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Morphology of the Nervous System of the Barnacle Cypris Larva (*Balanus amphitrite* Darwin) Revealed by Light and Electron Microscopy

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Abstract. The central nervous system of the cypris larva of *Balanus amphitrite* consists of a brain and posterior ganglion. The neuropil of the brain includes protocerebral and deutocerebral divisions, with nerve roots from the protocerebrum extending to the eyes and frontal filaments, and nerve roots from the deutocerebrum extending to the first antennae (antennules) and cement glands. The neuropil of the posterior ganglion includes subesophageal and thoracic divisions, with nerve roots from subesophageal divisions extending to the gut, and nerve roots from each of the six thoracic divisions extending to their corresponding thoracic appendage. The antennular nerve is the major peripheral extension of the nervous system and is composed in part by afferent fibers that innervate setae on the antennules. The cyprid nervous system is small, containing fewer than 2000 neurons, but is well organized for coordinating a response to settlement cues.

Introduction

The cyprid (cypris larva) is the final larval stage of the barnacle. Cyprids are specialized for settlement (Anderson, 1994), a behavioral process in which a site is selected for permanent attachment and metamorphosis (Anderson, 1994; Walker, 1995). Cyprid settlement is known to be mediated by specific environmental cues (Clare, 1995; Walker, 1995), but little is known about the mechanisms of cue detection and, in particular, how the detection of cues results in the centrally coordinated motor patterns of settlement behavior.

Cyprids are highly mobile and bear numerous sense organs (Walley, 1969). The nauplius eye (= median eye) is present during the cyprid stage and is remodeled into the adult ocelli during metamorphosis (Takenaka *et al.,* 1993). A pair of compound eyes are also present, which are unique to the cyprid. These develop during the final naupliar stage and are lost during metamorphosis (Walley, 1969; Hallberg and Elofsson, 1983). The compound eyes are closely associated with a pair of frontal filaments (Walker, 1974), and many setae are located on the antennules (Nott and Foster, 1969; Nott, 1969; Clare and Nott, 1994; Glenner and Høeg, 1995), thoracic appendages (Glenner and Høeg, 1995), caudal rami (Walker and Lee, 1976; Glenner and Høeg, 1995), and carapace valves (Walker and Lee, 1976; Jensen *et al.,* 1994; Glenner and Høeg, 1995). Many of these setae are thought to function as mechano- and chemoreceptors. Putative sensory structures located on the carapace include dermal pits, wheel organs (Elfimov, 1995), and lattice organs (Jensen *et al.,* 1994; Høeg *et al.,* 1998). Recently, cilia-type dendritic segments were shown to innervate the lattice organs, suggesting a chemosensory function (Høeg *et al.,* 1998).

To date, morphological studies of the cyprid have focused primarily on external structures (Elfimov, 1995), and particularly on the antennules because of the role played by these appendages during settlement (Nott and Foster, 1969; Nott, 1969; Moyse *et al.,* 1995). Fewer details are available on the internal organization of the cyprid. Walley (1969) described the larval development of *Semibalanus balanoides* (previously *Balanus balanoides*) and outlined the nervous system and major sense organs of both the cyprid and nauplius. Other studies have shown that the antennules (Nott and Foster, 1969), frontal filaments (Kauri, 1961; Walker, 1974), dermal pits (Walker and Lee, 1976), lattice organs (Høeg *et al.,* 1998), and cement glands (Walker, 1971; Okano *et al.,* 1996) are innervated, but the nerves

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associated with each of these structures have not been traced back to the central nervous system.

The cyprid is well equipped to detect settlement cues, but little is known about the underlying role of the nervous system. Recent studies have suggested that cyprid settlement behavior is affected by exposing cyprids to certain neuroactive substances (Clare *et al.,* 1995; Kon *et al.,* 1995; Yamamoto *et al.,* 1995, 1996; Okano *et al.,* 1996, 1998). Studies aimed at investigating the underlying mechanisms of settlement would benefit from a detailed account of the cyprid nervous system. We report here the results of an anatomical study of the central nervous system and the major sense organs of the cypris larva of *B. amphitrite,* gained from microdissection, semithin serial sections, and electron microscopy. We find that the central nervous system is made up of about 2000 neurons and that it contains regionalized neuropils, many of which are linked to peripheral sense organs. Although the cyprid nervous system is small, it is well organized, which is consistent with the cyprids' need to detect and respond to multiple cues for settlement.

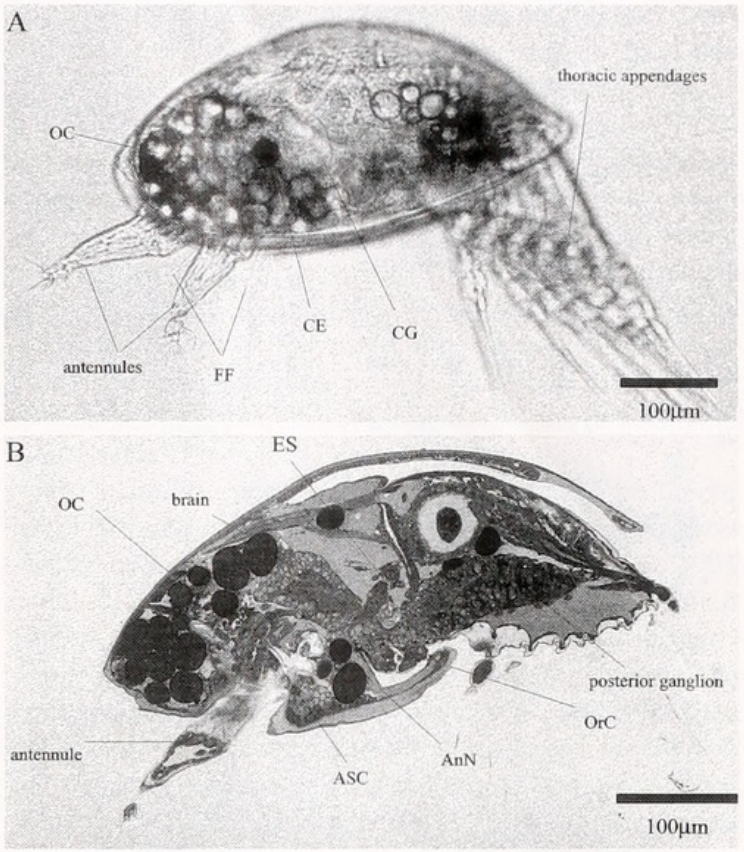

Figure 1. The cypris larva of *Balanus amphitrite.* (A) Light micrograph of a live cyprid with antennules and thoracic appendages extending from the bivalved carapace. Internal structures visible include a compound eye (CE), translucent oil cells (OC), and a cement gland (CG). Frontal filaments (FF) extend posterior to each antennule. (B) Longitudinal section of the cyprid showing the central nervous system, which consists of a brain and posterior ganglion. The brain connects *via* paired circumesophageal connectives (not visible in this section) to the posterior ganglion. A single antennular nerve (AnN) and its associated antennular soma cluster (ASC) are visible. The ASC contains the somata of bipolar sensory neurons. Also apparent in this section are densely stained oil cells, the oral cone, esophagus, and gut. The compound eyes and cement glands are located lateral to this plane and are not seen (0.5 μm section; stained with toluidine blue and osmium tetroxide).

Materials and Methods

Cyprids used in this study were obtained from a laboratory culture of *Balanus amphitrite* (see DeNys *et al.,* 1995). The selected individuals were between 1 and 3 days old (nauplius-cyprid molt = day 0), were active, and had clear (*i.e.,* non-milky) carapaces, obvious cement glands, and compound eyes. Dissection of the animals provided a useful overview of their structure, including the placement of the antennules and limbs within the bivalved carapace and the gross organization of internal organs. Specimens were placed on a stereomicroscope and dissected using tungsten microscalpels and pins (Conrad *et al.,* 1993). Individuals were placed in a calcium-free saline (in mmol · l^{-1}, 485 NaCl, 13 KCl, 10 $MgCl_2$, 10 HEPES, pH 7.4) to reduce movement and secured (ventral surface upward) to a silicon-coated microscope slide using either tungsten pins or a nontoxic, rapid-setting silicon adhesive (Kwik-Sil, World Precision Instruments). A cut along the ventral midline allowed separation of the carapace valves to expose the central nervous system and internal organs of the cephalon (see Figs. 1, 2). The carapace valves, antennules, and thoracic appendages were then secured with fine (<10 μm diameter) tungsten pins. The secured preparation was transferred to a fixed-stage Olympus BH-2 microscope and viewed and photographed using water immersion objectives.

