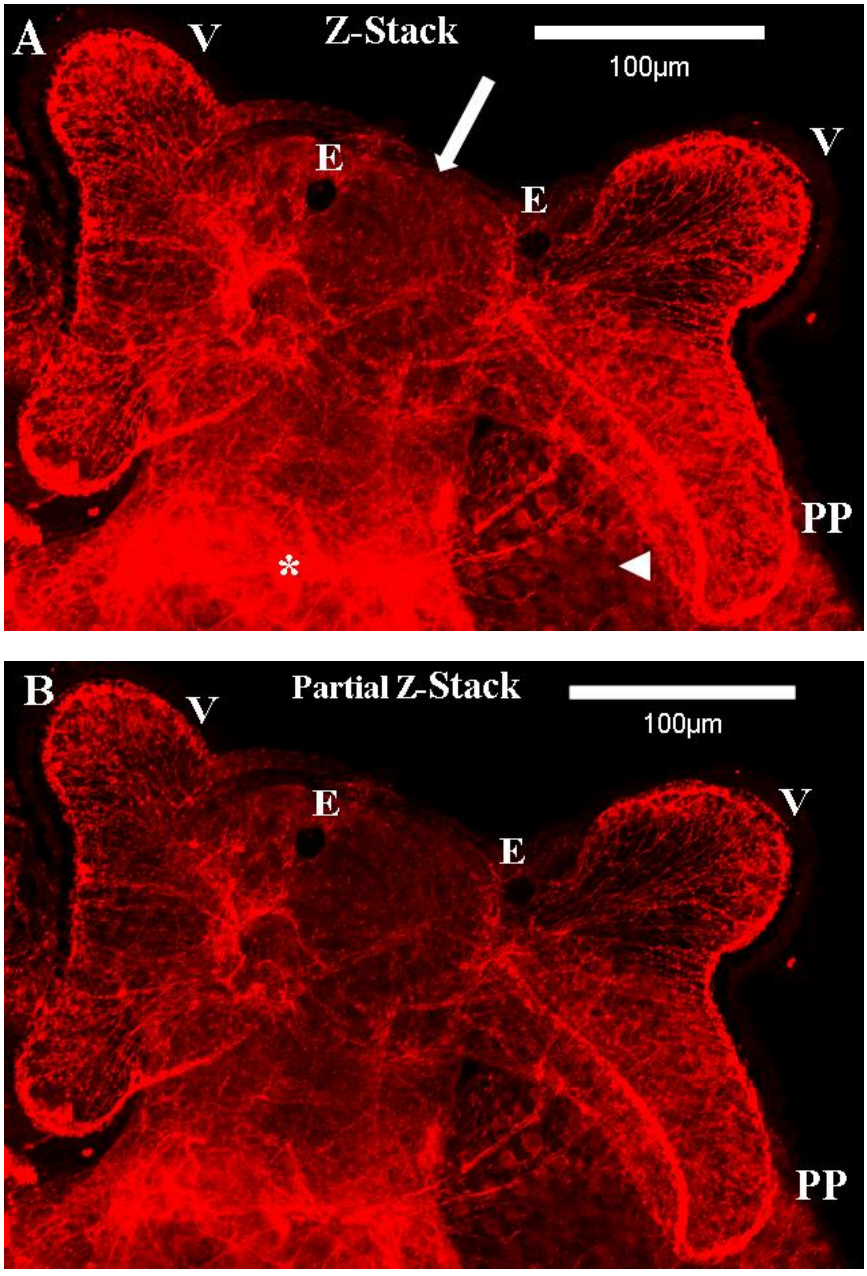

Figure 8. An Axonal Network Occurs Throughout the Larval Head. (A) Axons are visible radiating throughout the velar lobes (V) and into the head. There is also strong GABA-LIR in the pedal ganglia (*). Region of the apical ganglion shows a lack of IR (arrow). The propodium (PP) shows faint GABA-LIR (arrowhead). (B) An abbreviated z-stack of the first image reduces background and elucidates velar fibers, emphasizing the axonal network with no discernable cellular origin. E, eyespot; n=20. x290.

