Reference: *Biol. Bull.* **213:** 43–54. (August 2007)

Serotonin-like Immunoreactivity in the Central and Peripheral Nervous Systems of the Interstitial Acochlidean *Asperspina* sp. (Opisthobranchia)

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Abstract. Species of Acochlidea are common members of the marine interstitial environment and defined in part by their minuscule size and highly divergent morphology relative to other benthic opisthobranchs. Despite these differences, acochlideans such as species of *Asperspina* display many plesiomorphic characteristics, including an unfused condition of their neural ganglia. To gain insight into the distribution of specific neural subsets within acochlidean ganglia, a species of *Asperspina* was studied by using anti-serotonin immunohistochemistry and epifluorescence and confocal laser scanning microscopy. Results reveal similarities between *Asperspina* and larger opisthobranchs in the general distribution of serotonergic perikarya in the central nervous system. Specifically, the arrangement of perikarya into regional clusters within the cerebral and pedal ganglia and the absence of immunoreactive perikarya in the pleural ganglia are similar to the model species of *Aplysia californica, Pleurobranchaea californica,* and *Tritonia diomedea.* Moreover, serotonergic innervation of the rhinophores in all opisthobranchs, including *Asperspina* sp., originates from the cerebral ganglion instead of directly from the rhinophoral ganglion. Serotonergic innervation of the body wall, including the epithelium, muscles, and pedal sole, appears to arise exclusively from pedal and accessory ganglia. These observations indicate a general conservation of serotonin-like immunoreactivity in the central and peripheral nervous systems of acochlidean and other benthic opisthobranchs.

Received 9 September 2006; accepted 15 March 2007.
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Abbreviation: 5-HT-LIR, serotonin-like immunoreactivity.

Introduction

Microscopic gastropods of the marine interstitial environment have long fascinated malacologists and meiobenthologists alike, revealing a wealth of unusual species with unique biological adaptations distinct from other benthic molluscs (Swedmark, 1964, 1968). The late nineteenth and early twentieth centuries were especially important for the rise of systematic studies on meiobenthic molluscs, when numerous species of interstitial prosobranchs, opisthobranchs, and aplacophorans were described (*e.g.,* Kowalevsky and Marion, 1887; Kowalevsky, 1901a, b; Hertling, 1930; Odhner, 1937). By the middle of the twentieth century, attention turned to studies of ecology and evolution with a focus on the distribution, interactions, and adaptations of meiobenthic species, perhaps best summarized by Swedmark (1964, 1968). Swedmark (1968) noted the success of opisthobranchs in colonizing the interstitial environment and the abundance of morphological adaptations they possessed to this three-dimensional and space-restricted world. Specifically, interstitial opisthobranchs are renowned for their elongate bodies, capacity for contraction, adhesive ability, spicule-reinforced body wall, and simple internal anatomy. The simple anatomy of many interstitial species, hypothesized to be the result of regressive evolution, or pedomorphosis, make these opisthobranchs especially difficult to classify and therefore similar in many respects to other interstitial and meiobenthic animals with equally elusive origins (Swedmark, 1968). Swedmark emphasized the importance of developing novel methods to probe the anatomy of these microscopic molluscs, without which our understanding of even their basic anatomy would remain incomplete.

Recent investigations of interstitial opisthobranchs have

shown the importance of providing a detailed three-dimensional representation of anatomical structure, which allows for a better assessment of organ system homology and function (Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2006). As stated in both studies, previous investigations of opisthobranch anatomy have relied almost exclusively on traditional histological techniques and in some cases missed or misrepresented important details because of the miniscule size of the organ systems and the lack of fine structural information obtainable from paraffin sections. For these reasons, modern studies have relied on a combination of semithin and ultrathin resin sections, electron microscopy, and/or computer-based reconstruction of the major organ systems (Fahrner and Haszprunar, 2002; Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2006). To date, two species of the order Acochlidia, *Hedylopsis ballantinei* Sommerfeldt and Schrödl, 2005, and *Microhedyle remanei* (Marcus, 1953), have been examined using some combination of these techniques, revealing a wealth of new information on the structure of the digestive tract, reproductive system, and central nervous system. The structure of the nervous system is especially important because of its conservative nature in higher molluscan taxa (*e.g.,* Bargmann, 1930; Bishop, 1978; Haszprunar and Huber, 1990), and among acochlidean opisthobranchs may provide clues to their origins and phylogenetic status (Neusser *et al.,* 2006). To date, there is little evidence to suggest monophyly of the Acochlidea, as many of the hypothesized synapomorphies, such as the absence of a shell and the production of spicules, may be viewed as convergent adaptations to an interstitial lifestyle (reviewed by Swedmark, 1968). This view is reinforced by molecular sequence analysis, where neither complete 18S rRNA nor partial 28S rRNA provide robust support for monophyly (Vonnemann *et al.,* 2005), although monophyly does receive some support from a combined 18S/28S rRNA character set and is still considered plausible on morphological grounds (see Sommerfeldt and Schrödl, 2005; Wägele and Klussmann-Kolb, 2005). Still, the basal position of this unique assemblage within the Opisthobranchia has important implications for defining evolutionary trends and understanding the phylogeny of higher opisthobranch clades (Sommerfeldt and Schrödl, 2005).

In their study of opisthobranch relationships, Vonnemann *et al.* (2005) state that monophyly of Acochlidia—that is, monophyly receiving bootstrap support $> 50\%$—is dependent on the exclusion of *Hedylopsis spiculifera* (Kowalevsky, 1901), a member of the Hedylopsidae and a well-recognized taxon of the interstitial meiobenthos (Swedmark, 1968). Fahrner and Haszprunar (2002) consider the Hedylopsidae a basal taxon within the Acochlidia, and indeed, several species of *Hedylopsis* possess characters considered plesiomorphic within the Opisthobranchia, including a mantle cavity, two pairs of well-developed cephalic tentacles, and a prepharyngeal, epiathroid central nervous system with separate (unfused) ganglia, among other characters (see Sommerfeldt and Schrödl, 2005). However, few species of *Hedylopsis* or any other acochlidean opisthobranchs have been examined in detail, and with the exception of *H. spiculifera* (Wawra, 1989), *H. ballantinei* (Sommerfeldt and Schrödl, 2005), and *M. remanei* (Neusser *et al.,* 2006), little information exists on organizational or structural variation of the major organ systems, especially the nervous system. In particular, virtually nothing is known about the chemistry of the acochlidean nervous system, despite the importance of neurotransmitters in defining neural homology (Granzow and Rowell, 1981; Croll, 1987), identifying specific neural subsets (Moroz *et al.,* 1997; Sudlow *et al.,* 1998), and understanding behavioral patterns (Palovick *et al.,* 1982; McClellan *et al.,* 1994). Sommerfeldt and Schrödl (2005) report that the neuropeptide FMRFamide is present in the major ganglia of *H. balantinei,* but they do not present any additional data on its distribution.

The present study seeks to contribute additional information on the nervous system of acochlidean opisthobranchs through immunolocalization of serotonin (5-HT, 5-hydroxytryptamine) in a species of *Asperspina.* Specifically, the distribution of serotonin immunoreactivity in the major ganglia of the CNS and some aspects of serotonergic innervation of peripheral tissues are presented here using immunohistochemistry and confocal and epifluorescence microscopy. The objective of this contribution is to provide the first details on the serotonergic nervous system of an acochlidean opisthobranch and search for similarities in neural organization between *Asperspina* and the larger benthic opisthobranchs.