Fixation and embedding. Larvae were placed in equal volumes of 0.4 *M* $MgCl_2$ and 0.22 μm-filtered seawater (FSW) and gently agitated for up to 3 h, which had the effect of relaxing the carapace adductor muscles and exposing the antennules. Specimens were cooled to 4°C for 30 min, transferred to chilled (4°C) fixative consisting of 2.5% glutaraldehyde and 2.0% formalin in FSW (pH 8.2; 950 mosmol). The formalin used was prepared fresh from paraformaldehyde (37% w/v paraformaldehyde in H_2O). Microwave treatment was used to facilitate the penetration of fixative. For microwave fixation, specimens were placed in 20-ml glass vials filled with chilled fixative; the vials were secured in a beaker filled with chilled water, which in turn was placed in a beaker of crushed ice. Microwave treatment continued until the water in the beaker reached a temperature of 37°C (typically 50 s). Specimens were then removed from the oven and allowed to cool to ambient temperature; fixation continued overnight. The following day, specimens were rinsed in FSW for 1 h (three changes of 20 min each), post-fixed in 2% osmium tetroxide (in H_2O) for 30 min, 2% uranyl acetate (in H_2O) for 20 min, dehydrated through an ethanol series, cleared in propylene oxide, exposed to

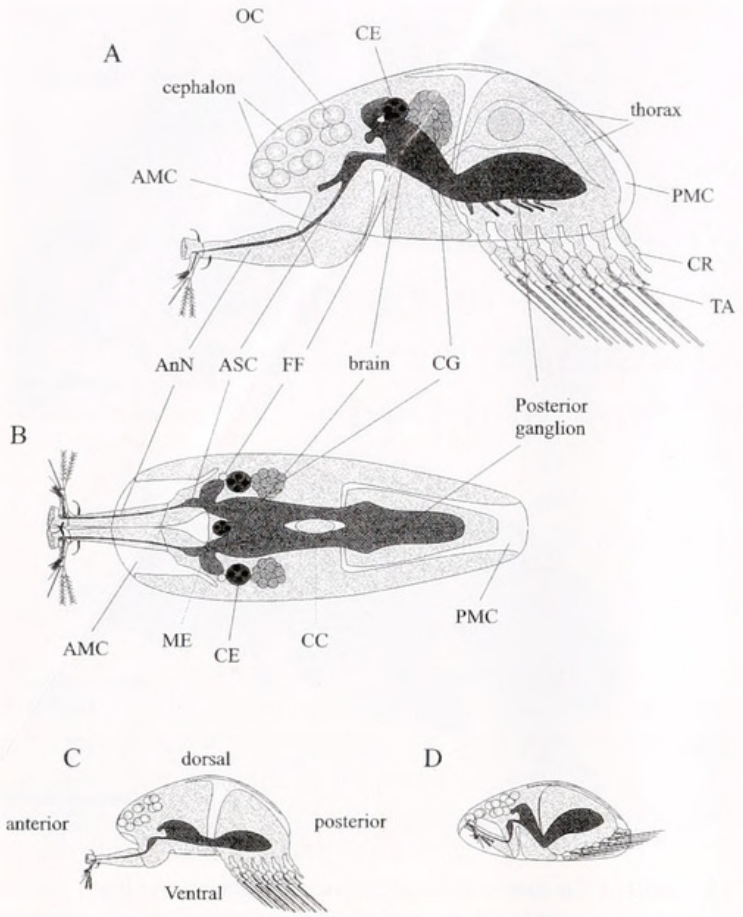

Figure 2. Schematic drawings of the cyprid nervous system and major organs in longitudinal and horizontal planes. (A–B) The body of the cyprid is organized in two main compartments, the cephalon and the thorax. The bivalve carapace encloses anterior (AMC) and posterior (PMC) mantle cavities about the cephalon and thorax respectively. The brain, compound eyes (CE), median eye (ME), and cement glands (CG) are contained within the cephalon. The antennules extend from the cephalon and bear the adhesive discs and putative chemoreceptive and mechanoreceptive sensilla. The brain connects with the posterior ganglion *via* circumesophageal connectives (CC). The posterior ganglion and gut are contained within the thorax. Six pairs of thoracic appendages (TA) and a pair of caudal rami (CR) extend from the thorax. (C–D) The orientation of neural structures in the cyprid depends on the relative position of the antennules and thoracic appendages, both of which extend beyond the carapace when the cyprid either swims or contacts the substratum (C), or can be withdrawn for protection (D). Planes are identified on the basis of the orientation of the nervous system in (C). Other abbreviations: ASC, antennular soma cluster; OC, oil cell; FF, frontal filament.

increasing concentrations of Araldite epoxy resin, and flat-embedded on microscope slides. Flat embedding allowed the orientation of the specimen to be determined using a light microscope. The Araldite was then removed from the slides by cold shock (using liquid nitrogen) and specimens cut from the blocks and remounted on Araldite stubs for sectioning.

Light microscopy. Twelve animals were serially sectioned at either 0.5 or 1.0 μm (six in sagittal plane, three frontal, and three horizontal—see Fig. 2 for orientation) with a Reichert-Jung ultramicrotome and diamond histology knife. Sections were transferred to microscope slides and stained with toluidine blue (1% in 6% borax, 0.6% boric acid, pH 8.3) or methylene blue (1% in 0.1% borax, pH 8.0); reconstructions were made from camera lucida drawings and photographs and with the aid of PC-based, Adobe Illustrator software.

Electron microscopy. Specimens for transmission electron microscopy were prepared as described above, sectioned at 60–70 nm on a Reichert-Jung ultramicrotome using a diamond knife, and viewed and photographed using a Hitachi H-7000 transmission electron microscope. For scanning electron microscopy, anesthetized and fixed animals were washed for 10 min in H_2O (three changes of 3 min each) with sonication during the first two steps, dehydrated, and transferred to acetone for critical-point drying. Dried specimens were mounted on microscope stubs with double-sided carbon tape, then gold coated and photographed on a Leica/Cambridge S-360 scanning electron microscope.

Results

General anatomy

Live cyprids of *B. amphitrite* typically measure 500–550 μm in length from the rostral to the caudal end of the carapace (Fig. 1A) (Glenner and Høeg, 1995), but minor size variations occurred in our cultured animals. When fixed, the average dimensions for 12 individuals were 480 μm in length, 220 μm in height, and 170 μm across the broadest part of the carapace.

The body of the *B. amphitrite* cyprid, like that of *S. balanoides* (Walley, 1969), is arranged as two separate compartments, the cephalon and thorax (Fig. 2). A bivalved carapace encloses anterior and posterior mantle cavities around the cephalon and thorax respectively (Fig. 2). The cephalon houses the brain, eyes, and cement glands, together with many large, densely staining oil cells (Figs. 1, 2), which are thought to supply energy for the lecithotrophic larva and its subsequent metamorphosis (Walley, 1969). First antennae (antennules) project anteriorly from the cephalon and can be extended well beyond the carapace during temporary attachment (Fig. 2C), or completely retracted within the anterior mantle cavity (Fig. 2D). Frontal filaments extend from the ventral surface of the cephalon, posterior to the antennules (Figs. 1A, 2A, 6C). The thorax houses the posterior ganglion and the undifferentiated gut (Figs. 1, 2). Six pairs of thoracic appendages and the caudal rami project from the ventral surface of the thorax, which may extend beyond the ventral edge of the carapace or be completely withdrawn within the posterior mantle cavity (Fig. 2C–D).

The central nervous system (CNS) can be seen in near-sagittal section (Fig. 1B) and is drawn schematically in Figure 2. The CNS consists of a cerebral ganglion, or "brain," linked by paired circumesophageal connectives to a

posterior ganglion. Nerve roots extend from central ganglia toward peripheral organs (Figs. 1, 2). The major peripheral nerves include the antennular nerve and thoracic nerve roots (Fig. 2A). The relative position of neural structures depends on the degree of contraction of the appendages. When the appendages are fully extended (*e.g.*, Fig. 2C), the central nervous system lies essentially flat along the ventral surface, and this orientation is used to identify planes throughout this study. When the appendages are withdrawn (*e.g.*, Fig. 2D), the central nervous system can bend to an angle of 60° relative to the longitudinal axis, and the antennular nerve bends accordingly to accommodate flexion of the antennule.

The brain and associated structures

The brain (Figs. 3, 4) is composed of centrally positioned neuropil surrounded by the somata of central cells (Figs. 3, 4). The neuropil is composed of fine fibers and nerve endings and on close inspection contains membrane-bound vesicles and densely staining clefts typical of invertebrate synapses (Fig. 4C). Central somata are relatively uniform in size, measuring 4–8 μm in diameter, and form an outer layer of between one and five cells thick (Figs. 3, 4). The somata of these cells typically contain lightly stained granular nuclei and have a relatively thin layer of cytoplasm between the nucleus and cell membrane (Fig. 3B–C). Distinct clusters project neurites together in bundles to the central neuropil (Fig. 3B–C); based on a calculation of soma volume, we estimate the total number of neuronal cells in the cyprid brain to be approximately 750.

The brain is enclosed in a thin sheath, but the broad perineural glial layer present beneath the sheath in decapod crustacean ganglia (Sandeman, 1982) is absent. Small, densely stained, spindle-shaped glial cells lie between the somata and central neuropil and delineate neuropil regions (Fig. 3C). Two broad areas of neuropil can be discerned in the cyprid brain, and the lateral lobes of each division link *via* transverse fiber tracts (Fig. 4). The anterior division is connected to the eyes and frontal filaments *via* the optic tracts, and the posterior division is connected to the antennules *via* the antennular nerves (Fig. 4A). These divisions appear similar to the protocerebral and deutocerebral divisions of the decapod brain. We find no evidence of a tritocerebrum in the cyprid, which is consistent with the absence of antenna II during this stage (Sandeman *et al.*, 1992).

