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Evolutionary Changes in the Olfactory Projection Neuron Pathways of Eumalacostracan Crustaceans

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ABSTRACT

Output from the olfactory lobe (primary olfactory center) of eumalacostracan crustaceans is transmitted to the medulla terminalis (MT) and hemiellipsoid body (HB) in the lateral protocerebrum (higher order center) by a large population of projection neurons. In eureptantian crustaceans (lobsters, crayfish, and crabs), these projection neurons also form the output pathway from an additional neuropil, the accessory lobe (higher order center), which appears to have arisen de novo in these animals. In a previous study of lobsters and crayfish we showed that whereas projection neurons innervating the olfactory lobe project primarily to the MT, those innervating the accessory lobe project exclusively to the HB (Sullivan and Beltz [2001a] J. Comp. Neurol. 441:9–22). In the present study, we used focal dye injections to examine the olfactory projection neuron pathways of representatives of four eumalacostracan taxa (Stomatopoda, Dendrobranchiata, Caridea, and Stenopodidea) that diverged from the eureptantian line prior to the appearance of the accessory lobe. These experiments were undertaken both to examine the evolution of the olfactory pathway in the Eumalacostraca and to provide insights into the changes in this pathway that accompanied the appearance of the accessory lobe. The innervation patterns of the olfactory projection neurons of the species examined were found to differ markedly, varying from that observed in the most basal taxon examined (Stomatopoda), in which the neurons primarily project to the MT, to those observed in the two highest taxa examined (Caridea and Stenopodidea), in which they primarily target the HB. These results suggest that substantial changes in the relative importance of the MT and HB within the olfactory pathway have occurred during the evolution of the Eumalacostraca. J. Comp. Neurol. 470:25–38, 2004.

Indexing terms: accessory lobe; olfactory pathway; olfactory projection neuron

Olfactory cues serve important functions in the biology of eumalacostracan crustaceans, playing central roles in feeding, mating, and territorial behaviors. Correspondingly, the primary olfactory neuropil, the olfactory lobe, is one of the most prominent neuropils in the brains of these animals. The lateral protocerebrum of eumalacostracan crustaceans has long been recognized as another important brain center in the olfactory pathway, as it is the target of the output tract from the olfactory lobe (Fig. 1; Hanström, 1924, 1925, 1931, 1947; Blaustein et al., 1988; Sandeman et al., 1993; Sullivan and Beltz 2001a, b). The importance of the lateral protocerebrum in the olfactory pathway has also been underlined by ablation experiments suggesting that this region is involved in discriminating food from nonfood items and in the control of feeding behaviors (Maynard and Dingle, 1963; Maynard and Yager, 1968; Maynard and Sallee, 1970; Hazlett, 1971). In addition to having strong connections with the olfactory lobe, the lateral protocerebrum also has connections with the optic neuropils (Hanström, 1925; Mellon, 2000; Sullivan and Beltz, 2001b) and is therefore thought to be an associative brain center.

The lateral protocerebrum is located proximal to the optic neuropils (lamina ganglionaris, medulla externa, and medulla interna) and is comprised of two main regions: the medulla terminalis and the hemiellipsoid body

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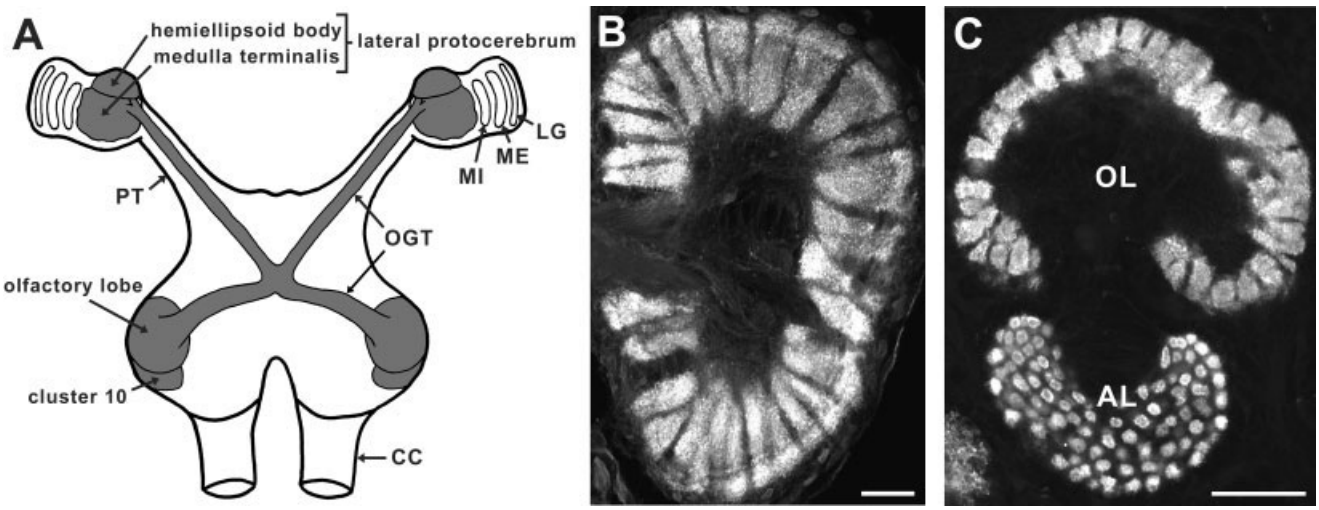


Fig. 1. Morphology of the brain of the eumalacostracan crustaceans examined in the present study. **A:** Schematic diagram outlining the central olfactory pathway. The axons of olfactory receptor neurons project ipsilaterally to the glomeruli of the OL. The OL is also innervated by a large population of projection neurons whose cell bodies are located lateral to the neuropils in a dense cluster, known as cluster 10. The axons of the projection neurons form a large tract, the OGT, which projects bilaterally to both the hemiellipsoid body and medulla terminalis. These two neuropils form the lateral protocerebrum. Not to scale. **B:** Synapsin labeling of the olfactory lobe of the caridean shrimp *Palaemonetes pugio* (Decapoda, Pleocyemata, Caridea). The olfactory lobe of *P. pugio*, as in other malacostracans, is comprised of columnar glomeruli arranged radially around the lobe. **C:** Synapsin labeling of the olfactory and accessory lobes of a eureptantian decapod, the ghost shrimp *Trypaea australiensis*. Abbreviations: AL, accessory lobe; CC, circumesophageal connectives; cluster 10, cell body cluster of the projection neurons innervating the olfactory lobe; LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; OGT, olfactory globular tract; OL, olfactory lobe; PT, protocerebral tract. Terminology from Hanström, 1947 and Sandeman et al., 1992. Scale bars = 100 μm in B,C.