Materials and Methods

Specimen collection

Specimens of *Asperspina* were collected from a sandy shoal about 3 miles offshore of Fort Pierce, Florida (27°26.52′N, 80°13.81′W) using the Smithsonian Marine Station's R/V *Sunburst* boat and a Van Veen grab. About 6 liters of sediment were collected from a depth of about 3 m in June 2005 and August 2006. Molluscs were removed from the sediment by using a decantation technique with 7% $MgCl_2$. Specimens of *Asperspina* were photographed alive on a Wild trinocular dissecting microscope with a Nikon Coolpix 8800 digital camera.

Microscopy and immunohistochemistry

Several specimens were processed for scanning electron microscopy with the following protocol: primary fixation for 2 h in 5% formalin in 0.1 mol l^{-1} phosphate buffer saline (PBS), a rinse in 0.1 mol l^{-1} PBS for 1 h, secondary fixation in 1% OsO_4 in 0.1 mol l^{-1} PBS for 1 h, and a final rinse in 0.1 mol l^{-1} PBS for 1 h. Dehydration proceeded

through an ethanol series (50%–100%) at 10 min per solution. Specimens were critical-point-dried with a Tousimis Samdri 790, sputter-coated with gold in a Technics Hummer II, and viewed on a JEOL 6400 scanning electron microscope.

For immunohistochemistry, 13 specimens were anesthetized and fixed in 4% paraformaldehye in 0.1 mol l^{-1} PBS for 24–72 h at 4 °C. Different time periods of fixation were chosen to test their effects on immunoreactivity. Specimens were rinsed three times over the course of 48 h in 0.1 mol l^{-1} PBS and then placed in IT Signal Enhancer (Invitrogen) for 1–2 h. IT Signal Enhancer prevents nonspecific staining that is normally blocked with bovine serum albumin or serum. Specimens were transferred to a primary antibody solution of rabbit anti-serotonin antibody (Sigma-Aldrich Corp.), diluted 1:200 in PBS with 0.5% Triton X-100 (PBT) in 1.5-ml centrifuge tubes at 4 °C on an orbital shaker for 48 h. Two specimens were used as controls and omitted from the primary antibody. Specimens were next rinsed three times in 0.1 mol l^{-1} PBS over 24 h and transferred to a solution of goat anti-rabbit Alexa Fluor 546 antibody (Sigma-Aldrich Corp.), diluted 1:200 in PBS with 0.5% Triton X-100 (PBT) in 1.5-ml centrifuge tubes, and placed in the dark at 4 °C and on an orbital shaker for 36 h. Specimens were then rinsed in 0.1 mol l^{-1} PBT for 24 h, stained in Alexa-Fluor 488 phalloidin (Invitrogen) for 1 h, and mounted in Fluormount G (Electron Microscopy Sciences) on glass slides. All slides were kept at 4 °C for 24 h prior to examination. All specimens retained their immunoreactivity even after several weeks of storage at 4 °C.

Specimens were examined on two microscopes: a Zeiss Axioimager M1 equipped with epifluorescence, digital Axiocam, and Zeiss software at the University of Massachusetts Lowell; and a confocal laser scanning microscope constructed of a Nikon Eclipse E800 compound microscope and a Biorad Radiance 2000 laser system at the Smithsonian Marine Station in Fort Pierce, Florida. Lasersharp 2000 ver. 4.0 software was used to collect a series of 0.1-μm optical sections with maximum intensity projection along the *z*-axis. Confocal images were imported into Confocal Assistant ver. 4.02 and converted into TIF files. Additional digital files were imported into Volocity ver. 4.0 (Improvision) to render 3-D images and create X-Y-Z rotations (TIF, AVI files). No manipulations of the original images were made other than changes of color (false color or grayscale) or cropping. The program Carnoy V 2.0 (© 2001 Peter Schols) was used to measure neurons in some digital images.

Results

General description

All specimens fit the taxonomic definition of the monotypic genus *Asperspina* as described by Rankin (1979). Crawling specimens are up to 2 mm long, while anesthetized specimens are 780 μm to 1.1 mm long. The body is divided into an anterior head-foot complex and posterior visceral sac (Figs. 1, 2). The head-foot complex constitutes roughly 35% of the total body length in both living and fixed specimens (Fig. 1). Total body coloration is white-pink, with the head-foot complex noticeably more translucent then the visceral sac (Fig. 1B). Small pink glands line the entire animal, and small patches of cilia cover the head-foot complex (Fig. 2). Pigmented eyes are absent. Rhinophores and oral tentacles are present anteriorly (Figs. 1, 2). The rhinophores are slightly longer (70–78 μm) than the more ventrally placed oral tentacles (48–56 μm). Observations of the radula are incomplete, but there appear to be 40–41 radular tooth rows. A ciliated sperm groove is

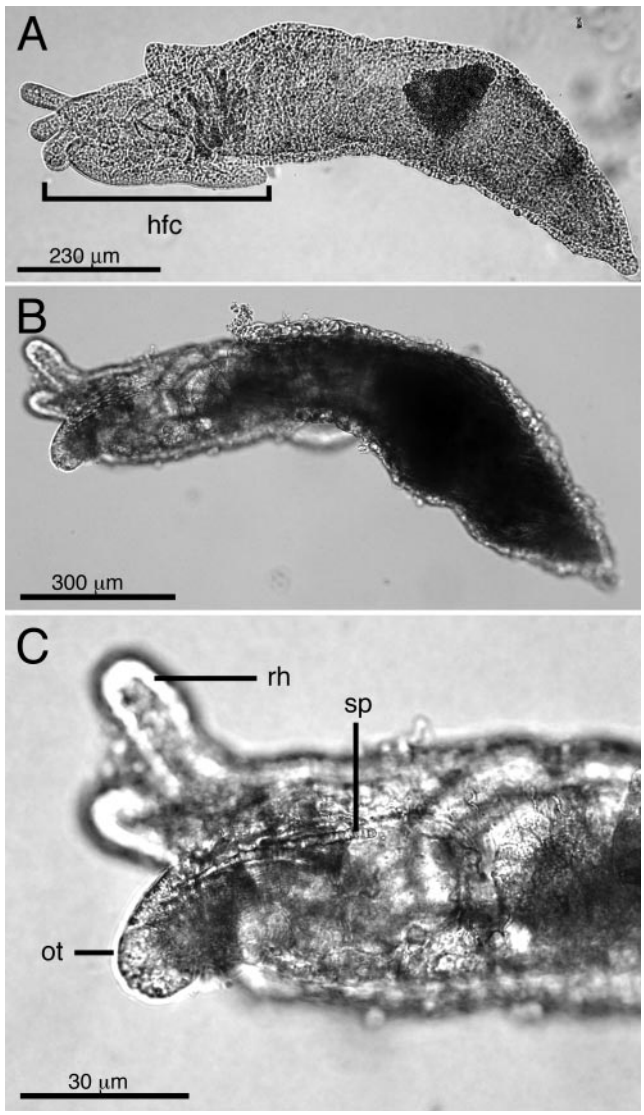

Figure 1. Light micrographs of *Asperspina* sp. (A) Fixed wholemount specimen. (B) Live specimen. (C) Anterior end of live specimen. *Abbreviations:* hfc, head-foot complex; ot, oral tentacles; rh, rhinophores; sp, spicules.