The protocerebrum. The protocerebrum can be subdivided into three regions, based on connections with peripheral sense organs and delineation by spindle-shaped glia (Fig. 5). We refer to these regions as the dorsofrontal neuropil, optic lobe neuropil, and median protocerebral neuropil. The dorsofrontal neuropil is dorsal to the protocerebral commissure (Fig. 5) and receives input from the median eye. The optic lobe neuropils are located within the anterolateral extensions of the brain and are linked to more posterior regions of the protocerebrum *via* the optic tract (Figs. 4, 5). Each optic lobe neuropil receives input from the adjacent frontal filament and compound eye (Figs. 4, 5). The median protocerebral neuropils elongate along the anteroposterior axis of the brain (Fig. 5) and are not directly linked with peripheral sense organs. Neurites from surrounding somata project into the median protocerebral neuropils (Figs. 3B, C) and longitudinal fibers that extend from the posterior regions of these neuropils contribute to the circumesophageal connectives (Fig. 5). Lateral lobes of the median protocerebral neuropil connect *via* the protocerebral commissure (Figs. 4, 5).

The nauplius eye (= median eye) is located on the anterodorsal margin of the brain. The nauplius eye has been studied in *B. amphitrite hawaiiensis* (Takenaka *et al.*, 1993) and in *S. balanoides* and *B. crenatus* (Kauri, 1961). In the *B. amphitrite* cyprid, the nauplius eye is composed of three pigment "cups" (two lateral and one ventral), with each cup containing four retinular cells. Axons from each of the three pigment cups were traced to the dorsofrontal neuropil. In one of three preparations in which these axons were traced, however, some axons appeared to bypass the dorsofrontal neuropil and contribute directly to the protocerebral commissure.

A frontal filament is attached to the anteromedial margin of each compound eye. The fine structure of the frontal filament in the nauplius of *S. balanoides* has been described previously (Walker, 1974). In the relaxed cyprid, the filaments extend beyond the carapace margin (Figs. 1A, 6C) and each contains large internal vesicles in its basal region (Fig. 6B). A frontal filament tract connects each frontal filament to its adjacent optic lobe neuropil (Figs. 5, 6B).

The structure of *the compound eye* in *B. amphitrite* is consistent with that described for *S. balanoides* (Walley, 1969; Hallberg and Elofsson, 1983). Each eye is located within a lateral "pocket" of the cephalon and composed of radially arranged ommatidia, each with a spherical lens and underlying retinular cells (Figs. 2, 5, 6A, B). Retinular cell axons converge to form a short optic nerve (Figs. 6A, B), which emerges from the medial surface of each compound eye and projects anteriorly to the optic lobe neuropil (Figs. 5, 6A, B).

The deutocerebrum. The deutocerebrum can be subdivided into two distinct regions, which we call the circular deutocerebral neuropils and median deutocerebral neuropils (Figs. 3B, 4A, 5). All peripheral nerves associated with the deutocerebrum travel within the antennular nerves. The circular deutocerebral neuropils are located lateral and slightly posterior to the brain-antennular nerve junction (see Fig. 3B). These neuropils are clearly delineated by glial cells and, based on their position and shape, are possible candidates for olfactory lobes. However, glomeruli that characterize the olfactory lobes in many animals (Hallberg

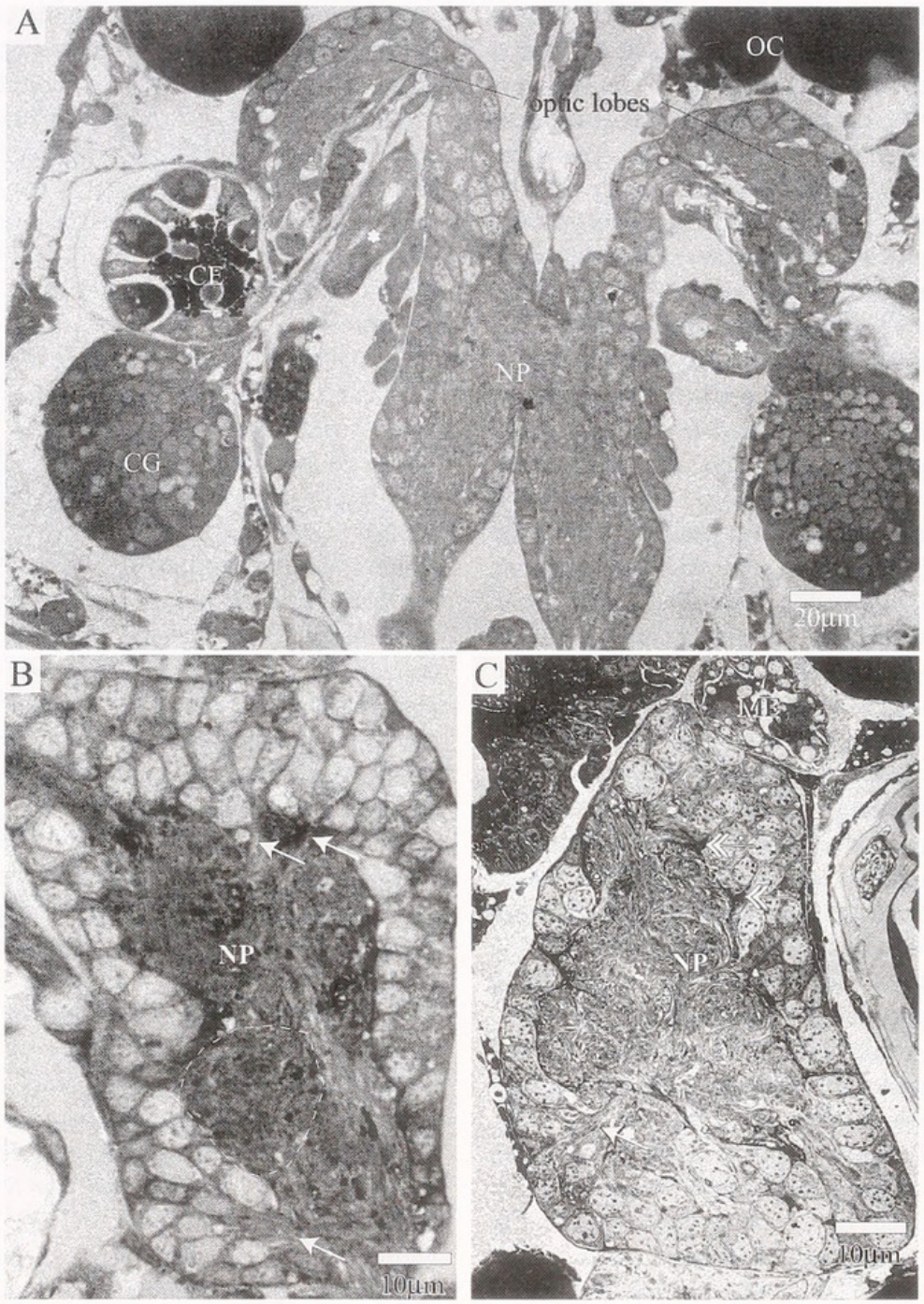

Figure 3. (A) Horizontal section through the cyprid brain. The brain is composed mainly of centrally positioned neuropil (NP) and surrounding cell somata. The anterior portion of the brain extends laterally to form the optic lobes. Also apparent are the compound eyes (CE), cement glands (CG), and cross-sections of the cement ducts (*), which extend to the adhesive discs of the antennules (see also Figure 7). OC, oil cell. (B) Longitudinal section of the cyprid brain showing clusters of somata with primary neurites (arrows) that project to the neuropil (NP). The circular deutocerebral neuropil (see also Fig. 5) is outlined. (C) Electron micrograph of the brain in near sagittal section highlighting projections of neurons (single arrow) into the central neuropil (NP) and the delineation of the neuropil by spindle-shaped glia (double arrows). ME, median eye.

et al., 1992; Hildebrand and Shepherd, 1997) were not seen in this region. Lateral lobes of the median deutocerebral neuropil are linked by the deutocerebral commissure and receive primary neurites from surrounding cell somata, particularly those located ventrolaterally to this neuropil. Posterior projections from the median protocerebral neuropils contribute to the circumesophageal connectives and travel in bundles distinct from those associated with the median protocerebral neuropil (Fig. 5).

The antennules and associated cement glands are innervated by the antennular nerves, which link to the deutocerebral neuropil (Fig. 4A, B). Detailed morphological descriptions of the cyprid antennule are available for both *B. amphitrite* (Clare and Nott, 1994; Glenner and Høeg, 1995)