(Fig. 1A; Sandeman et al., 1992, 1993). The medulla terminalis is a complex of several interconnected neuropil regions, some of which are glomerular in structure whereas others are unstructured (Hanström, 1925, 1931, 1947; Blaustein et al., 1988; Sullivan and Beltz, 2001a). The hemiellipsoid body was first described in the stomatopod *Squilla mantis* by Bellonci (1882) as the *corpo emielissoidale*. Although hemiellipsoid bodies have subsequently been described in a number of other eumalacostracan taxa, both the size and anatomy of this neuropil can differ markedly between species (Hanström, 1924, 1925, 1931, 1947; Blaustein et al., 1988; Sandeman et al., 1993; Strausfeld, 1998; Sullivan and Beltz, 2001a). In most species, however, the hemiellipsoid body is recognizable as a hemispherical complex of two or more layered or glomerular neuropil regions situated medial to the medulla terminalis.

The olfactory pathway of eumalacostracan crustaceans originates in the olfactory organ which is comprised of arrays of specialized sensilla, known as aesthetascs, located along the lateral flagella of the first antennae. The axons of the olfactory receptor neurons innervating these sensilla project ipsilaterally to the deutocerebrum where they terminate within the highly structured, glomerular neuropil of the olfactory lobe (Fig. 1B). The olfactory lobe glomeruli are sites of synaptic contact between the receptor neurons, local interneurons, and projection neurons. The main output pathway from the olfactory lobe is provided by the axons of the projection neurons, whose somata lie adjacent to the lobe in a densely packed cluster, known as cluster 10 (Sandeman et al., 1992). Upon leaving the olfactory lobe, the axons of these neurons form a large tract, known as the olfactory globular tract (OGT; Hanström, 1925), which bifurcates at the midline of the brain before projecting bilaterally to the lateral protocerebrum (Fig. 1A).

In eureptantian decapods (lobsters, crayfish, ghost shrimp, and crabs) an additional deutocerebral lobe, known as the accessory lobe, is also involved in the processing of olfactory inputs (Fig. 1C; Arbas et al., 1988; Mellon and Alones, 1994; Wachowiak et al., 1996). Accessory lobes appear to have arisen de novo in the Eureptantia (Fig. 2; Sandeman et al., 1993; Derby et al., 2003) and are among the most prominent neuropils in the brains of lobsters, crayfish, and ghost shrimp (Blaustein et al., 1988; Sandeman et al., 1993; Sandeman and Scholtz, 1995). The accessory lobe is comprised of small, spherical glomeruli (Fig. 1C; Hanström, 1925; Maynard, 1971; Sandeman and Luff, 1973; Blaustein et al., 1988; Sandeman et al., 1993) and is not innervated by the processes of either primary sensory neurons or motoneurons (Blaustein et al., 1988; Sandeman et al., 1992, 1995).

The anatomy and physiology of neurons innervating the accessory lobe have been most extensively examined in spiny lobsters and crayfish. These studies have shown that the accessory lobe receives olfactory inputs from local interneurons innervating both the accessory lobe and the ipsilateral olfactory lobe (Arbas et al., 1988; Mellon and Alones, 1994; Wachowiak et al., 1996). The bilateral accessory lobes are also joined by a large tract, known as the deutocerebral commissure, which is comprised of the axons of a large population of interneurons that provide the lobes with visual, tactile, and olfactory inputs (Sandeman et al., 1995; Wachowiak et al., 1996). In contrast, therefore, to the olfactory lobe, which receives primary olfactory inputs, the accessory lobe appears to be involved in the processing of higher order multimodal inputs.

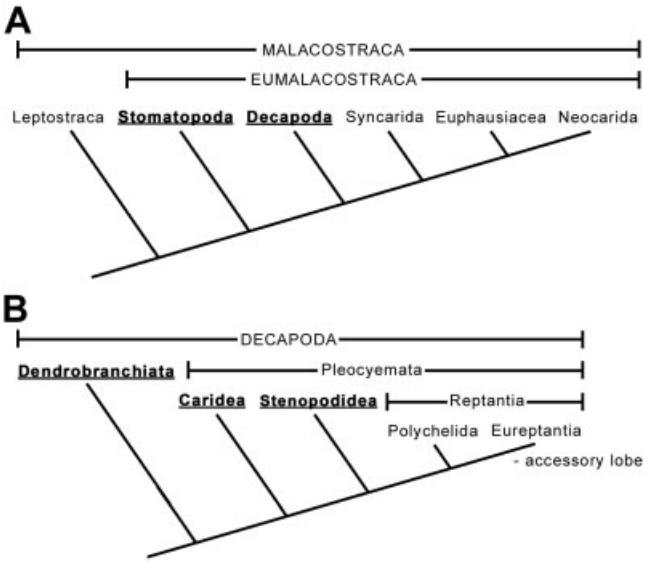


Fig. 2. Phylogenetic relations within the Malacostraca (**A**) and Decapoda (**B**) according to Scholtz and Richter (1995) and Richter and Scholtz (2001). Representatives of the taxa shown in bold were examined in the present study. The Neocarida is comprised of the Pancarida, Mysidacea, and Amphipoda + Mancoida.

Anatomical studies have shown that the accessory lobe is also innervated by projection neurons whose somata reside in cluster 10 and whose axons comprise part of the OGT (Mellon et al., 1992; Wachowiak and Ache, 1994; Schmidt and Ache, 1996; Wachowiak et al., 1996; Sullivan et al., 2000). In eureptantian decapods, therefore, the OGT forms the output pathway from both the olfactory and accessory lobes. Recent anatomical tracing studies in the brains of crayfish (*Procambarus clarkii* and *Orconectes rusticus*) and clawed lobsters (*Homarus americanus*) have shown that the output pathways from the olfactory and accessory lobes target different regions of the lateral protocerebrum (Sullivan and Beltz, 2001a). In all three species, projection neurons innervating the accessory lobe were found to project exclusively to the hemiellipsoid body. In contrast, the projection neuron pathway from the olfactory lobe innervates neuropil regions of the medulla terminalis adjacent to the base of the hemiellipsoid body. The olfactory lobe projection neuron pathway of *H. americanus* has an additional branch that projects into the hemiellipsoid body (Sullivan and Beltz, 2001a).