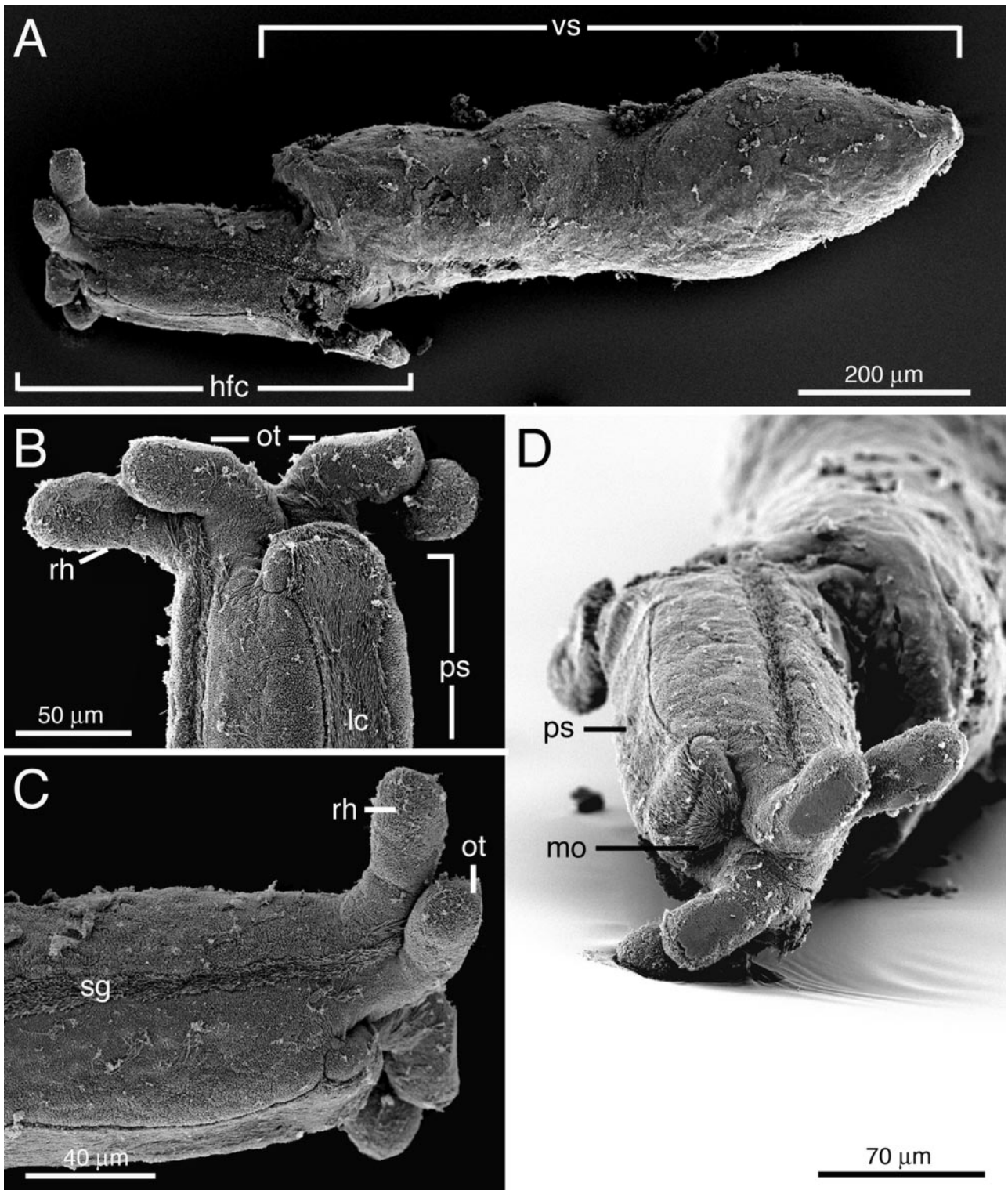

Figure 2. Scanning electron micrographs of *Asperspina* sp. (A) Lateral view of the right side of a specimen. (B) Ventral view of anterior end. (C) Lateral view of anterior end. (D) Anterior view. *Abbreviations:* hfc, head-foot complex; lc, locomotory cilia; mo, mouth; ot, oral tentacle; ps, pedal sole; rh, rhinophore; sg, sperm groove; vs, visceral sac.

present on the right side of all specimens and runs longitudinally from a region between the rhinophore and oral tentacle to the visceral sac (Fig. 2C). A column of locomotory cilia (20–26 μm wide) is present on the pedal sole of the head-foot complex (Fig. 2B, D). Coarse spicules are present in the head-foot complex and visceral sac. The spicules of the head-foot complex are small ($<$ 50 μm), sparsely distributed, and generally located posterior to the rhinophores (Fig. 1C). The spicules of the visceral sac are up to 105 μm long, arranged diagonally to the longitudinal body axis, and contribute to a loose test-like appearance. A single copulatory spicule was observed in one specimen.

Anti-serotonin immunohistochemistry

Central nervous system. Serotonin-like immunoreactivity (5-HT-LIR) was present throughout the central nervous system of all specimens, regardless of length of fixation. Control and experimental specimens showed some nonspecific staining in the epidermal glands that line the visceral sac (Fig. 3B, C). Several specimens were heavily contracted, and most specimens were unintentionally mounted left-side down, making comparative observations of neural symmetry (left *vs.* right) difficult. For this reason, most observations pertain to the right side of the nervous system only. Ganglia are identified according to their location and connections and based on descriptions in previous literature (Wawra, 1989; Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2005).

Most 5-HT-LIR in the central nervous system was located around the buccal mass (bm), a large muscular organ containing the radula (Figs. 3A, 4). The buccal mass from a single specimen (900 μm long) was about 89 μm in height (ventral to dorsal) and 102 μm in length. The right cerebral ganglion (cg) was positioned on the dorso-anterior surface of the buccal mass (Figs. 3B, C; 4 B–H). The borders of the right cerebral ganglion were easily visualized in all specimens (*e.g.,* Figs. 3C, 4C), and measured 63–71 μm long. The right cerebral ganglion of all specimens contained 12–15 immunoreactive perikarya in the 7.2–8.6-μm size range. Most 5-HT-LIR perikarya were located at the dorsoposterior border of the cerebral ganglion (Figs. 3B, C; 4F–H; 5B, C). At the center of the cerebral ganglion was a region of high varicosity (Fig. 5B, C); it is unknown whether these varicose regions represent neuropils. Similar varicosities were present in all the major ganglia and appeared to line the neurites in and around the ganglia (Figs. 4H, 5). Several small neurites projected from the cerebral ganglion toward the anterior body region (described below; Figs. 6, 8). A short cerebro-rhinophoral connective (crc) projected from the antero-dorsal surface toward the presumed rhinophoral ganglion (rg) (Fig. 5B). This ganglion was 18–20 μm in diameter and contained numerous varicosities but no obvious 5-HT-LIR perikarya (Figs. 3B, 6, 8).