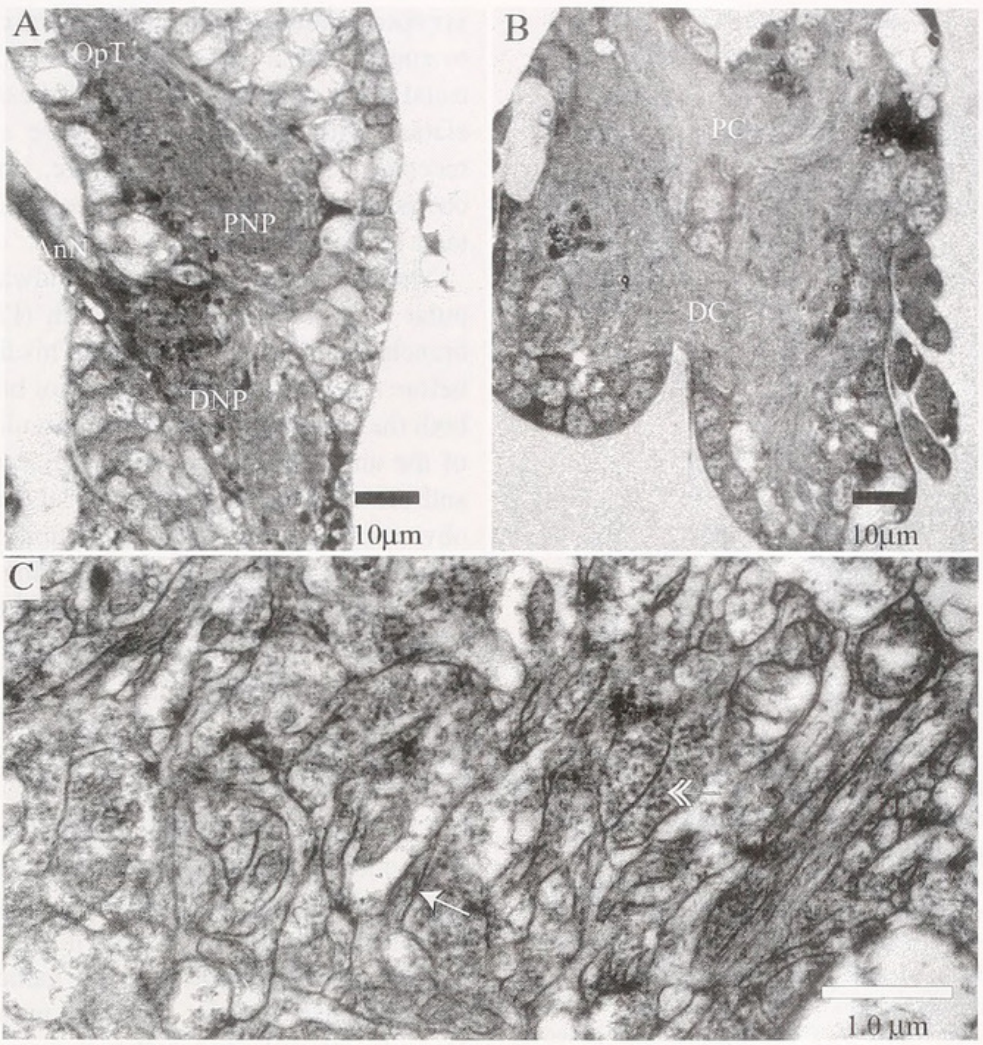

Figure 4. Two distinct neuropil divisions in the cyprid brain as seen in (A) longitudinal and (B) horizontal planes. (A) The anterior, or protocerebral, neuropil (PNP) is associated with the optic tract (OpT); the posterior, or deutocerebral, neuropil (DNP) is associated with the antennular nerve (AnN). (B) Protocerebral and deutocerebral commissures (PC, DC respectively) are the transverse fiber tracts that link lateral lobes of these two major neuropil divisions. (C) Electron micrograph of the neuropil showing many dark clefts (*e.g.,* single arrow) and membrane-bound vesicles (*e.g.,* double arrow) indicative of synapses.

and *S. balanoides* (Nott and Foster, 1969). We provide a brief description here to account for the neural innervation of this appendage. The antennule, represented schematically in Figure 7, consists of four articulating segments. Segment I projects ventrally from the cephalon and attaches to the slightly longer and slender segment II. Segment III functions as an adhesive disc and is used for attachment to the substratum (Nott and Foster, 1969; Nott, 1969; Walker, 1971). Segment IV is the terminal segment and extends laterally from the disc. Cuticular setae project from the antennular segments, particularly from the disc (Nott and Foster, 1969; Moyse *et al.,* 1995) and from segment IV (Nott and Foster, 1969; Gibson and Nott, 1971; Clare and Nott, 1994; Glenner and Høeg, 1995). Two large cement glands are associated with the antennules (Fig. 7). These are located within the cephalon, posterior to the compound eyes (Figs. 1, 2, 7; Walker, 1971), and ducts from these glands extend the length of the antennule to open through the adhesive disc. A muscular sac surrounds each duct (Walley, 1969) near the base of the antennule. In addition to the cement glands, antennulary glands are present in segment II of the antennule, which are thought to mediate the controlled release of adhesive used for temporary attachment (Nott and Foster, 1969; Walker, 1971).

The antennular nerve extends from the ventrolateral margin of the brain to the distal region of the antennule (Fig. 7). Distally, the antennular nerve is composed almost exclusively of neural processes associated with the distal setae (Figs. 8, 9). The external morphology of setae on segment IV has been described previously (Clare and Nott, 1994; Glenner and Høeg, 1995). There are nine setae on the fourth segment, which are arranged in terminal and subterminal rows (Fig. 8); their associated neural processes can be seen in cross-section of segment IV (Fig. 9A). Most neural processes in this segment are between 0.5 and 1.0 μm in diameter and contain between one and three mitochondria

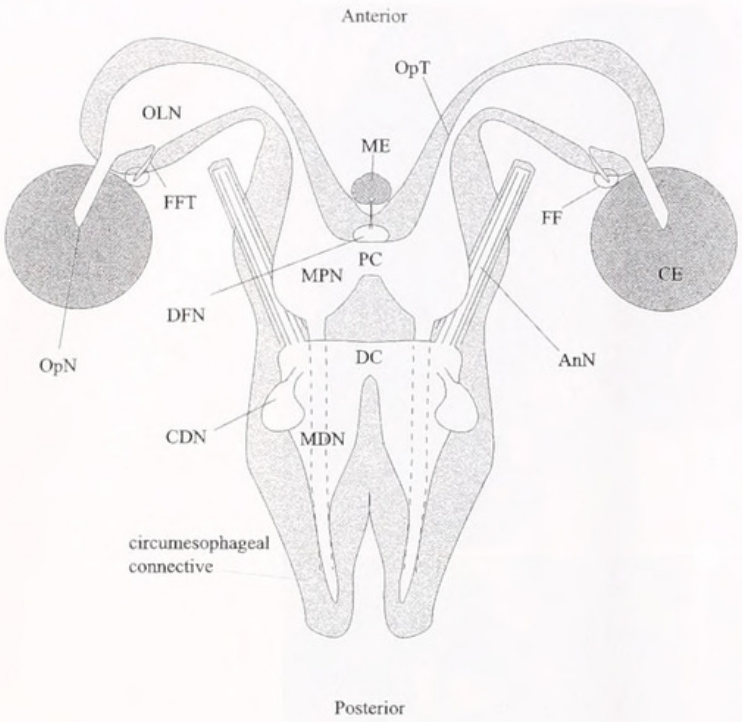

Figure 5. Schematic drawing of the neuropils and fiber tracts in the cyprid brain, showing further possible subdivision. The protocerebrum includes the paired optic lobe neuropils (OLN), median protocerebral neuropils (MPN), and an unpaired dorsofrontal neuropil (DFN). Optic lobe neuropils receive input from compound eyes (CE) *via* the optic nerve (OpN), and from frontal filaments (FF) *via* the frontal filament tract (FFT). The small dorsofrontal neuropil (DFN) lies anterior to the PC and receives input *via* the median eye (ME). Median protocerebral neuropils are not directly linked to peripheral sense organs, but the lateral lobes are connected by the protocerebral commissure (PC). The deutocerebrum includes paired circular and median deutocerebral neuropils (CDN, MDN, respectively). Peripheral nerves from the deutocerebrum form the antennular nerve (AnN), and lateral lobes of the median deutocerebral neuropil are connected by the deutocerebral commissure (DC). Longitudinal fiber tracts extend from the posterior of both the MPN and MDN and contribute to the circumesophageal connectives.

(Fig. 9B). Narrow "cilia-type" dendritic profiles (0.1–0.2 μm in diameter), which can be identified by a $9 \times 2 + 2$ microtubule arrangement, are also present (Fig. 9C). The larger processes can be traced as far as the distal portion of this segment, whereas the outer dendritic segments of smaller cilia-type processes extend into each of the four short subterminal setae (Fig. 9C).

Approximately 50 μm from the brain, the antennular nerve is associated with a cluster of neurons, which we refer to as the *antennular soma cluster* (Fig. 10). This group of cells was referred to as the antennular "ganglion" by Walley (1969), who proposed that the somata were those of motorneurons that had migrated out from the brain. From light micrographs, the cells in this cluster appear to be bipolar, with processes extending both proximally to the brain and distally along the antennular nerve. The somata, which measure 6–8 μm in length, have large nuclei and a relatively thin layer of cytoplasm (Figs. 10B, 3C). From electron micrographs, we found no evidence of branching or of synapses within the antennular soma cluster, which leads us to conclude that these cells do not form a ganglion in the usual sense. The morphology of these cells and their association with the antennular nerve suggest that they are receptor cells and are, therefore, possible candidates for chemoreceptors or mechanoreceptors whose dendrites extend to the distal setae.

The antennular nerve splits midway between the antennular soma cluster and the brain (Fig. 7), sending a fine branch toward the *cement duct.* This fine branch splits again before reaching the duct, and minor branches project toward both the *cement gland* and the muscular sac. These branches of the antennular nerve travel adjacent to the cement duct and are difficult to trace in serial section. They are more obvious, however, during dissection of this region and will typically separate from the collecting duct following slight enzymatic treatment (0.01 mg $\cdot$ ml^{-1} trypsin for 5 min). We were unable to trace projections of these fine branches beyond the muscular sac.

The posterior ganglion and associated structures

The posterior ganglion is composed largely of centrally positioned neuropil and fiber tracts surrounded by neuronal somata (Fig. 11A). The somata in this region, like those in the brain, measure 4–8 μm in diameter, contain lightly stained granular nuclei, and are gathered into clusters with neurites that project together to the central neuropil. Densely stained glial cells are present and delineate the neuropil (Fig. 11A, B).