Gross anatomical studies of the brains of non-eureptantian eumalacostracans, such as stomatopods and shrimp, have shown that in a number of species the OGT terminates in both the medulla terminalis and the hemiellipsoid body (Bellonci, 1882; Hanström, 1931, 1947). As these crustaceans do not possess accessory lobes, the OGT in these animals is the output pathway of the olfactory lobe alone. The hemiellipsoid bodies of these animals may function, therefore, primarily as second-order olfactory neuropils, whereas in crayfish and lobsters the hemiellipsoid body primarily receives higher order multimodal inputs via the OGT. In the present study, we used focal dye injections into the olfactory lobes of representatives of four eumalacostracan taxa that diverged from the eureptantian line before the appearance of the accessory lobe (Fig. 2) to examine the projection patterns of the OGT in these animals. These experiments were undertaken both to examine the evolution of the olfactory pathway within the Eumalacostraca and to gain insights into the changes in this pathway that accompanied the appearance of the accessory lobe.

We show that marked differences exist between eumalacostracan species in the projections of the OGT to the medulla terminalis and hemiellipsoid body. These differences suggest that substantial changes in the relative importance of these two neuropils in the olfactory pathway have occurred during the phylogeny of these animals.

MATERIALS AND METHODS

Animals

In this study, we examined the olfactory pathways of a representative species of the Stomatopoda (*Gonodactylus bredini)*, a basal eumalacastracan taxon (Fig. 2), as well as species representing each of the three major non-reptantian decapod taxa (Fig. 2): the Dendrobranchiata (*Penaeus duorarum*), the Caridea (*Palaemonetes pugio*), and the Stenopodidea (*Stenopus hispidus*). Male and female specimens of the four species were obtained from commercial suppliers in the USA. Mantis shrimp, *Gonodactylus bredini*, and banded coral shrimp, *Stenopus hispidus*, were obtained from Carolina Biological Supply Company (Burlington, NC), pink shrimp *Penaeus duorarum* from Gulf Specimen Marine Laboratories (Panacea, FL), and grass shrimp *Palaemonetes pugio* from the Aquatic Resources Center at the Marine Biological Laboratories (Woods Hole, MA).

Neuroanatomy of the lateral protocerebrum

To examine the anatomy of the lateral protocerebral neuropils of the four species, their brains were stained immunocytochemically by using an antibody against synapsin, a protein found in neuronal terminals. This antibody selectively labels neuropil and was selected both to characterize the morphology of the lateral protocerebrum and to aid in determining the boundaries between the hemiellipsoid body and the medulla terminalis, as there is no distinct border between these two regions in many decapod species (Hanström, 1947). In characterizing the lateral protocerebra of the species examined in the present study we have utilized the distinguishing features outlined by Sandeman et al. (1993): the medulla terminalis is a complex neuropil that is comprised of a number of contiguous subregions but is not geometrically organized, whereas the hemiellipsoid body is a hemispherical complex of layered or glomerular neuropil regions positioned medial to the medulla terminalis.

Brains, including the optic ganglia, were removed from the animals under cold saline, desheathed, and fixed for 1–2 days in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at 4°C. Subsequently, preparations were rinsed for 4 hours in 0.1 M PB, suspended in 6% Noble agar (DIFCO, Detroit, MI), and sectioned at 100 μm on a vibratome (Technical Products, St. Louis, MO). Sections were rinsed in PB containing 0.3% Triton X-100 (PBTx) for 4 hours and then incubated for 2 days in the SYNORF1 antibody (mouse anti-*Drosophila* synapsin; 1:50; provided by E. Buchner, Universität Würzburg, Germany) diluted in PBTx for 2 days at 4°C. Following incubation in the primary antibody, tissues were rinsed over several hours in PBTx and then incubated overnight at

4°C in an Alexa 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR) diluted 1:50 in PBTx. Subsequently, the sections were rinsed for 3 hours in PB, mounted in Gelmount (Biømedia, Foster City, CA), and viewed using a Leica scanning confocal microscope.

Projection neuron labeling

Animals were anesthetized by immersion in crushed ice for 5–10 minutes and then decapitated.

The entire brain, including the optic ganglia, was dissected free from the excised head in cold saline and desheathed. Isolated brains were placed in a well of cold saline on a slide coated with poly-L-lysine (0.01% in ddH_2O; Sigma) and viewed by using a fixed-stage Nikon compound microscope equipped with Nomarski optics. The morphology of the olfactory lobe projection neurons within the lateral protocerebrum was examined by focal injections of the lipophilic tracer DiA (Molecular Probes) into the olfactory lobe. Microelectrodes were backfilled with a saturated solution of DiA in 100% ethanol, which was then pressure injected into the lobe. Following the dye injections, brains were fixed for 24 hours in 4% paraformaldehyde in 0.1 M PB (pH 7.4) at room temperature. The preparations were then placed in fresh fixative and incubated in the dark at 37°C for 10–30 days to allow the dyes to travel along the lengths of the projection neurons. Subsequently, the brains were rinsed for 4 hours in 0.1 M PB, suspended in 6% Noble agar, and sectioned at 100 μm on a vibratome. Sections were then mounted in Gelmount and viewed by using a Leica scanning confocal microscope.

Confocal microscopy and image processing

Specimens were viewed with a Leica TCS SP laser-scanning confocal microscope equipped with an argon laser. Serial optical sections were taken at intervals of 1.5 μm and were saved as both three-dimensional stacks and two-dimensional projections. Images were processed to adjust brightness and contrast using Paint Shop Pro 4.12 (JASC) and Adobe Photoshop 5.0 (Adobe Systems).

RESULTS

The lateral protocerebral neuropils of the four species examined are illustrated schematically in Figure 3. Although the morphologies and orientations of these neuropils were ascertained by examination of the synapsin-labeled sections that follow (Figs. 4, 6, 8, 10), we have placed this figure before them as a reference to aid in the interpretation of these images.

Gonodactylus bredini (Stomatopoda, Gonodactylidae)—stomatopod/ mantis shrimp

Neuroanatomy of the lateral protocerebrum. The neuroanatomy of the lateral protocerebrum of a stomatopod was first described for *Squilla mantis* by Bellonci (1882). Bellonci identified four distinct neuropil regions within the lateral protocerebrum: the *corpo emielissoidale* (hemiellipsoid body), *corpo allungato*, *corpo reniforme*, and the *massa centrale*. The first three of these neuropil regions are readily identifiable, based on their relative positions and morphologies, in synapsin-labeled sections of the lateral protocerebrum of *Gonodactylus bredini* (Figs. 3A, 4). Bellonci (1882) did not provide a description of the anatomy of the *massa centrale*, and it is unclear from his drawings whether this term was meant to describe the remainder of the lateral protocerebrum, i.e., the medulla terminalis, or a particular neuropil region therein. This term is therefore not used in the present description of the lateral protocerebrum of *G. bredini*.