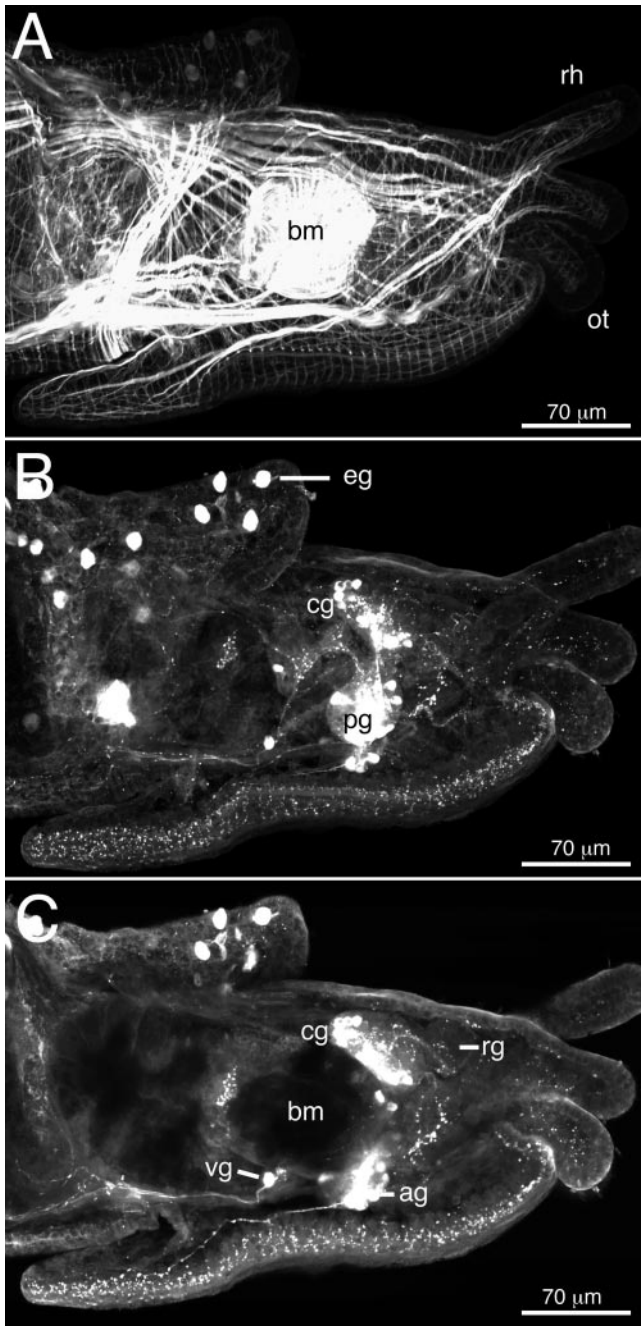

Figure 3. Epifluorescence images of the musculature and anti-serotonin-like immunoreactivity (5-HT-LIR) in the anterior head-foot complex of a specimen of *Asperspina* sp., right side view. (A) Phalloidin-stained musculature. (B) 5-HT-IR revealed in a shallow focal plane. (C) 5-HT-IR revealed in a deeper focal plane. *Abbreviations:* ag, accessory ganglion; bm, buccal mass; cg, cerebral ganglion; eg, epidermal gland; ot, oral tentacle; pg, pedal ganglion; rg, rhinophoral ganglion; rh, rhinophore, vg, visceral ganglion.

A small ganglion, the right pleural ganglion (pg), was present posterior to the cerebral ganglion and directly on the buccal mass (Figs. 4B, 5, 7, 8). This ganglion measured about 19–20 μm in diameter and did not contain

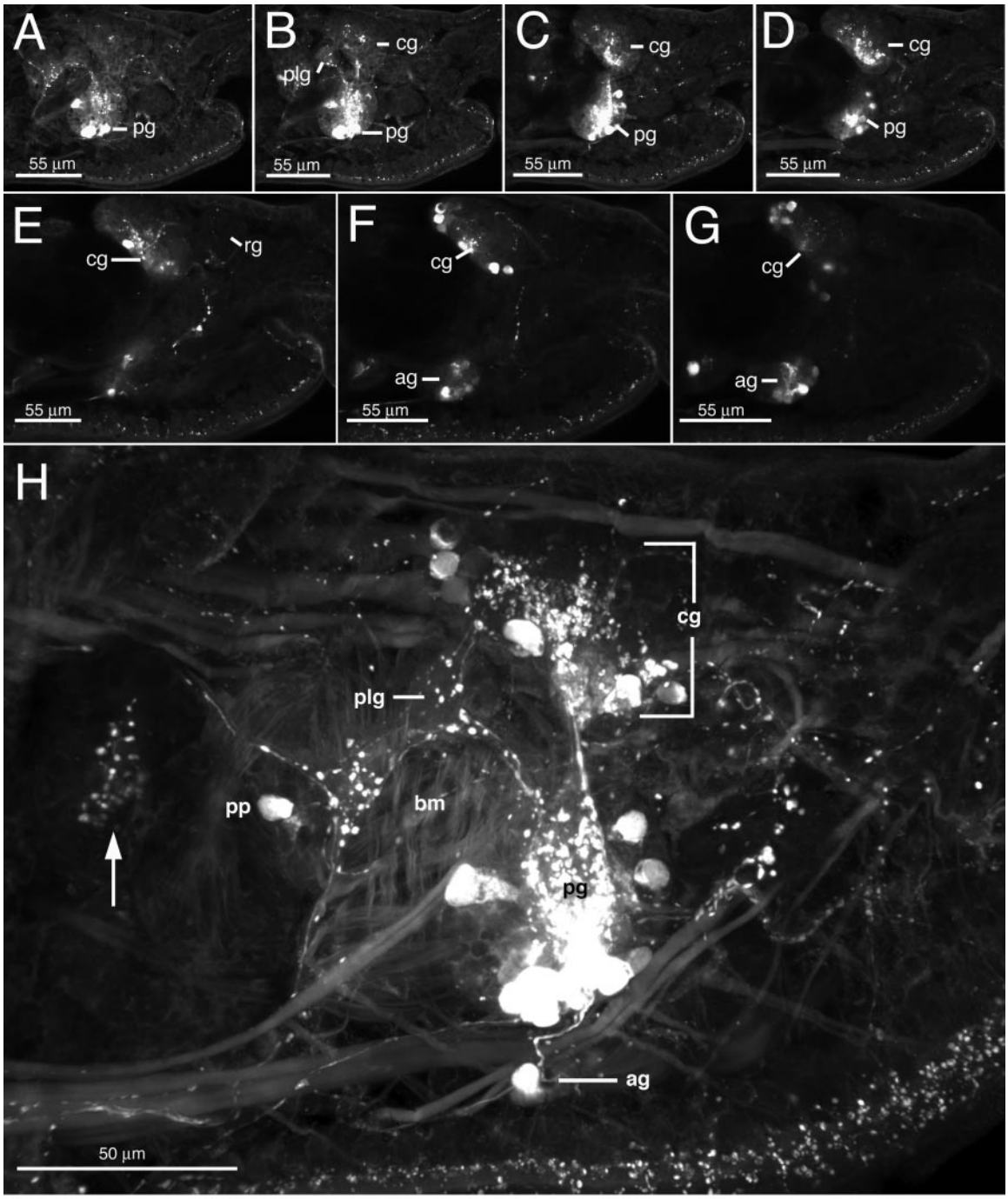

Figure 4. Series of optical sections through a specimen of *Asperspina,* revealing 5-HT-LIR in the head region (anterior is to the right). (A–G) Focus is from shallow to deep. (H) Z-projection of 5-HT-LIR in the head region just posterior of the tentacles. A patch of ill-defined immunoreactivity (arrow) is present posterior of the buccal mass. The accessory ganglion (ag) is not completely shown. *Abbreviations:* ag, accessory ganglion; bm, buccal mass; cg, cerebral ganglion; pg, pedal ganglion; plg, pleural ganglion; pp, posterior perikaryon; rg, rhinophoral ganglion.