The posterior ganglion is composed of several fused divisions. Longitudinal fiber tracts extend through the length of this ganglion, and individual divisions can be discerned by the presence of transverse commissures (Fig. 11A). We identified six divisions in the posterior portion of this ganglion as thoracic divisions on the basis that paired nerve roots extend ventrally from each division toward the corresponding thoracic appendage (Figs. 2, 11A–C). We were unable to determine whether a seventh thoracic division, which might be expected in cirripedes (see Grygier, 1987), was present in the cyprid. Individual divisions are more difficult to distinguish in the anterior portion of the ganglion, which elongates laterally and is compressed longitudinally (Fig. 11A). However, we identified three divisions in this region (from two of the three preparations sectioned in the horizontal plane), which might reflect the presence of the three pairs of gnathopods that can be seen with either scanning electron microscopy or light microscopy (Fig. 12B).

Thoracic appendages and the caudal rami. Six pairs of thoracic appendages (thoracopods) and the paired caudal rami extend from the ventral surface of the thorax. The extrinsic muscles of thoracic appendages and the caudal rami attach dorsally in the thorax and can be seen in both

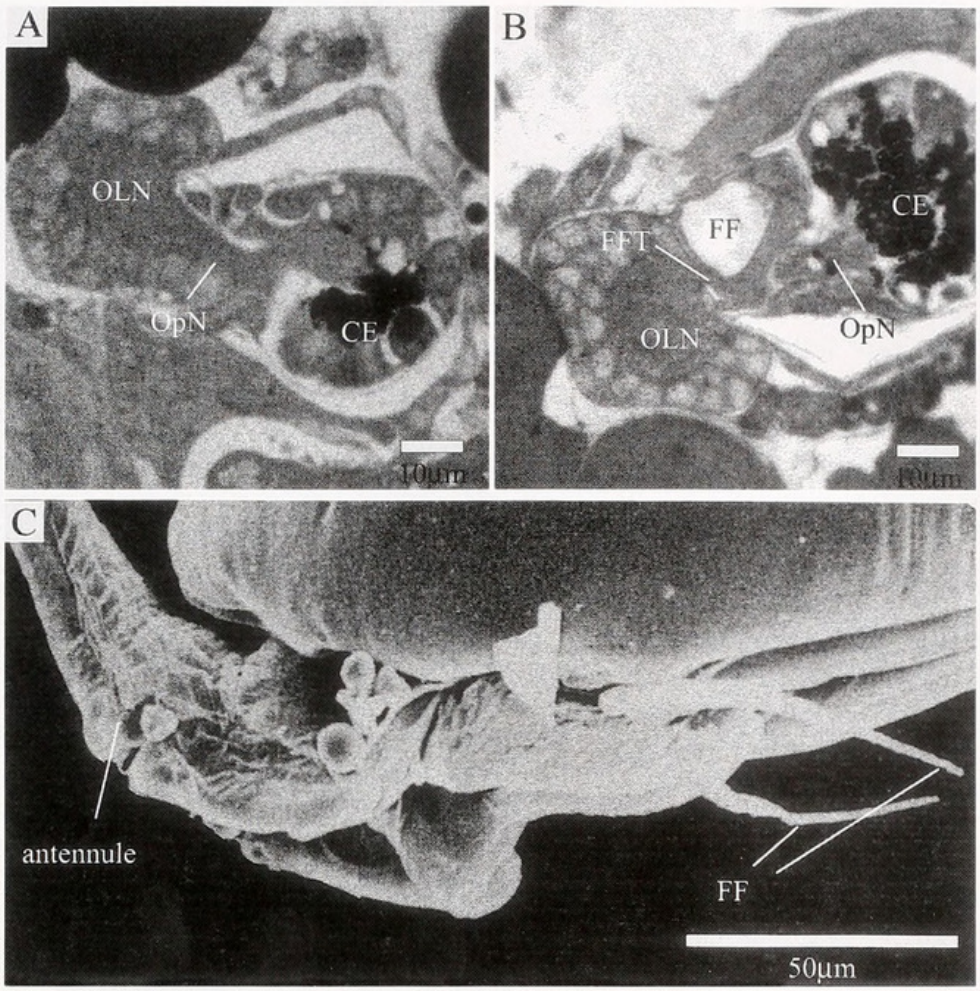

Figure 6. Compound eyes and frontal filaments. (A) The optic nerve (OpN) connects the compound eye (CE) to the optic lobe neuropil (OLN). (B) Frontal filaments are closely associated with compound eyes, and a transverse section through the base of a frontal filament (FF) is shown. The frontal filament tract (FFT) connects the frontal filament with the optic lobe neuropil proximal to the point of entry of the optic nerve (OpN) (see Fig. 5). (C) Scanning electron micrograph of the anterior ventral surface of a cyprid in which the cephalon is extended from the bivalve carapace. Frontal filaments (FF) extend from the ventral surface of the cephalon, posterior to the antennules.

horizontal and longitudinal sections (Fig. 11A, B). Paired nerve roots to the thoracic appendages extend from each thoracic division (Fig. 11B, C). Paired nerve roots extend to the caudal rami, but unlike those to thoracic appendages, are not associated with an obvious ganglionic division. Instead, these nerve roots appear to extend from a terminal loop of the longitudinal fibers (Fig. 11A, D).

Oral cone. The gnathopods of the cyprid form an oral cone, which opens to the ventral surface of the cephalon (Figs. 1, 2, 12A). The cyprid does not eat, and gnathopods are rudimentary during this stage (Walley, 1969). There are no obvious nerves connecting the gnathopods to the central nervous system in *B. amphitrite.* However, the posterior ganglion extends laterally in the region adjacent to the oral cone. It is likely that the three ganglionic divisions located adjacent to the oral cone reflect the presence of three pairs of gnathopods and are, therefore, referred to as subesophageal divisions.

Esophagus and digestive system. The digestive system of the cyprid is not fully developed (Walley, 1969). The esophagus has an oral opening (Fig. 12A), but we found no evidence of a rostral opening of the digestive system. In some sections the esophagus appears to be closed in the region where it passes between the cephalon and thorax (Fig. 12A, B), but it remains possible that this represents a sectioning artifact. Fine nerves can be traced from the dorsal surface of the subesophageal ganglionic divisions to the esophagus and midgut (Fig. 12B). These nerves are most obvious when they converge to pass between the cephalon and thorax, but they disperse among the cells surrounding the midgut (Fig. 12C).

Discussion

Our results show that the cypris larva of *B. amphitrite* has a well-developed nervous system that, in spite of being relatively small, contains the full complement of neural elements necessary for mediating complex interactions with