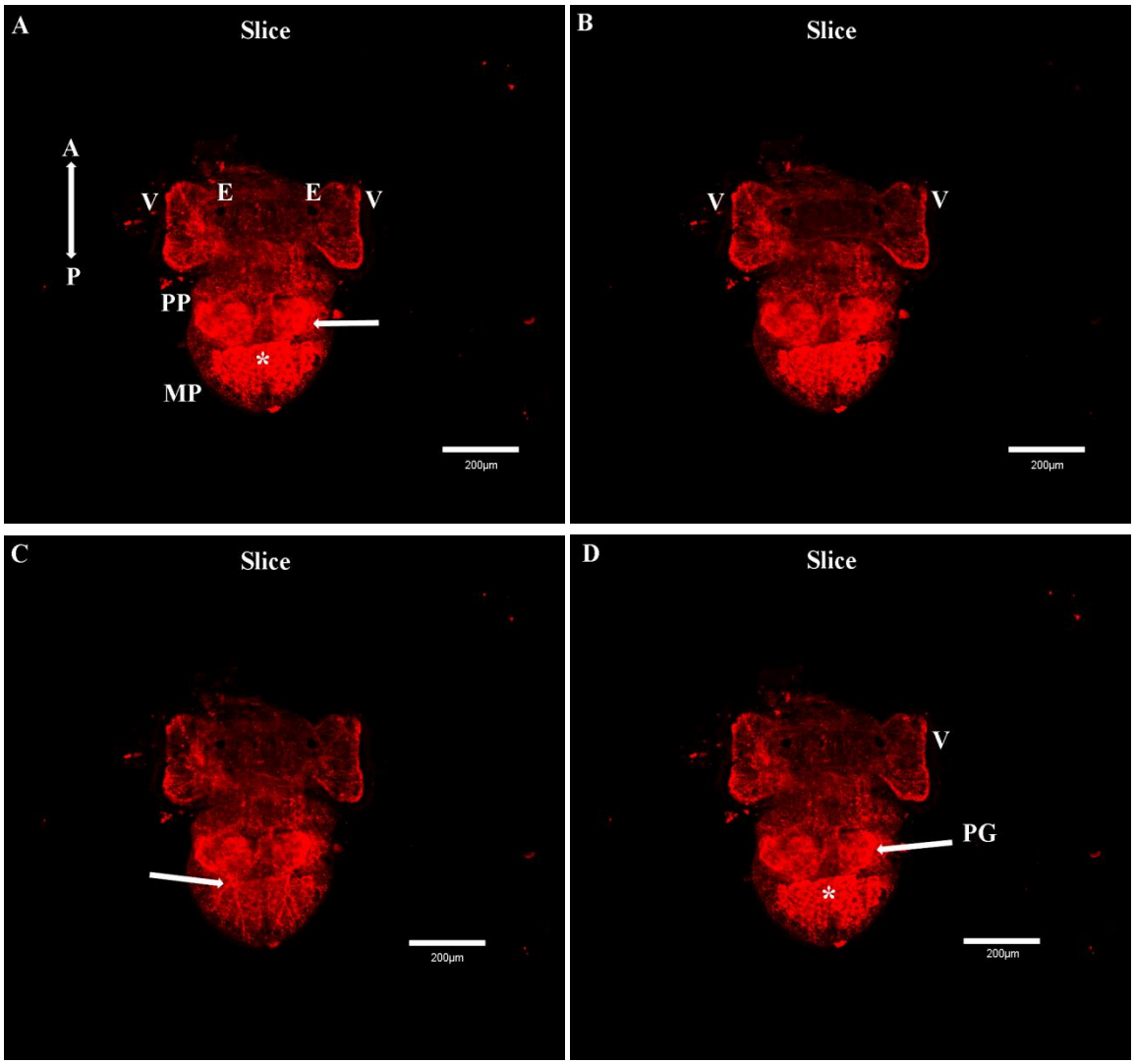

Figure 9. Sections From a Z-stack allow Visualization of the Nervous System Throughout One Specimen. (A) GABA-LIR occurs in the foot, in the pedal ganglia of the propodium (arrow, PP) and glandular regions (*) of the metapodium (MP). Velar lobes (V) contain radiating fibers. (B) Fibers in the velar lobes (V) are discernable in a deeper section. (C) Typically conserved pedal nerves extend from the pedal ganglia towards the metapodium (arrow). (D) Deeper into the organism, the pedal fibers are obscured by gland cells (*). Pedal ganglia (PG) and velar IR is still visible. Double headed arrow in (A) indicates anterior to posterior axis. E, eyespots; n=12. x50.