any 5-HT LIR perikarya, but it did receive significant innervation in the form of a cerebro-pleural connective (cplc) (*ca.* 6 μm long; Fig. 5) and a pedal-pleural connective (pplc) (17–19 μm long; Fig. 5). One 5-HT-LIR nerve projected from the posterior margin of the pleural ganglion (Fig. 3C).

The right pedal ganglion (pg) was the second largest ganglion in the CNS, measuring 50–55 μm in diameter in

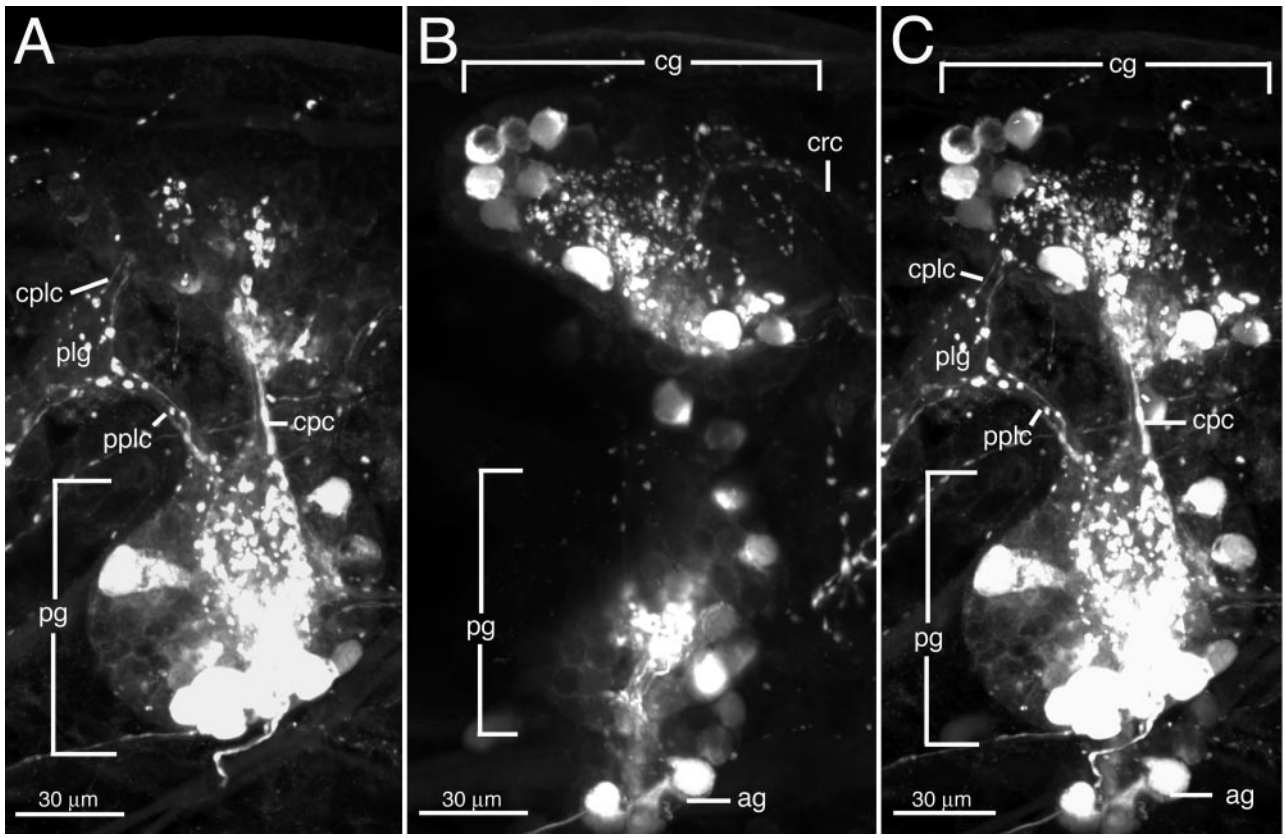

Figure 5. Z-projections of 5-HT-LIR in the anterior right-side ganglia of *Asperspina* sp. (A) Shallow focus revealing ganglia and connectives positioned on the outer right-side wall of the buccal mass. (B) Deeper focus revealing the distribution of serotonergic perikarya. (C) Cumulative z-projection of anti-serotonin immunoreactivity on the right side of the central nervous system. *Abbreviations:* ag, accessory ganglion; cg, cerebral ganglion; cpc, cerebro-pedal connective; cplc, cerebro-pleural connective; crc, cerebro-rhinophoral connective; pg, pedal ganglion; plg, pleural ganglion; pplc, pedal-pleural connective.

all specimens. The ganglion was positioned postero-ventrally to the cerebral ganglion and about 100 μm posterior to the mouth (Figs. 3B, 4, 5, 7, 8). The pedal ganglion contained 9–11 5-HT-LIR cells, mostly distributed around its base. The smallest cells were oval and 7.4–7.6 μm in diameter, and the larger cells were teardrop-shaped and up to 14 μm long. The pedal ganglion received innervation from the cerebro-pedal connective (cpc) and the pleuro-pedal connective (plpc) (Fig. 5). A series of small neurites projected from the pedal ganglion to a smaller accessory ganglion (ag) just ventral of the buccal mass (Figs. 3C, 4F, 5B, 7, 8). This ganglion contained 8–10 5-HT-LIR perikarya about 7.4–8.6 μm in diameter.

Two 5-HT-LIR perikarya were present posterior to the major ganglia in all specimens. One perikaryon (pp) was positioned toward the posterior margin of the buccal mass and appeared to receive innervation from neurites that exited the pleural ganglion (Figs. 4H, 7A). A second 5-HT-LIR perikaryon (vg) was positioned at the ventro-posterior margin of the buccal mass and received similar innervation from the pleural ganglion. This latter perikaryon was always surrounded by numerous varicosities (Figs. 4G, 7, 8). One or two neurites projected from the region of the IR perikaryon (undetermined whether the perikaryon itself was the source of the neurites) toward the visceral sac (Fig. 7).

Peripheral nervous system. The peripheral nervous system was composed of numerous fine neurites that innervated the epidermis of the visceral sac, pedal sole, dorsal body wall, mouth, rhinophores, and oral tentacles. All neurites contained numerous varicosities and followed similar routes in all specimens examined ($n = 11$). In some cases, the sites of innervation were difficult to determine with accuracy.