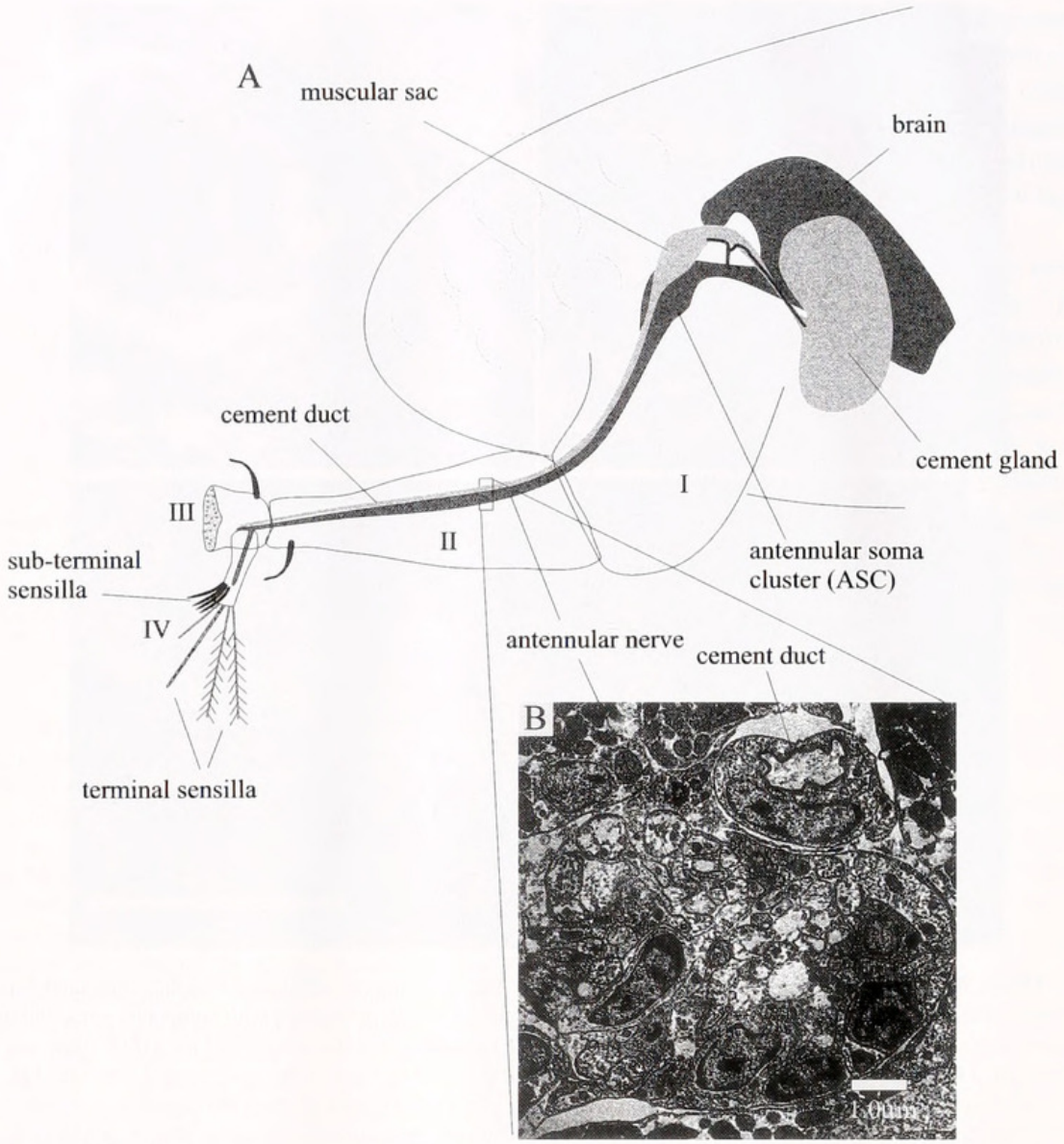

Figure 7. (A) Schematic drawing of the antennule to show the extension of the antennular nerve and its association with the collecting duct of the cement gland. The antennule has four segments (I–IV). The antennular nerve consists of neural processes associated with the distal setae (innervation of setae not drawn here, but see Figs. 8, 9). The antennular nerve is also expected to contain the motor neurons to the antennular musculature. The antennular soma cluster (ASC), located near the base of the antennule, contains bipolar cells, which are candidates for receptor cell somata whose dendrites innervate the distal setae (discussed in text). Midway between the ASC and the brain, a small branch of the antennular nerve extends to the muscular sac, a node of muscle surrounding the collecting duct of the cement gland, and a separate branch extends toward a cement gland.

the environment. The presence of regionalized neuropils, some of which clearly receive input from peripheral sensory structures, suggests a level of neural integration that goes beyond simple reflexive responses.

Observations of complex behavior displayed by cyprids during settlement support the claim that the nervous system has the capacity for more than simple reflex responses. For example, cyprids of *S. balanoides* settle gregariously in response to a proteinaceous cue associated with the cuticle of conspecifics (Crisp and Meadows, 1962; Gabbott and Larman, 1987). Upon encountering this cue, however, the cyprids' response is not indiscriminate. For example, in "favored" areas (those containing conspecifics), individual cyprids will still conduct a meticulous inspection phase and reject the substratum if the immediate barnacle density is too high or if the surface topography is inadequate (Crisp, 1961).

The cyprids' need to settle is reflected by the fact that the barnacle nervous system is most "complete" during the cyprid stage. The cyprid has a well-developed brain and a large investment in cephalic sense organs, whereas the brain is greatly reduced in the naupliar stages and almost completely absent in the adult barnacle (Walley, 1969). The "upgrade" from the nauplius to the cyprid nervous system is

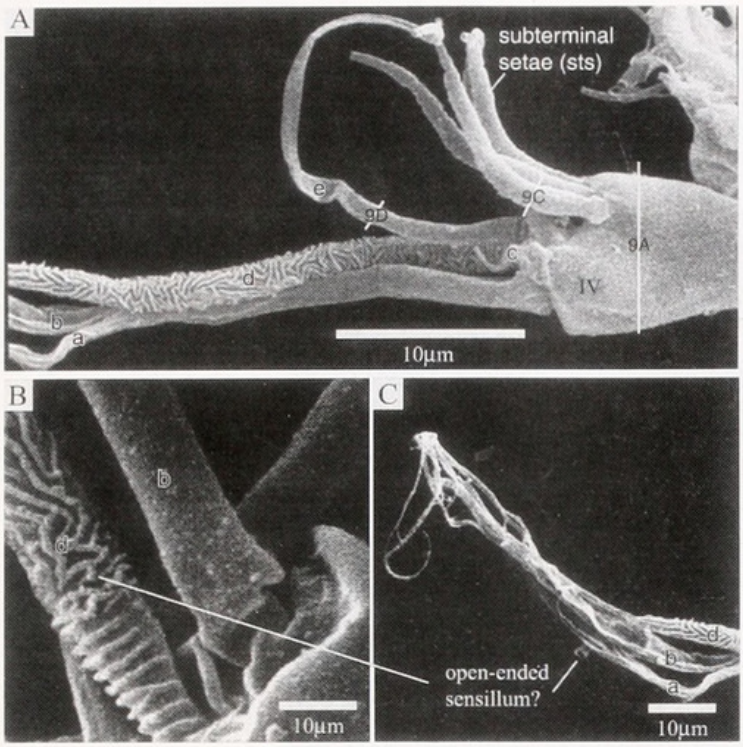

Figure 8. (A) The setae on the fourth antennular segment (IV) are arranged in terminal and subterminal rows. (A) The four subterminal setae (sts) do not articulate at the base. These are short and taper toward the tip with a general morphology similar to that of the aesthetasc (olfactory) sensilla described in other crustaceans. The terminal row consists of two plumose setae (a, b), a short, sickle-shaped seta (c), a longer "sculptured" seta with many cuticular ridges along its length (d), and a shorter unsculptured seta (e). Terminology following Clare and Nott (1994) and Glenner and Høeg (1995). (B) Higher power micrograph highlighting the articulating bases of two of the terminal setae (b, d). (C) High power of the distal tips of setae a, b, and d. The setules of a and b are folded back on themselves, and seta d appears open-ended. 9A, 9C, 9D in (A) refer to cross-sectional planes shown in Figure 9.

consistent with the cyprids' need to detect and actively respond to settlement cues. The restructuring to the adult nervous system (Walley, 1969), in which most of the anterior neuropil regions and cephalic sensory structures that we describe here degenerate, is presumably an adaptation to sedentary life.

The nervous system and associated structures

Neural input from cephalic sense organs is structurally organized in discrete neuropils within the cyprid brain (see Fig. 5). The cephalic sensory input in the cyprid can be summarized as follows: primary nerves from the median eye project to the dorsofrontal neuropil; primary nerves from each compound eye form an optic tract and project to the optic lobe; primary nerves from each frontal filament form a frontal filament tract and project also to the optic lobe; and primary nerves that innervate setae on the antennule project to the deutocerebrum.

Eyes and frontal filaments. Optic nerves connect the compound eyes with their adjacent optic lobe neuropils (Figs. 5, 6A, B). Under the light microscope, this neuropil appears to be unstructured, lacking the geometrically ordered segments seen in the optic lobes of decapods (Dahl, 1965). Nevertheless, compound eyes are morphologically well developed in the cyprid (Hallberg and Elofsson, 1983), and the fact that these eyes are present only during the cyprid stage is suggestive of a significant role in settlement. The exact function of these eyes is not yet known. Crisp (1955) argued, on the basis of the simple structure of these eyes, that image formation was unlikely, but suggested a role in mediating responses to fine-scale topographic features such as cracks and grooves. It is likely that compound eyes enable the detection of reflected light levels (Yule and Walker, 1984), thereby mediating light-guidance behavior (Barnes *et al.*, 1951). However, this function might equally be attributed to the median eye.

The frontal filament tract connects each of the frontal filaments with the adjacent optic lobe (Fig. 6B), proximal to the point of entry of the optic nerve (Figs. 5, 6A–B). The optic lobe of decapods includes neuropil divisions of the lamina ganglionaris, external and internal medullae, terminal medulla, and hemiellipsoid body (Dahl, 1965). We were unable to identify these divisions in the cyprid and have therefore chosen to use the general term of optic lobe. The exact nature of the frontal filaments and their function remains a contentious issue. Frontal organs are found in many Crustacea but, to date, frontal filaments of barnacle larvae have been considered only as pressure sensors on the basis of their suspected homology with the SPX organs (or organ of Bellonci) of Pericarida (Kauri, 1964; Walker, 1974).

Antennules. The antennules play a role as attachment organs during exploration and settlement (Nott and Foster, 1969) and have been implicated in the detection of chemical and physical cues (Nott and Foster, 1969; Walker, 1971; Clare *et al.*, 1994; Clare and Nott, 1994; Walker, 1995). The antennular nerve extends through the length of the antennule (Fig. 1B) and *during dissection* can be dissected into smaller individual bundles. Recently, electrical activity has been recorded from this nerve in response to chemical and mechanical stimulation of the distal segments of the antennule (Harrison, 1998).