The anatomy of the hemiellipsoid body of *G. bredini* resembles that of the lobster *H. americanus* (Sullivan and Beltz, 2001a) in being comprised of a hemielliptical, concave sheet of neuropil (cap neuropil) surmounting an inner region of neuropil (core neuropil; Fig. 4A,B). The cap neuropil of *G. bredini* is a thin, crenulated layer of neuropil that is closely opposed to the much larger core neuropil, which is composed of a number of layers exhibiting different intensities of synapsin staining. As in *S. mantis*, the *corpo allungato* of *G. bredini* is a prominent neuropil region located at the caudal margin of the lateral protocerebrum. The *corpo allungato* of *G. bredini* has a distinctive "S" shape in cross sections (Fig. 4D) and is associated with several smaller, satellite neuropils (Fig. 4C,D). The *corpo reniforme* of *G. bredini* is not as prominent as that of *S. mantis* but is located in the same location, lateral to the medulla terminalis (Fig. 4C).

Neuroanatomy of the olfactory lobe output pathway. Focal injections of DiA into the olfactory lobe of *G. bredini* showed that the projection neuron tract from this lobe bifurcates in the center of the brain before projecting bilaterally to the lateral protocerebrum. Within the lateral protocerebrum, the tract initially arborizes extensively within a rostral region of the medulla terminalis adjacent to the medulla interna (Fig. 5A,B). The branching of the projection neuron tract within this region is generally diffuse, but fine branches do arise that innervate discrete neuropilar regions nearby (Fig. 5A,B,D). An additional fine branch of the OGT projects medially to the rostral base of the hemiellipsoid body from where it projects caudally, innervating a peripheral layer of the hemiellipsoid body core neuropil (arrowheads in Fig. 5B,C). No asymmetries were observed in the bilateral projections of the tract, as were observed in the crayfish *P. clarkii* (Sullivan and Beltz, 2001a).

Penaeus duorarum (Decapoda, Dendrobranchiata)—pink shrimp

Neuroanatomy of the lateral protocerebrum. In contrast to that of *G. bredini*, the lateral protocerebrum of *P. duorarum* is poorly differentiated (Figs. 3B, 6). Discrete, identifiable neuropil regions, such as the *corpo allungato* and *corpo reniforme* of stomatopods, are not readily apparent in synapsin-labeled sections of the lateral protocerebrum of *P. duorarum*. The hemiellipsoid body of *P. duorarum* is also poorly differentiated, with the border between the hemiellipsoid body and the medulla terminalis only discernable in cross sections of the lateral protocerebrum (Fig. 6B). The hemiellipsoid body is located ventromedially and is comprised of a single region of unstructured neuropil.

Neuroanatomy of the olfactory lobe output pathway. Focal injections of DiA into the olfactory lobe of *P. duorarum* showed that, as in *G. bredini*, the output tract from this lobe bifurcates in the center of the brain before projecting bilaterally to rostrodorsal regions of the medulla terminalis where it branches diffusely (Fig. 7A). Two branches of the tract then arise that project dorsally, one medial and the other lateral to a circular neuropil region, and extensively innervate the hemiellipsoid body (Fig. 7C,D). The medial and lateral branches appear to inner-