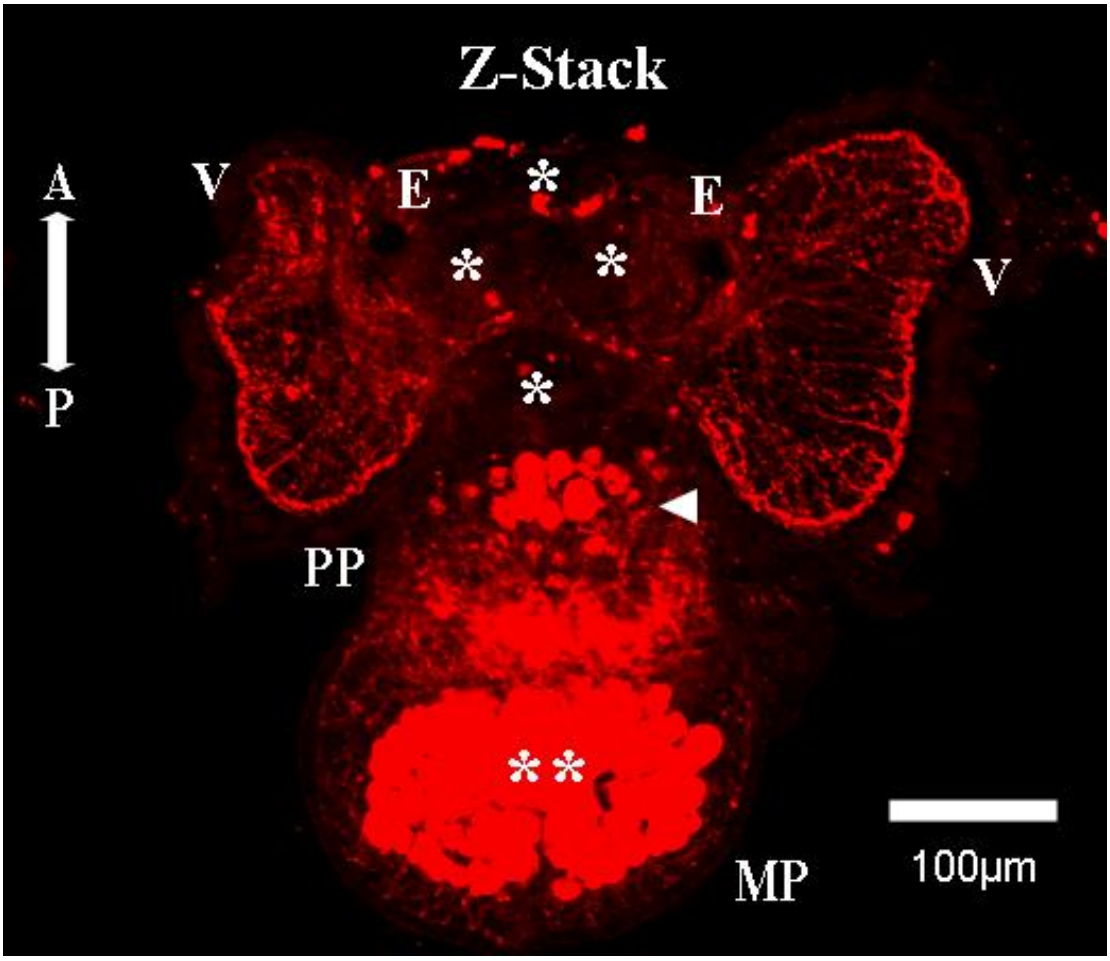

Figure 10. Chloroform Dehydration of Larval *N. obsoletus*. Chloroform dehydration preserves radiating axons in the velar lobes (V), and displays more intense GABA-LIR in the foot. Some details in the foot are lost in the bright glandular IR of the metapodium (MP, **), and although distinct cells occur in the propodium (arrowhead), no cell bodies are evident in the more anterodorsal parts of the CNS (*). Double headed arrow indicates anterior to posterior axis. E, eyespot; n=9. x215.