The setae on the fourth antennular segment, and the nerves that innervate them, vary considerably in their morphology (Figs. 7–9). The functional properties of individual setae on this segment are not known. However, the innervation of segment IV suggests that these are sensilla, and some appear to be morphologically similar to chemoreceptors and mechanoreceptors of other Crustacea (Nott and Foster, 1969; Bush and Laverack, 1982; Heimann, 1984; Schmidt, 1989; Clare and Nott, 1994). The external morphology of the four subterminal setae, for example, is similar to that of the olfactory

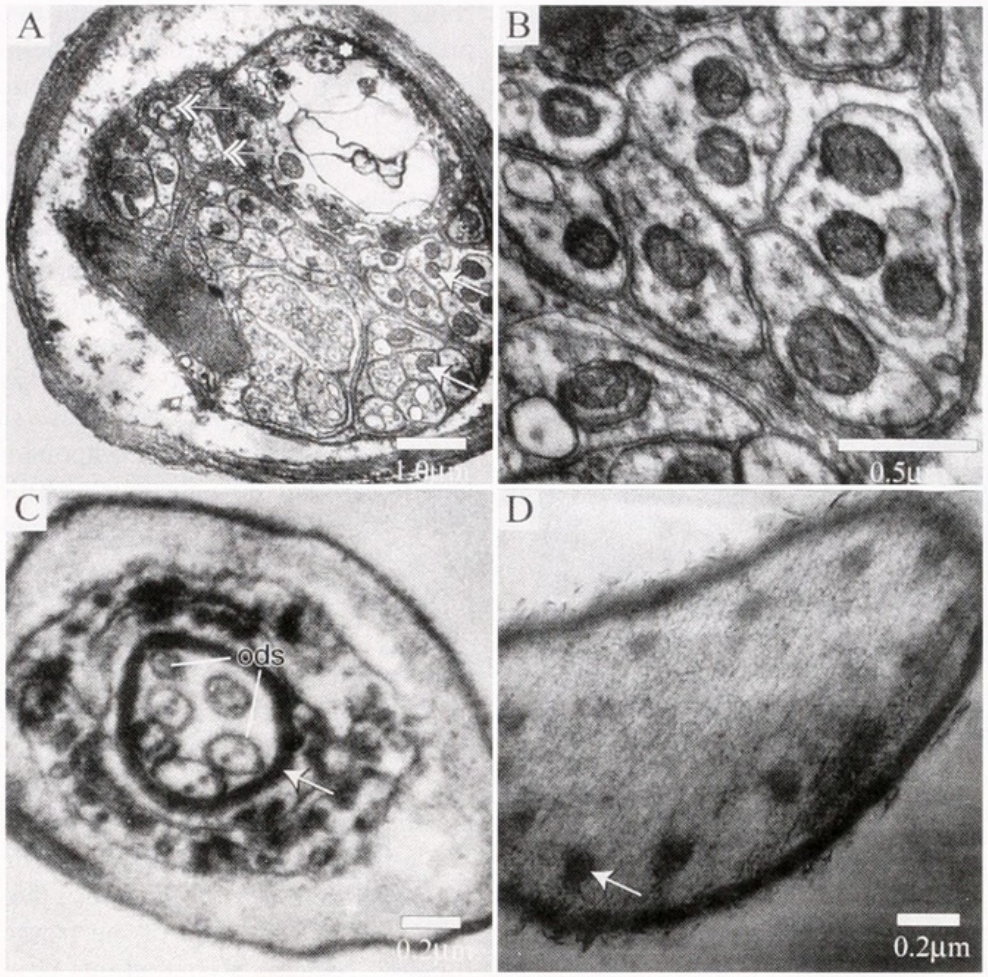

Figure 9. Innervation of antennular segment IV (see Fig. 8A for orientation). (A) The dendritic profiles in segment IV vary in diameter. Most profiles are between 0.5 and 1.0 μm in diameter and contain between one and three mitochondria (single arrow). Smaller-diameter processes (typically 0.1–0.3 μm in diameter) are also present (double arrows), and in some cases a 9 × 2 + 2 pattern of microtubules is apparent (*). (B) Enlargement of part of (A) to highlight the variation in the number of mitochondria in neural profiles. (C) Transverse section through a single subterminal seta showing the outer dendritic segments (ods) that extend into each of the subterminal setae. At this level, the dendrites are contained within an electron-dense tube (arrow), which is encircled by two glial cells. (D) A transverse section through seta e (see Fig. 8a) reveals densely stained peripheral structures (arrow) that possibly support this seta. Neural processes were not observed to extend into this seta, nor into any of the other terminal setae.

aesthetascs of Decapoda (Hallberg *et al.,* 1992; Clare and Nott, 1994). Furthermore, our results show that up to six outer dendritic segments (each 0.1–0.2 μm in diameter) project into the lumen of each subterminal seta (Fig. 9C). The outer dendritic segments are contained within a central cavity bordered by electron-dense material and a pair of ensheathing cells. This arrangement is similar to that reported for olfactory aesthetascs of crayfish (Tierney *et al.,* 1986).

It is generally accepted that the setae on the antennule include both chemoreceptors and mechanoreceptors (Clare and Nott, 1994; Clare, 1995), but the location of the somata of receptor cells has not been shown. In an effort to locate receptor cell somata, we traced serial sections of the antennule and were led to the bundle of cells that form the antennular soma cluster (Fig. 10). The cells in this bundle are 6–8 μm in length, 4–6 μm in width, and are located about 150 μm from the fourth segment of the antennule. Interestingly, the size and shape of these soma is again consistent with olfactory receptor neurons of many decapods (Laverack and Ardill, 1965; Snow, 1973; Tierney *et al.,* 1986; Hallberg *et al.,* 1992). These cells, however, are located at the base of the antennule in the cyprid and not, as in decapods, at the base of the sensilla.

The antennules are used for temporary attachment during surface exploration, which enables the cyprid to "walk" across the substratum (Nott and Foster, 1969; Walker, 1971). This involves the controlled release of cement from the adhesive disc *via* the antennulary glands (Nott and Foster, 1969; Walker, 1971; Okano *et al.,* 1996) and the coordination of motor activity. Motor neurons to the antennular musculature are expected to travel in the main branch of the antennular nerve. The

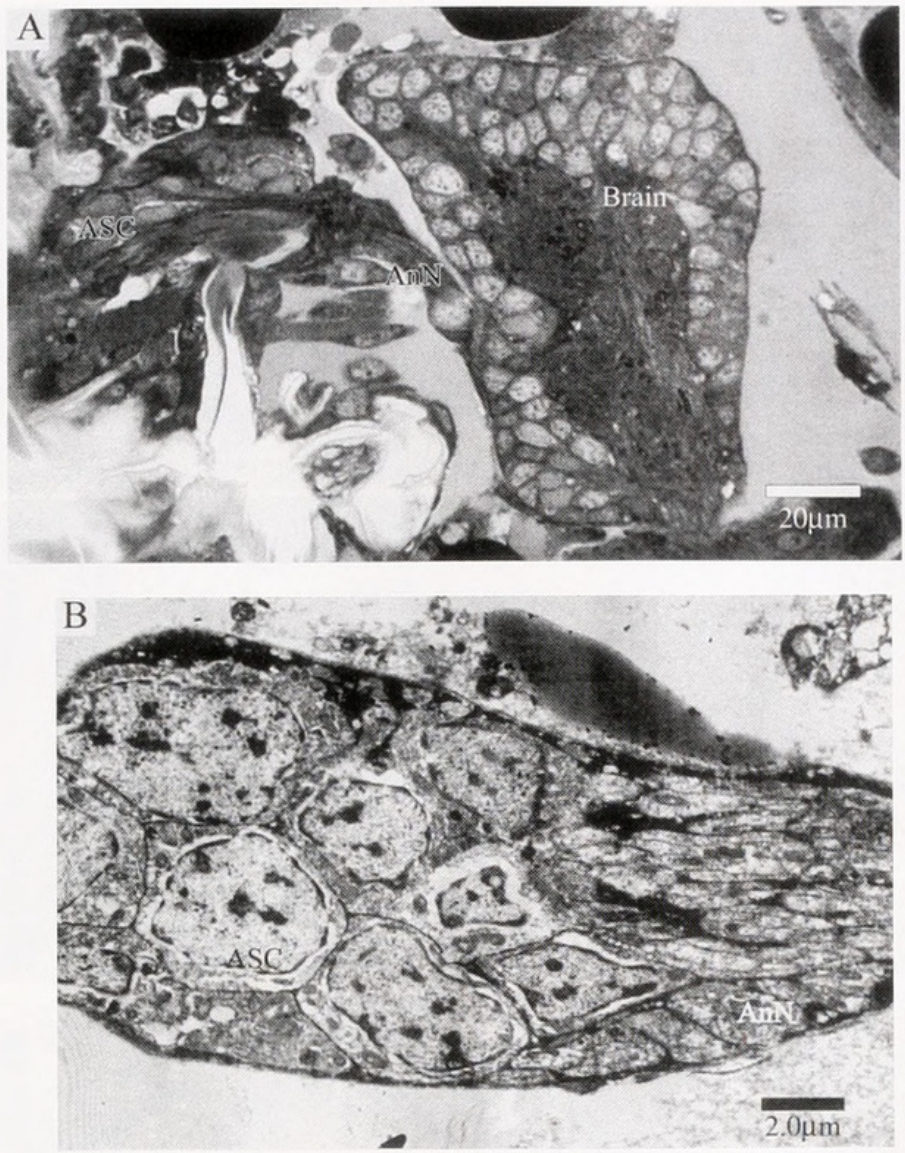

Figure 10. Longitudinal sections of the antennular soma cluster (ASC). (A) Light micrograph of the ASC shows its position relative to the brain, and association with the antennular nerve (AnN). The bipolar cells in this cluster are likely candidates for receptor cells that project to distal setae. (B) Electron micrographs of the ASC do not reveal synapses in this region, as would be expected for motor neurons, suggesting that these cells do not form a ganglion of antennular motorneurons as previously suggested (discussed in text).

location of efferent cell soma within the central nervous system is not known, but cells located ventrolateral to the median protocerebral neuropil that project anteriorly in the deutocerebrum are possible candidates (see Fig. 3B). The location of cells that control the release from the antennular glands for temporary attachment and the explosive release from cement glands for permanent attachment remains to be shown.