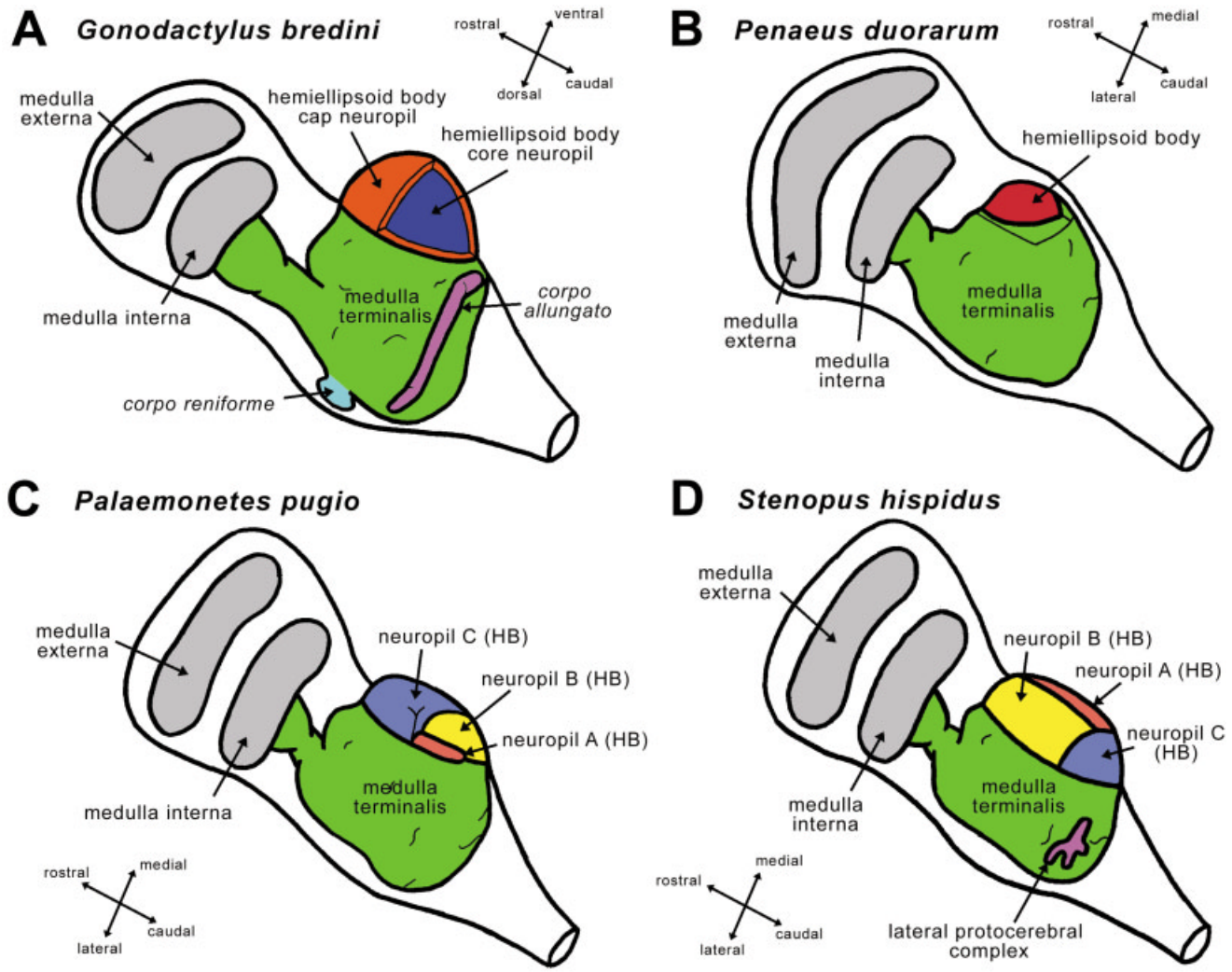


Fig. 3. Schematic diagrams illustrating the arrangement of the lateral protocerebral and optic neuropils of *Gonodactylus bredini* (**A**), *Penaeus duorarum* (**B**), *Palaemonetes pugio* (**C**), and *Stenopus hispidus* (**D**). The rostral-most optic neuropil, the lamina ganglionaris, is not shown. HB, hemiellipsoid body.

vate two distinct regions of the hemiellipsoid body (Fig. 7D), suggesting that this neuropil may possess some substructure that is not apparent in synapsin-labeled sections. The innervation pattern resembles that of the crayfish *Procambarus clarkii* and *Orconectes rusticus*, whose hemiellipsoid bodies are comprised of two lobes that lie adjacent to one another in the dorsoventral plane. The hemiellipsoid body of the crayfish, however, is innervated by the output tract from the accessory lobe (Sullivan and Beltz, 2001a).

Palaemonetes pugio (Decapoda, Pleocyemata, Caridea)—grass shrimp

Neuroanatomy of the lateral protocerebrum. The hemiellipsoid body of *Palaemonetes pugio* is a prominent structure comprised of three interconnected neuropil regions that lie medial to the medulla terminalis (Figs. 3C, 8). The arrangement and anatomy of these neuropils differ markedly from those previously described in other crustaceans, and we have therefore adopted a new nomenclature to describe the neuropils, naming them neuropils A, B, and C. The largest of the three neuropils is neuropil C, which lies rostral to neuropils A and B and exhibits prominent bands of neuropil showing different intensities of synapsin labelling (Fig. 8). Neuropil A is the smallest neuropil and lies on the dorsal surface of the lateral protocerebrum, rostral to neuropil C. Neuropil A is characterized by a band of lightly synapsin stained neuropil flanked by two bands of intensely labeled neuropil (arrowheads in Fig. 8A,B). Neuropil B is situated ventrally, rostral to neuropil C, and contains regions that differ in the intensity of their synapsin labeling but do not form the banded patterns characteristic of neuropils A and C (Fig. 8A,C,D).

Neuroanatomy of the olfactory lobe output pathway Dye injections into the olfactory lobe of *P. pugio* showed that the olfactory lobe output pathway in this shrimp primarily targets the hemiellipsoid body (Fig. 9). As in the previous species examined, the OGT of *P. pugio* bifurcates in the center of the brain and then projects bilaterally,