Several thin neurites projected from the cerebral ganglion toward the rhinophoral ganglion; many of these neurites did not appear to be part of the larger cerebro-rhinophoral connective (Fig. 6). In general, these neurites lined the periphery of the rhinophoral ganglion but did not appear to enter it. At least one neurite innervated the dorso-posterior region of the head-foot complex, and a second neurite innervated the dorsal epithelium that composed the rhinophores. Additional neurites appeared to innervate the center

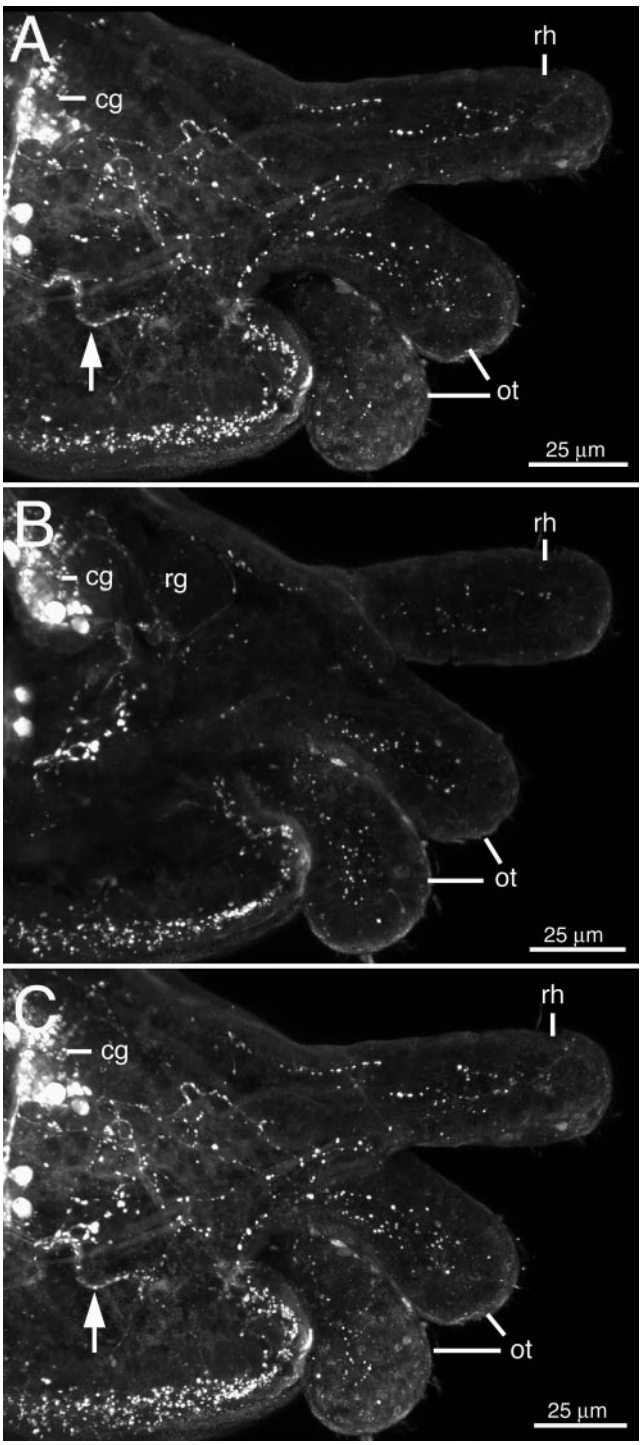

Figure 6. Z-projections of 5-HT-LIR in the head tentacles of *Asperspina* sp. (A) Shallow focus revealing neurites that innervate the body wall and tentacles. (B) Deeper focus revealing the rhinophoral ganglion. (C) Cumulative z-projection. Arrow points to thick nerve that projects from the right pedal ganglion. *Abbreviations:* cg, cerebral ganglion; ot, oral tentacles; rg, rhinophoral ganglion; rh, rhinophore.

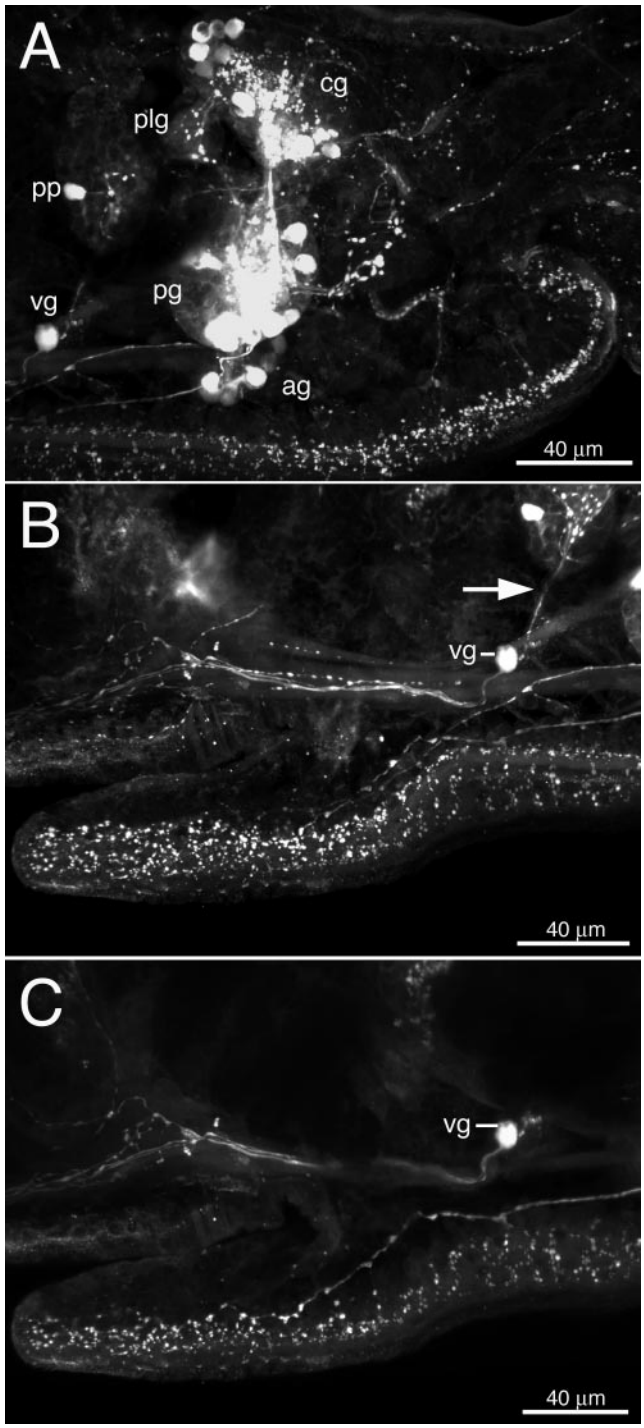

Figure 7. Peripheral 5-HT-LIR in the head-foot complex of *Asperspina* sp. (A) Major ganglia of the CNS. (B) Innervation of the ventral epithelium by the accessory and pedal ganglia. Arrow points to visceropleural connective. (C) Innervation of the posterior body region by the visceral ganglion. *Abbreviations:* ag, accessory ganglion; cg, cerebral ganglion; pg, pedal ganglion; plg, pleural ganglion; pp, posterior perikaryon; vg, visceral ganglion.