Thoracic appendages and caudal rami. The nerves that project to the thoracic appendages are, together with the antennular nerve, the most obvious peripheral extensions from the central nervous system. Thoracic appendages are used for swimming and bear many setae (Glenner and Høeg, 1995). These appendages, however, serve a natatory function, and it is not known whether the setae play a sensory role. Setae are also present on the caudal rami (Walker and Lee, 1976; Glenner and Høeg, 1995), and behavioral observations suggest that caudal rami might play a sensory role (Crisp and Barnes, 1954).

The nervous system and settlement

Cyprids settle in response to a range of environmental cues. It follows that the cyprid nervous system must sort and process input from various sense organs, and coordinate an appropriate behavioral response. We have traced neural connections between the central nervous system and many of the peripheral sense organs, but connections to the lattice organs (Jensen *et al.,* 1994b; Høeg *et al.,* 1998) and other sensory structures on the carapace remain to be shown. The small size of the cyprid raises questions about the behavioral capacity of this

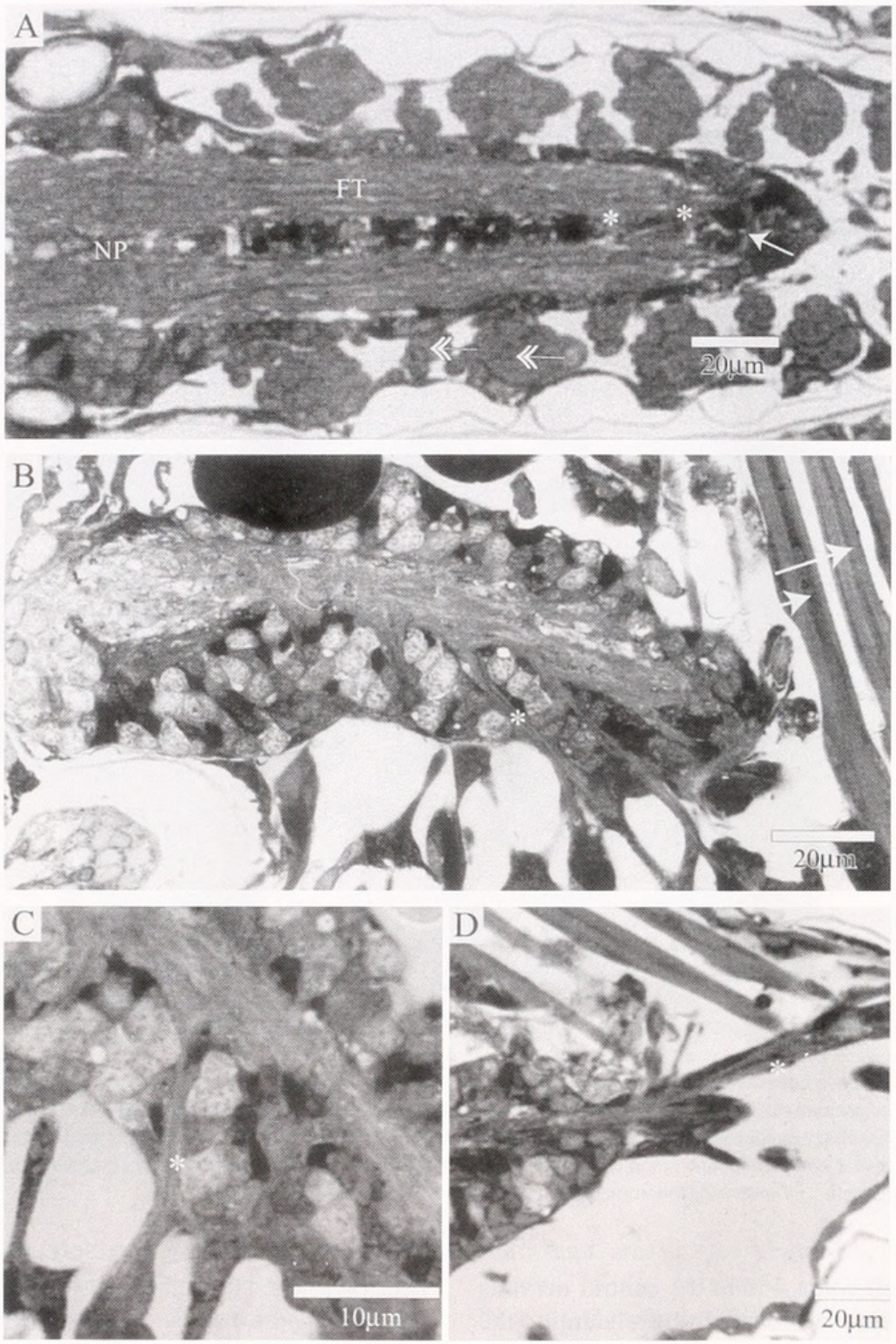

Figure 11. Light micrographs of the posterior ganglion. (A) Horizontal section showing regions of neuropil (NP), parallel fiber tracts (FT) and transverse fiber tracts (*). The transverse fibers distinguish individual divisions of this ganglion. The parallel fiber tracts end in a terminal loop (arrow). Extrinsic muscles of the thoracic appendages are seen in cross-section (double arrows). (B) A longitudinal section through the posterior ganglion. Paired nerve roots (*) extend from the ventral portion of each ganglionic division to the corresponding thoracic appendage. Extrinsic muscles of the thoracic appendages are seen (arrows). (C) Higher power of a thoracic nerve root (*) extending ventrally from the posterior ganglion. (D) Paired nerve roots (*) extend from the terminal loop of the transverse fiber tract to the caudal rami.

organism (Rittschof *et al.*, 1998). However, the large investment in sensory structures, each of which links to a discrete neuropil within the brain, suggests that the cyprid nervous system has the capacity for a relatively sophisticated level of neural processing.

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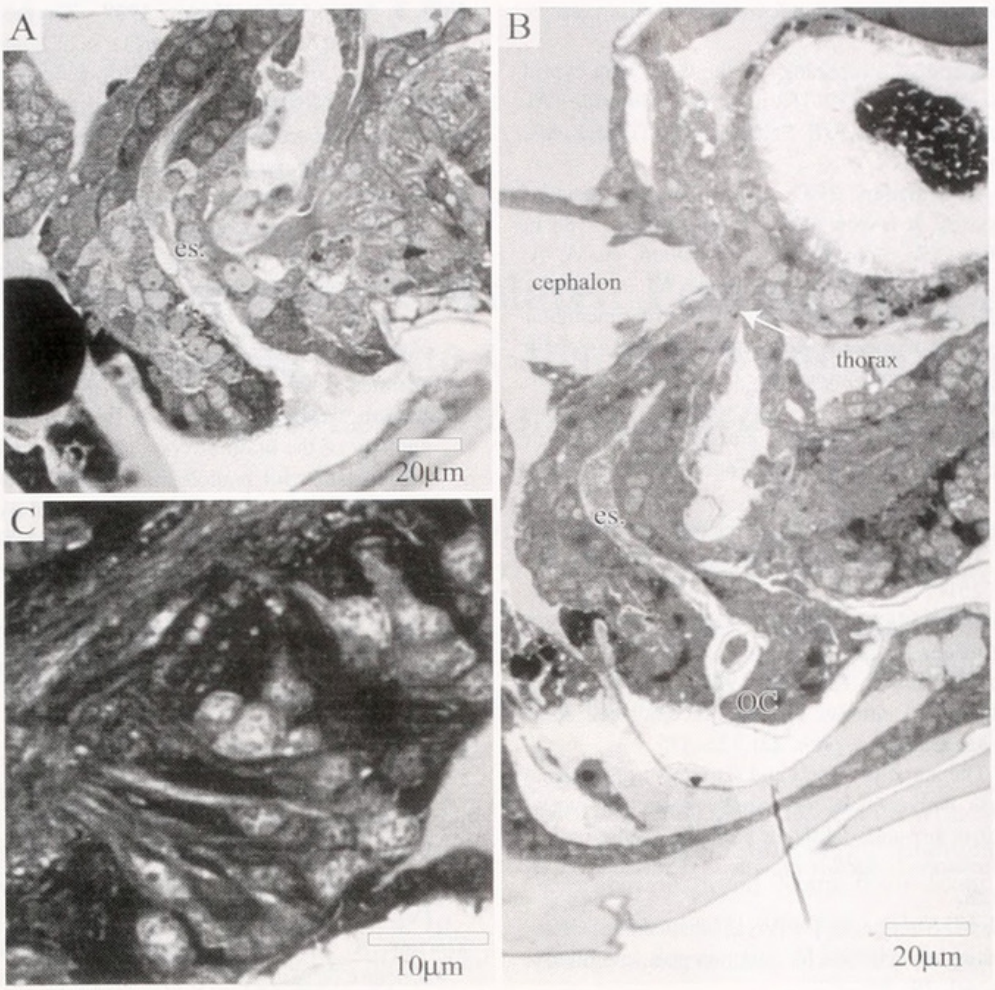

Figure 12. Light micrographs showing possible innervation of the esophagus and gut. (A–B) The esophagus of the cyprid opens on the ventral surface through the oral cone (consisting of vestigial mouthpart appendages). In these sections the esophagus appears to be closed in the region where it passes between the cephalon and thorax. Nerve roots arising from the anterior ganglionic divisions can be traced along the length of the esophagus and extend toward the gut (arrows). Innervation of mouthpart appendages was not observed. (C) The neurites of ventrally located cells project to the neuropil of anterior ganglionic divisions, but few nerves could be traced out of this region.

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