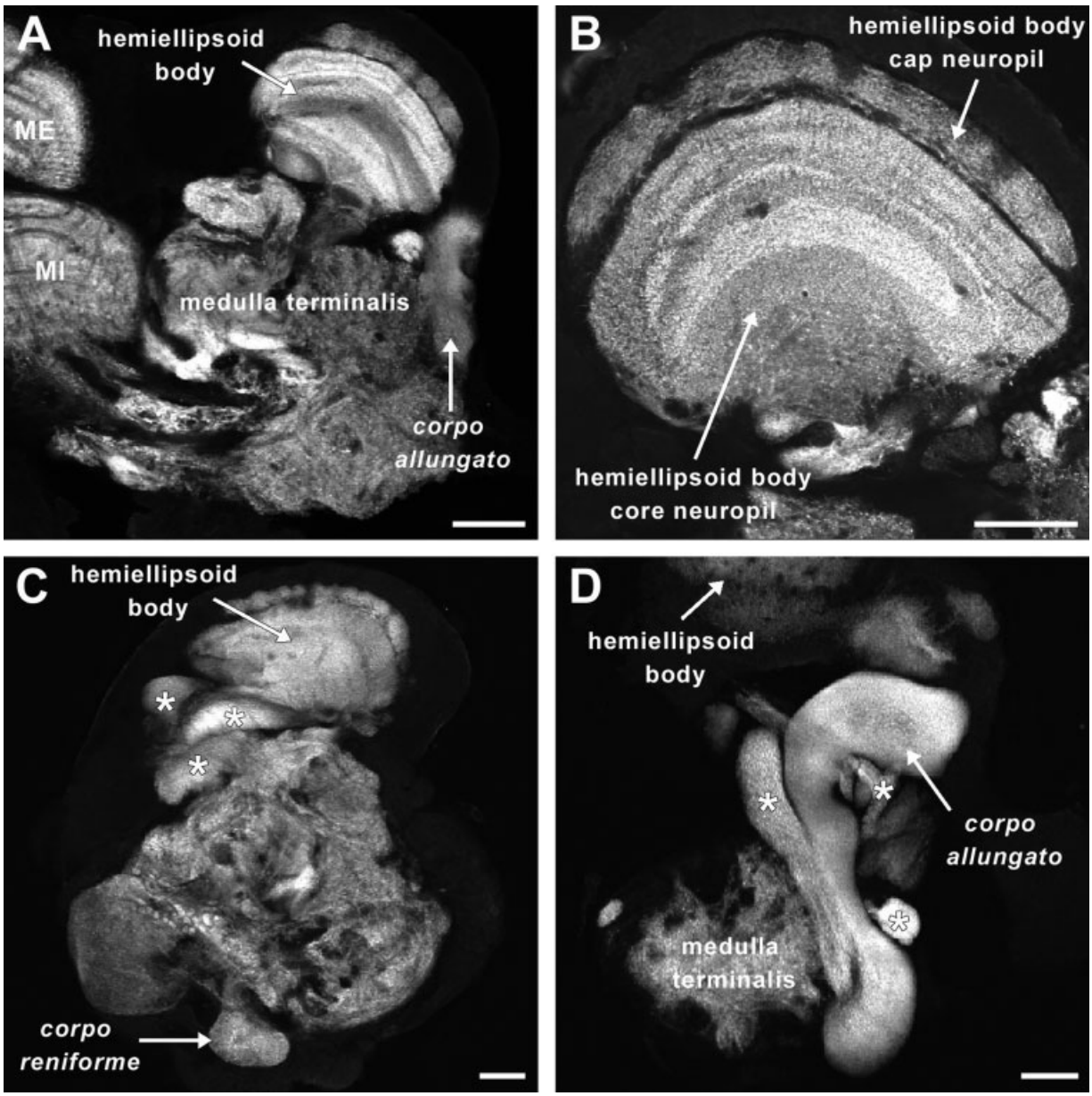


Fig. 4. Neuroanatomy of the lateral protocerebrum of the stomatopod *Gonodactylus bredini* (Stomatopoda, Gonodactylidae). The orientation of the lateral protocerebrum of *G. bredini* differs from that of most eumalacostracans in that the hemiellipsoid body lies ventral to the medulla terminalis rather than medial. **A,B:** Longitudinal sections through the lateral protocerebrum of *G. bredini* stained by using an antibody against *Drosophila* synapsin. Rostral is to the left and ventral is at the top. **C,D:** Synapsin immunoreactivity in cross sections midway through (C) and at the caudal margin of (D) the lateral protocerebrum of *G. bredini*. The satellite neuropils of the *corpo allungato* are indicated by the white asterisks. Ventral is at the top. Abbreviations: ME, medulla externa; MI, medulla interna. Scale bars = 100 μm in A–D.

entering the lateral protocerebrum dorsally. Unlike in *G. bredini* and *P. pugio*, the labeled projection neuron tract does not branch extensively in the medulla terminalis but rather projects primarily to the hemiellipsoid body, where it extensively innervates neuropil C and peripheral regions of neuropil B (Fig. 9). No innervation of neuropil A was observed.

Stenopus hispidus (Decapoda, Pleocyemata, Stenopodidea)—banded coral shrimp

Neuroanatomy of the lateral protocerebrum. The hemiellipsoid body of *S. hispidus* resembles that of *P. pugio*, being comprised of three distinct neuropil regions exhibiting layers of differing synapsin immunoreactivity (Figs. 3D, 10). As in *P. pugio*, two of these neuropils (neuropils A and B) lie adjacent to one another in the dorsoventral plane, whereas the third neuropil (neuropil C) is situated adjacent to the two others in the rostrocaudal plane. The hemiellipsoid body neuropils of *S. hispidus*, however, are rotated 180° relative to those of *P. pugio* such that neuropil C is caudal to neuropils A and B and neuropil A lies ventral to neuropil B. The relative sizes of the three neuropils also differ from those in *P. pugio*, with

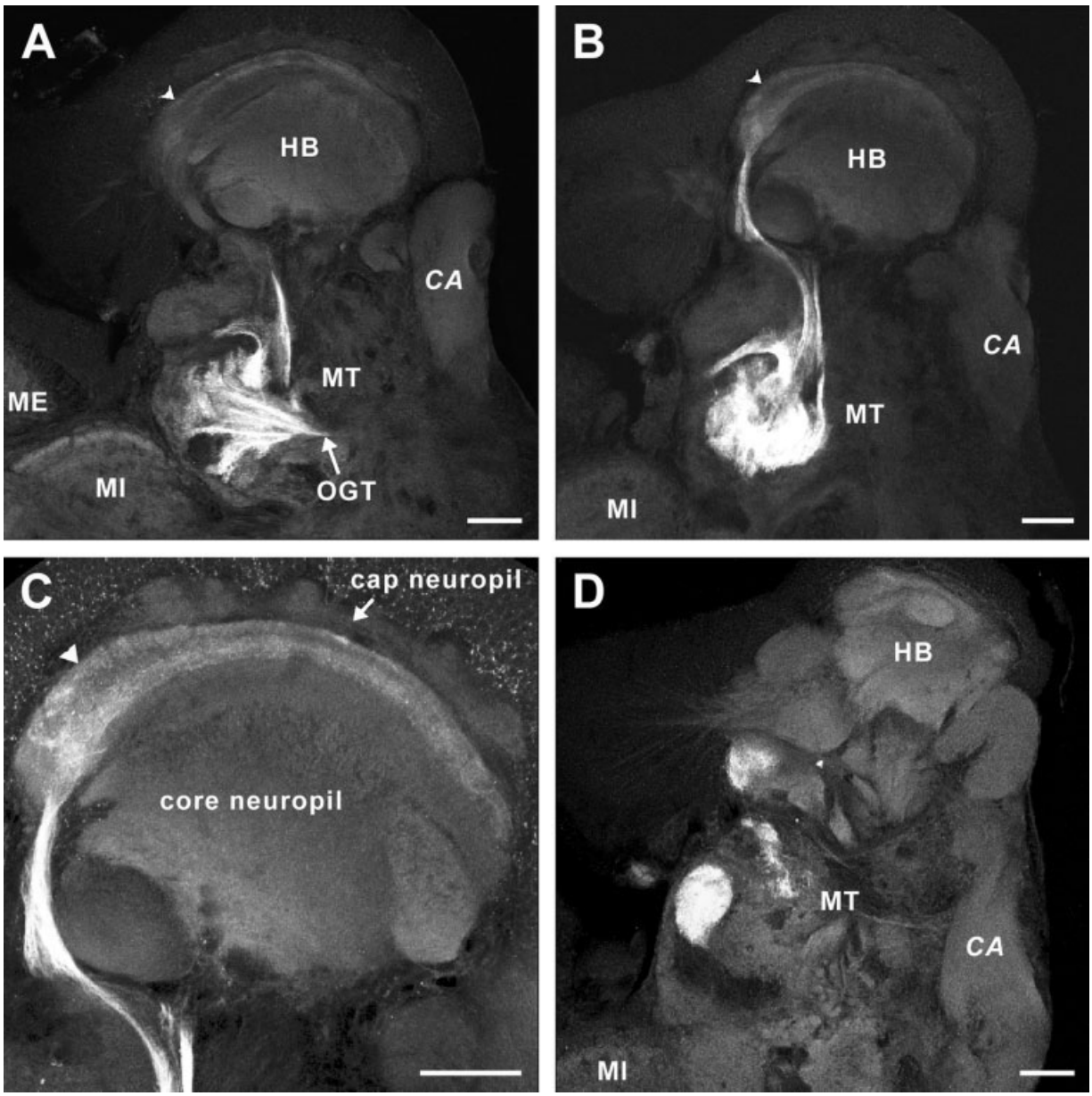


Fig. 5. Dye injections into the olfactory lobe of *Gonodactylus bredini* reveal that the projection neurons from this lobe primarily innervate the medulla terminalis with a further branch innervating an outer layer of the core neuropil of the hemiellipsoid body. **A–D:** Stacked confocal images of longitudinal sections through the lateral protocerebrum of *G. bredini* in which the ipsilateral olfactory lobe was injected with DiA. The white arrowheads indicate labeled projections within a peripheral layer of the core neuropil of the hemiellipsoid body. Rostral is to the left and ventral is at the top. Abbreviations: CA, *corpo allungato*; HB, hemiellipsoid body; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; OGT, olfactory globular tract. Scale bars = 100 μm in A–D.