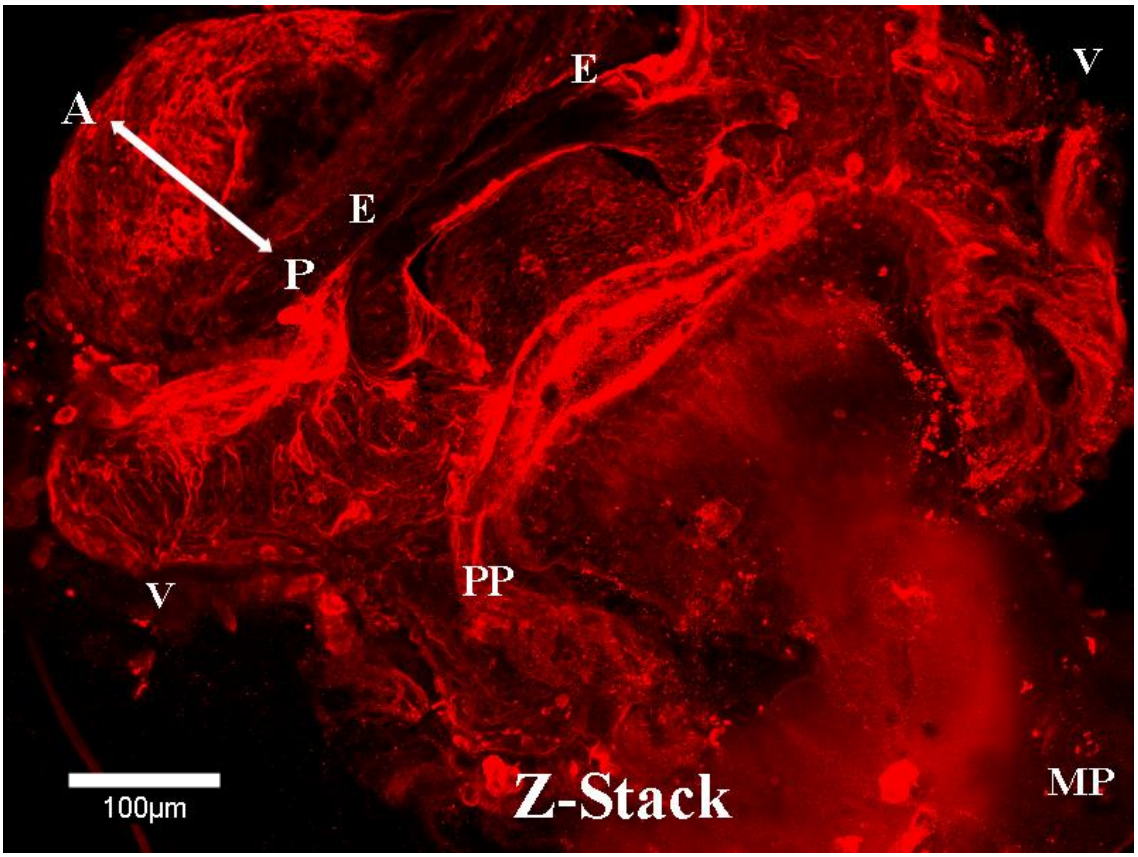

Figure 11. Hexane Dehydration of Larval *N. obsoletus*. Hexane dehydration yielded minimal GABA-LIR results, with many hallmark features of the nervous system missing, including the radiating fibers in the velar lobes (V) and cell populations in the propodium (PP) and metapodium (MP). No GABA-LIR is evident in the CNS. Double headed arrow indicates anterior to posterior axis. E, eyespot; n=9. x210.