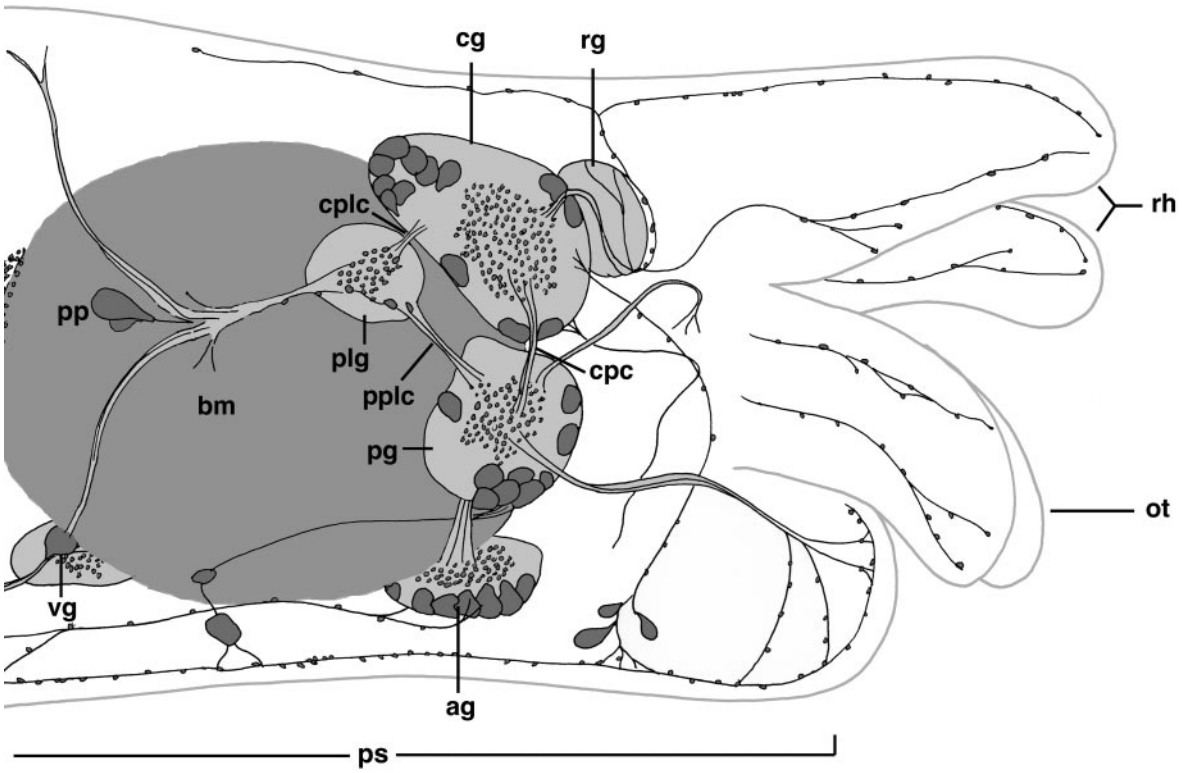

Figure 8. Diagram of the central and peripheral nervous systems of *Asperspina* sp. based on observations of 11 specimens stained with anti-serotonin antibodies and visualized on the right side of the body. *Abbreviations:* ag, accessory ganglion; bm, buccal mass; cg, cerebral ganglion; cpc, cerebro-pedal connective; cplc, cerebro-pleural connective; ot, oral tentacle; pg, pedal ganglion; plg, pleural ganglion; pp, posterior perikaryon; pplc, pedal-pleural connective; ps, pedal sole; rg, rhinophoral ganglion; rh, rhinophore; vg, visceral ganglion.

of the rhinophores, but their origin from the cerebral ganglion could not be confirmed. 5-HT-LIR neurites also innervated the oral tentacles, but it was undetermined whether their origin was the cerebral ganglion or the pedal ganglion.

The right cerebral, pedal, and accessory ganglia all innervated the ventral body wall (Figs. 7, 8). A single nerve (containing several neurites) from the cerebral ganglion projected ventrally to the pedal sole and branched prior to innervating the epithelium (not shown). Three or four 5-HT-LIR cell bodies were present at the base of the epithelium. The pedal ganglion gave rise to several neurites that innervated the pedal sole and body wall. One thicker nerve appeared to branch extensively prior to innervating a region around the mouth (Figs. 6, 7A). The accessory ganglion gave rise to one or two thin neurites that innervated the posterior region of the foot (Fig. 7). This ganglion appeared to be the primary source of pedal sole innervation, with its neurites branching extensively within the ventral epithelium and forming a complex network that ranged the length of the sole.

Several fine neurites projected from the posterior margin of the pleural ganglion along the buccal mass. A large varicose region of 5-HT-LIR was present at the midpoint of the buccal mass; from this region, several fine neurites projected dorsally toward the body wall and posteriorly toward the visceral sac (Figs. 7A, 8). Only one of these neurites innervated the perikaryon (vg) beneath the buccal mass (Figs. 7A, 8).

Discussion

The organization of the central nervous system of *Asperspina,* revealed here in part using immunohistochemistry, conforms to the known structure of the CNS in other interstitial acochlidean opisthobranchs (Rankin, 1979; Wawra, 1989; Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2006). And while several details of CNS structure remain to be determined for this species (*e.g.,* bilaterality, presence of buccal and parietal-intestinal ganglia), the current study reveals the presence of an extensive serotonergic network throughout the head-foot complex. In particular, serotonin-like immunoreactivity is present in several ganglia—most notably the cerebral and pedal ganglia—and one accessory ganglion, with lesser immunoreactivity in the pleural and visceral ganglia. Peripheral tissues are also highly innervated, and an extensive serotonergic plexus exists in the

sensory tentacles, pedal sole epithelium, and general body wall.

In *Asperspina,* 5-HT-LIR perikarya are generally restricted to the major ganglia of the CNS, where cell bodies form clusters in specific regions of each ganglion, similar to the distribution of serotonergic perikarya in the ganglia of other larger opisthobranchs (Langley and Langley, 1986; Moroz *et al.,* 1997; Sudlow *et al.,* 1998). For example, in the notaspid *Pleurobranchaea californica* MacFarland, 1966, and the nudibranch *Tritonia diomedea* Bergh, 1894, serotonergic cells cluster in the dorsal region of the cerebro-pleural ganglia (Sudlow *et al.,* 1998). A similar condition occurs in *Asperspina,* where about 8–10 5-HT-LIR perikarya reside in the dorsal region of the right cerebral ganglion compared to few cells in the ventral and anterior regions—the latter region is the site where the cerebral ganglion abuts the presumed rhinophoral ganglion. 5-HT-LIR in the cerebro-pleural ganglia of the larger opisthobranchs is generally restricted to fewer than 60 cells total, with some asymmetry in quantity and position between left and right cerebral lobes (Sudlow *et al.,* 1998). Based on analyses of *P. californica*, at least one subgroup of these serotonergic neurons contributes to a pattern generator pathway involved in the swimming escape response (Jing and Gillette, 1999). Interestingly, no serotonergic perikarya occupy the pleural region of the cerebro-pleural ganglia in *P. californica* or *T. diomedea;* similarly, no serotonergic perikarya are present in the pleural ganglia of the anaspid *Aplysia californica* Cooper, 1863 (Langley and Langley, 1986), and none were detected in the right pleural ganglion of *Asperspina* (see Fig. 5).