neuropil B being the largest neuropil of the hemiellipsoid body of *S. hispidus*, rather than neuropil C. As in *P. pugio*, neuropil A in *S. hispidus* is characterized by the presence of two bands of strongly labeled neuropil (arrowheads in Fig. 10A,C) that border a band of lightly stained neuropil. Neuropils B and C are both comprised of several layers that differ in both their size and the intensity of their synapsin labeling (Fig. 10B–D).

In addition to the medulla terminalis and hemiellipsoid body, the lateral protocerebrum of *S. hispidus* also contains a complex of neuropils that resemble in their location and synapsin labeling the *corpo allungato* of *G. bredini*. We have named this complex the lateral protocerebral complex (LPC). The LPC is comprised of several neuropils and is located at the caudal margin of the lateral protocerebrum, lateral to the base of neuropil C of the hemiellipsoid body. The dense synapsin labeling of the LPC neuropils differs from the diffuse labeling observed in the medulla terminalis but closely resembles that observed in the *corpo allungato* (Fig. 4D). The largest of the LPC neuropils is an elongated neuropil with several arms (asterisks in Fig. 10D), which, like the *corpo allun-*

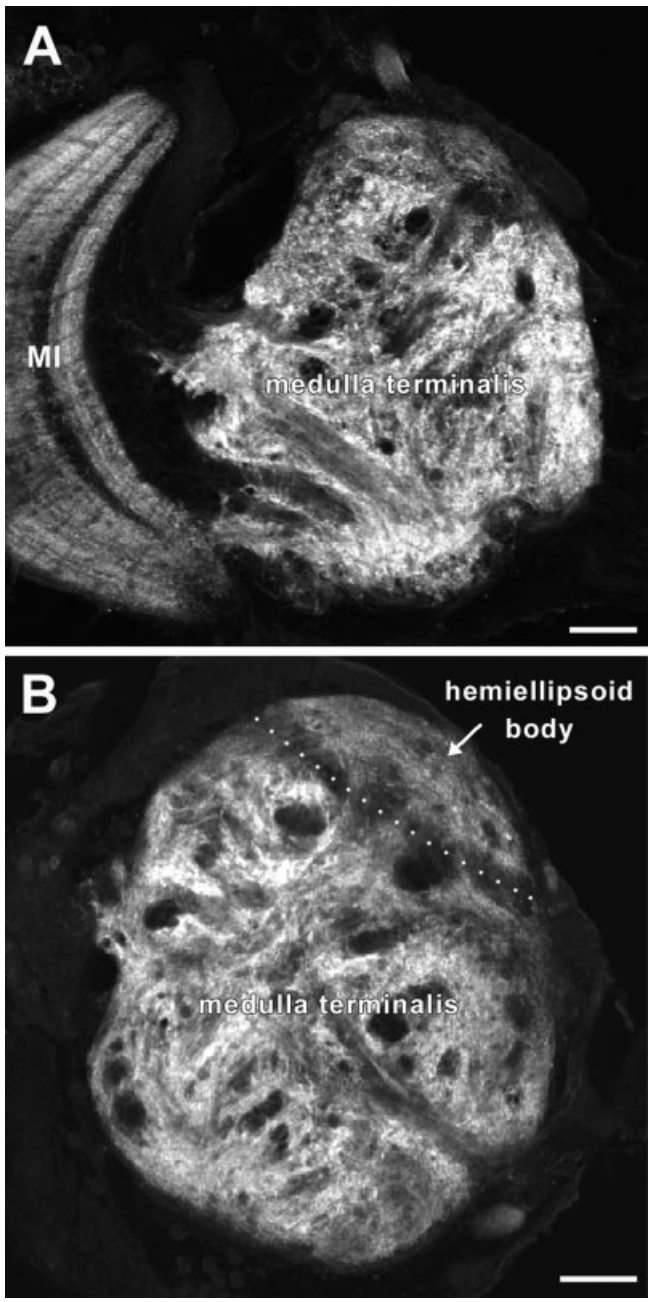


Fig. 6. Neuroanatomy of the lateral protocerebrum of the pink shrimp *Penaeus duorarum* (Decapoda, Dendrobranchiata). **A:** Horizontal section through the lateral protocerebrum of *P. duorarum* stained by using an antibody against *Drosophila* synapsin. Rostral is to the left. **B:** Synapsin immunoreactivity in a cross section through the lateral protocerebrum of *P. duorarum*. The dotted line indicates the boundary between the hemiellipsoid body and the medulla terminalis. Ventral is to the left. Abbrevation: MI, medulla interna. Scale bars = 100 μm in A,B.

gato, is surrounded by several, smaller satellite neuropils (Fig. 10D).

Neuroanatomy of the olfactory lobe output pathway. Dye injections into the olfactory lobe of *S. hispidus* showed that, as in *P. pugio*, the olfactory lobe output tract in this species primarily targets the hemiellipsoid body (Fig. 11). The OGT enters the hemiellipsoid body at the dorsal margin of neuropil C and branches within this neuropil region before projecting to both neuropils B and A (Fig. 11). Within neuropil B the projection neuron axons primarily innervate one neuropil layer (Fig. 11A), whereas in neuropil A the branches are separated into two major arbors (Fig. 11B). In addition to the arbors within the hemiellipsoid body, a small number of projection neurons also innervate a region of the medulla terminalis adjacent to the base of neuropil A (arrowheads in Fig. 11B). The extent of the labeled arbors within this region of the medulla terminalis varied between preparations. Although in some preparations only a small number of labeled projection neurons was observed innervating the medulla terminalis (Fig. 11B), the same region was densely innervated in others (Fig. 11B, inset). These observations suggest that the dendritic arbors of projection neurons innervating the medulla terminalis may not be evenly distributed throughout the olfactory lobe. Although the targets of the OGT were identical bilaterally, the ipsilateral labeling was more intense, suggesting that more projection neurons project to the ipsilateral lateral protocerebrum than project contralaterally.