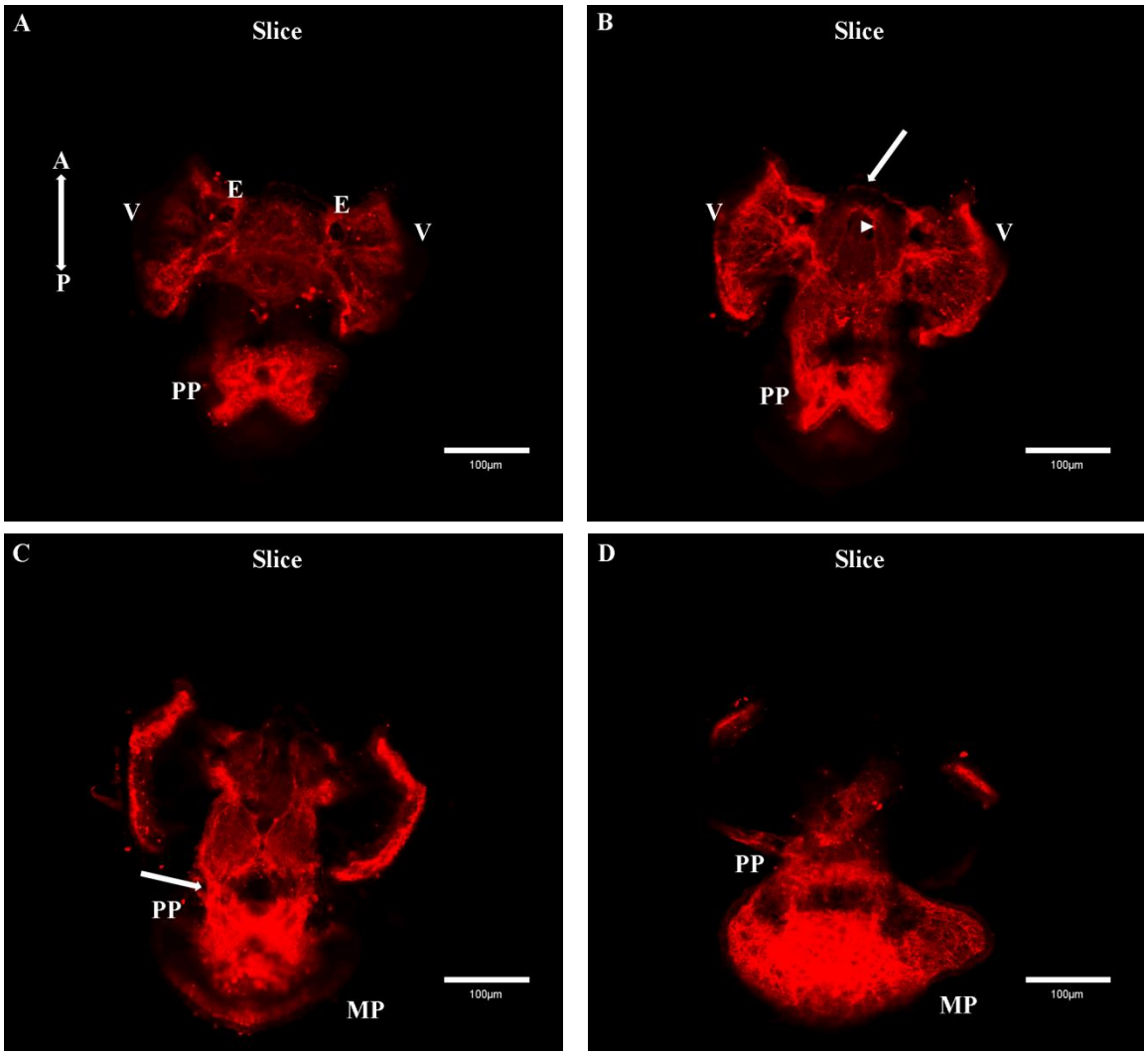

Figure 12. A Z-stack of a Specimen Following Heated Hexane Dehydration. All images are slices from one z-stack composite of a single specimen after hexane dehydration at 60ºC. (A) Anterior-most image shows GABA-LIR in the propodium (PP), with radiating axons throughout velar lobes (V). (B) Deeper section shows radiating velar fibers but only an outline of the CNS (arrowhead). Lack of IR in the apical ganglion is apparent (arrow). (C) GABA-LIR in pedal ganglia. GABAergic nerves descend into the propodium (arrow). (D) Metapodium (MP) shows diffuse IR, indicating ruptured cells and poor quality of heated hexane dehydration. Double headed arrow in (A) indicates anterior to posterior axis. E, eyespot; n=18. x110.