Serotonergic innervation by the right cerebral ganglion of *Asperspina* sp. appears to be restricted to the right pedal, pleural, and rhinophoral ganglia, and to a lesser extent, some regions of the body wall. Noticeably absent from this species are distinct IR neurons that correspond to the labial and rhinophoral nerves described from histological sections of *Hedylopsis ballantinei* (Sommerfeldt and Schrödl, 2005) and *Microhedyle remanei* (Neusser *et al.,* 2006). Although there is obvious serotonergic innervation of the rhinophores, oral tentacles, and mouth region, this innervation does not arise from a single and obvious source such as a labial or rhinophoral nerve, but instead occurs as a diffuse plexus of independent and delicate neurites. Some of these neurites appear to exit the rhinophoral ganglion, but this requires additional observations for confirmation. This ganglion itself displays little immunoreactivity in the form of cell bodies or fibers, and it may be that cerebral neurites pass through the rhinophoral ganglion on their way to innervate peripheral tissues such as the dorsal epithelium of the head. A similar condition is present in notaspids and nudibranchs, where cerebro-pleural axons pass through the rhinophoral ganglia and into the rhinophores (Moroz *et al.,* 1997). In these opisthobranchs, the rhinophoral ganglia are a rich source of innervation for both the musculature of the head tentacles and the chemosensitive epithelium that lines them (Moroz *et al.,* 1997). In the nudibranch *Phestilla sibogae* (Bergh, 1870), serotonergic neurons terminate on sensory cells that line the tentacular epithelium and therefore modulate sensory input to the cerebro-pleural ganglia (Croll *et al.,* 2003). In *Asperspina,* the fine neurites that innervate the head region are extremely difficult to trace with accuracy, but many appear to terminate both short of the head tentacles and just beneath the tentacular epithelium.

The right pedal ganglion is of similar size to the cerebral ganglion and displays comparable levels of immunoreactivity in the form of perikarya and neurites. Most of the immunoreactive perikarya are present on the ganglion's ventral margin, while most neuronal pathways exit the ganglion on it dorsal side. Anteriorly, at least three neurites project from the ganglion to the body wall: one neurite innervates a site posterior to the oral region, perhaps synapsing on muscles; a second neurite innervates the ventral mouth margin; and a third neurite innervates the ventral epithelium of the foot. In other acochlideans, at least two nerves exit the right pedal ganglion to innervate peripheral tissues (Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2006). In *H. ballantinei,* a third nerve arises antero-dorsally and innervates the posterior body region. Unfortunately, there are no details about the sites of innervation for these pedal nerves. In *Asperspina,* those nerves that innervate the body wall form an extensive network of fine serotonergic fibers. Many of these fibers line the ventral epithelium and contact the subepidermal musculature. Interestingly, both the region around the mouth and the ventral body wall are also extensively lined with cilia, forming a single column of locomotory cilia along the pedal sole (see Fig. 7). The close spatial relationship between the fine serotonergic plexus and the locomotor cilia hints at a functional relationship, as serotonin is a well-recognized modulator of ciliary activity in adult nudibranchs (Audesirk *et al.,* 1979) and molluscan embryos (Diefenbach *et al.,* 1991; Uhler *et al.,* 2000). Similar observations were noted for other opisthobranchs—serotonergic somata are clustered at the base of the pedal ganglion, and there is an extensive innervation of the ciliated epithelium of the foot by the pedal ganglia (Sudlow *et al.,* 1998).

One additional ganglion showed significant immunoreactivity in *Asperspina:* the right accessory ganglion. This accessory ganglion has a clear serotonergic connection to the right pedal ganglion and contains 7–10 perikarya positioned along its ventral margin. This ganglion also displays significant innervation of the pedal sole and may therefore regulate the activity of the locomotory cilia. Comparable accessory ganglia are absent in *H. ballantinei,* whereas a variety of accessory ganglia are known for other species and are generally associated with their cerebral ganglia (Marcus, 1953; Marcus and Marcus, 1954; Rankin, 1979). Only in *M.*

remanei are there two accessory ganglia present in a similar position to that of *Asperspina* sp., however, nothing is known about their connections with the CNS or their peripheral innervation (Neusser *et al.,* 2006).

Additional IR-perikarya were positioned close to the major identifiable ganglia of the CNS, but it is undetermined whether these perikarya are homologous with cells or ganglia described from other species. For example, one or two perikarya (vg) are located beneath the buccal mass in a similar position to ganglia in related species—for example, the parietal-subintestinal ganglion of *H. ballantinei* (Sommerfeldt and Schrödl, 2005) or the subintestinal ganglion of *H. spiculifera* (Neusser *et al.,* 2005). These perikarya (vg) have high immunoreactivity, are surrounded by significant varicosities, and receive innervation from the pleural ganglion. Intriguingly, these perikarya appear to be a major source of serotonergic innervation to the posterior body region where a diffuse network of delicate fibers innervates different portions of the visceral sac. Immunohistochemical observations of other specimens of Acochlidea would help to clarify the homology of these perikarya and determine whether they are part of a larger ganglionic mass.

Although much of the peripheral innervation in *Asperspina* was identifiable with respect to the CNS (*i.e.,* could be associated with an evident ganglion), some patches of tissue also exhibited immunoreactivity with no or limited contact with the CNS. For example, in all specimens, a small patch of tissue directly posterior to the buccal mass showed consistent varicosity in the absence of perikarya or neurites (Fig. 4H). This patch of immunoreactivity was not present in control specimens.

Conclusions

Despite a strong research focus on model opisthobranchs, several species of lesser-known opisthobranchs are beginning to receive increasing attention, particularly the interstitial species of Acochlidia (Rankin, 1979; Wawra, 1989; Fahrner and Haszprunar, 2002; Sommerfeldt and Schrödl, 2005; Neusser *et al.,* 2006). Up to now, several species from both suborders Hedylopsacea and Microhedylacea have been the subject of anatomical investigation, but only the current study has revealed the presence of an extensive serotonergic network in the CNS of an acochlidean. Although the microscopic size of these opisthobranchs makes electrophysiological studies of neuron homology difficult, future immunohistochemical research may provide additional details on neuron presence (*e.g.,* metacerebral giant), location, size, neurotransmitter content, and development to allow for comparisons with the larger model species. This information, together with detailed anatomical descriptions like those of Sommerfeldt and Schrödl (2005) and Neusser *et al.* (2006), should permit a better understanding of opisthobranch diversity and evolution and provide valuable information on evolutionary trends in the gastropod central nervous system.

Acknowledgments

I thank Dr. Michael Schrödl for his generosity in providing copies of necessary publications. I am also grateful to two anonymous reviewers for their critical comments that have greatly improved this manuscript. Thanks are due to the staff and scientists of the Smithsonian Marine Station in Fort Pierce, Florida, for their help in specimen collection and for the use of their facilities during part of this research's tenure. I also thank those malacologists at the 2006 SICB conference for discussions on this research. This research received financial support from the University of Massachusetts Lowell and from the Sumner Gerard Foundation. This is Smithsonian Marine Station at Fort Pierce contribution #686.

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