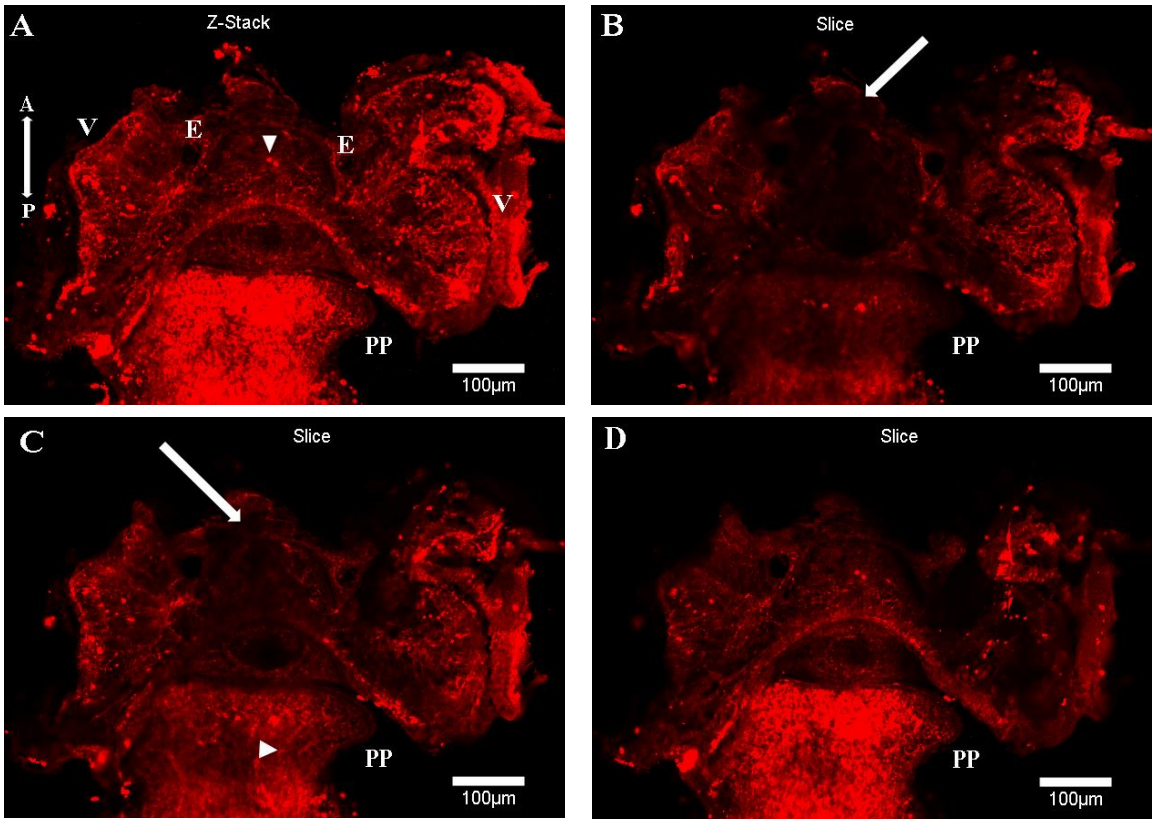

Figure 13. Z-stack Series of a Specimen Following Xylene Dehydration. Images obtained from a z-stack composite of a single specimen after xylene dehydration. (A) Full z-stack shows GABA-LIR in propodium (PP), some IR across the velar lobes (V) and possible IR in the CNS (arrowhead). (B) and (C) show, in individual slices, that the CNS, particularly the region of the apical ganglion (arrows), is not IR. Some axons can be seen in the propodium in (arrowhead, C). (D) IR cell bodies in the propodium corroborate earlier findings of distinct populations. Double headed arrow in (A) indicates anterior to posterior axis. E, eyespot; n=12. x90.

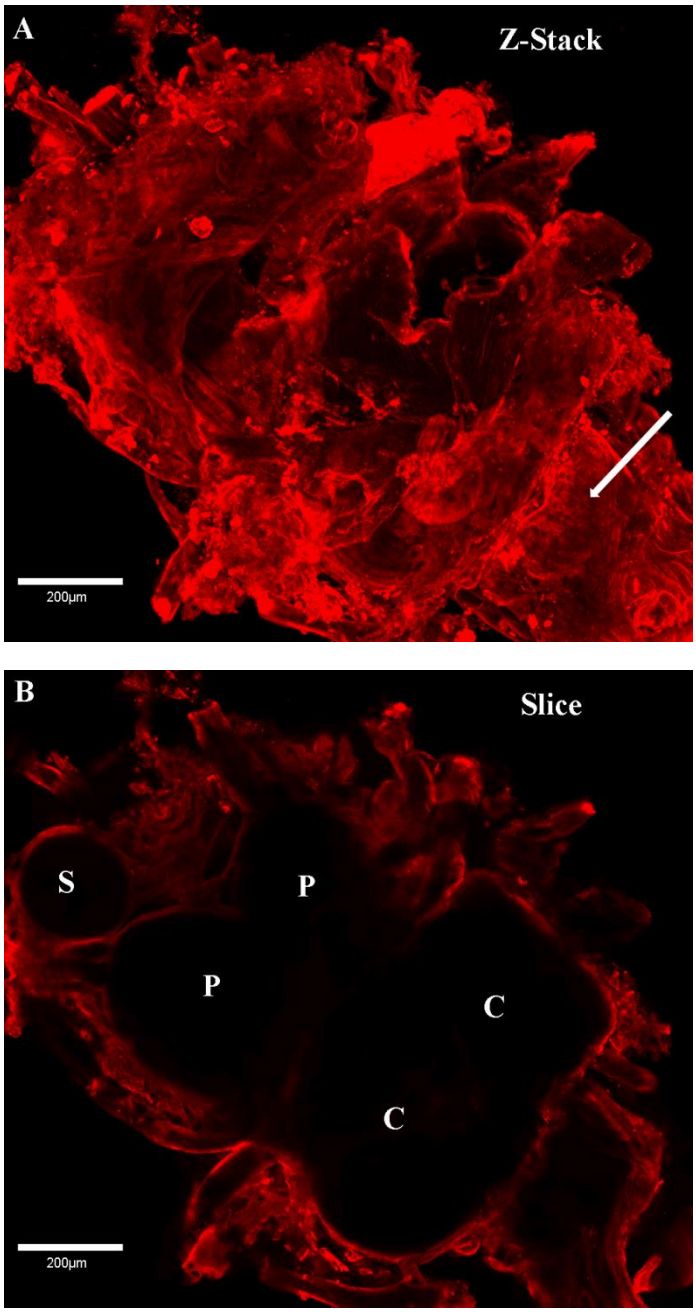

Figure 14**.** Adult CNS in a Specimen Processed by the CLARITY Protocol. (A) In a Z-stack composite of a specimen, individual cells are rarely visible (arrow). Most fluorescence is due to improperly cleared gel that interferes with typical IR. (B) A slice from the z-stack shows lack of IR in the middle of the cerebral (C) and pedal ganglia (P). S, statocyst; n=8. x70.