DISCUSSION

Evolutionary changes in the projection patterns of the olfactory lobe output pathway

In eumalacostracan crustaceans, information about olfactory cues is transmitted to higher order neuropils, after primary processing within the olfactory lobe, by the axons of a large population of projection neurons (Fig. 1; Mellon et al., 1992; Wachowiak and Ache, 1994; Wachowiak et al., 1996; Mellon and Wheeler, 1999; Mellon, 2000). In the animals examined in the present study, the tract formed by these axons, the OGT, functions solely as the output pathway from the olfactory lobe. Previous anatomical studies have shown that, in most eumalacostracan taxa, the OGT projects to the lateral protocerebrum, where it innervates both the medulla terminalis and the hemiellipsoid body (Bellonci, 1882; Hanström, 1931, 1947; present study). Although the specific targets of the OGT appear, therefore, to have been conserved during the phylogeny of these animals, the major finding of the present study is that the relative extent to which the OGT innervates these two neuropil regions can vary markedly between species. The projection patterns of the OGT were observed to vary from that present in the stomatopod *G. bredini*, in which the projection neurons primarily arborize within the medulla terminalis, to those observed in the pleocyematan decapods *P. pugio* and *S. hispidus*, in which the major arbor of the projection neuron tract is within the hemiellipsoid body (Fig. 12).

The marked variability observed among these species in the extent of innervation of the medulla terminalis and hemiellipsoid body suggests that the relative importance of these two neuropils within the olfactory pathway has changed during the evolution of these animals. A phylogenetic comparison of the projection patterns of the OGT in the species examined suggests that the hemiellipsoid body became an increasingly integral part of the olfactory pathway during the phylogeny of the Eumalacostraca and that the role of the medulla terminalis concomitantly diminished (Fig. 12). In the most basal species examined,

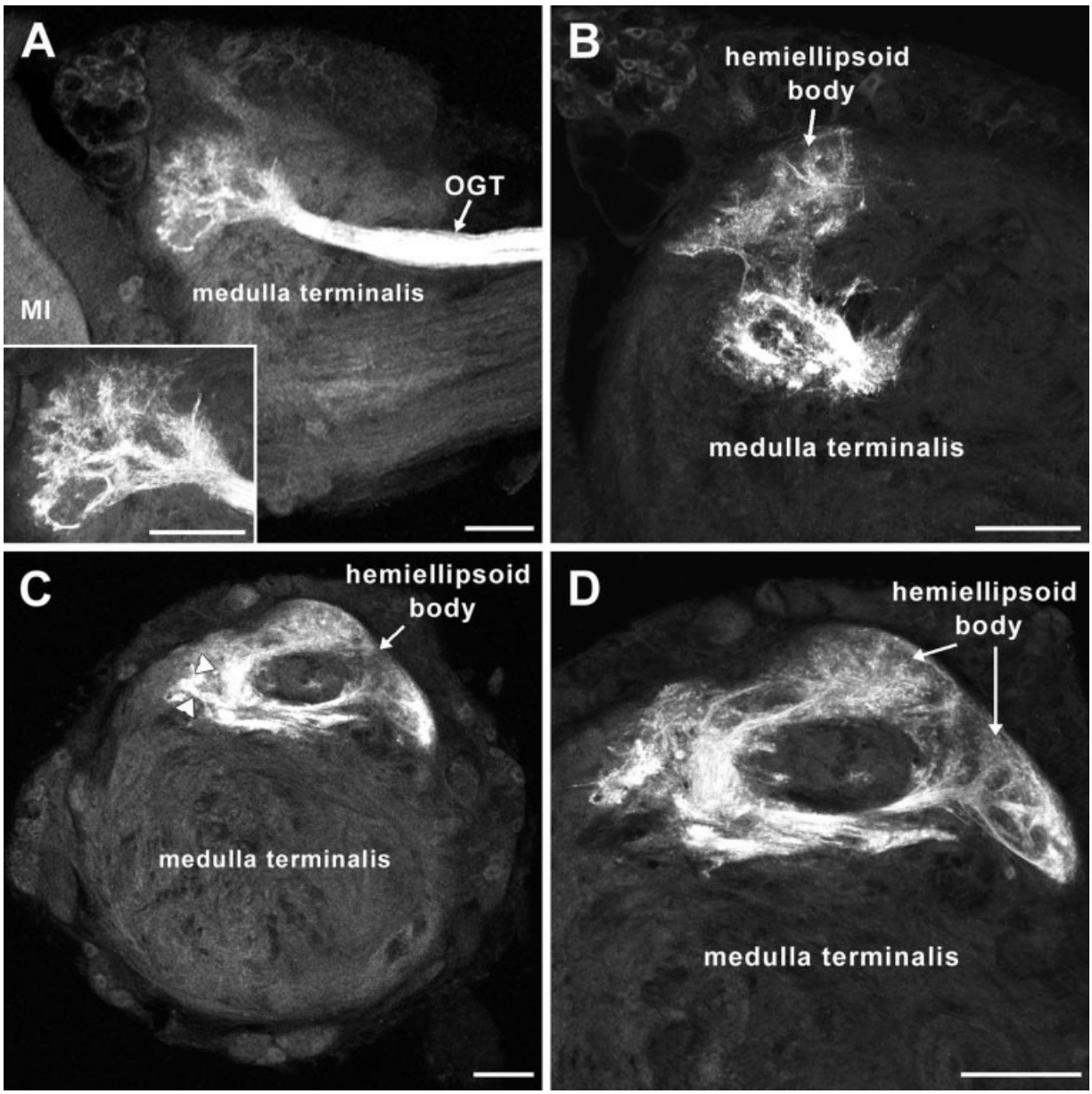


Fig. 7. Dye injections into the olfactory lobe of *Penaeus duorarum* show that the output tract arborizes in both the medulla terminalis and the hemiellipsoid body. **A–D:** Stacked confocal images of horizontal (A,B) and cross (C,D) sections through the lateral protocerebrum of *P. duorarum* in which the ipsilateral olfactory lobe was injected with DiA. The section shown in A is dorsal to that shown in B. **Inset, A:** Higher magnification image of the labeled projection neuron arbors within the medulla terminalis. Rostral is to the left in A and B. Ventral is to the left in C and D. Abbreviations: MI, medulla interna; OGT, olfactory globular tract. Scale bars = 100 μm in A–D.

the stomatopod *G. bredini*, the medulla terminalis appears to be the most important second-order olfactory neuropil, with the hemiellipsoid body receiving only minimal innervation from the OGT. In *P. duorarum*, a representative of the most basal decapod taxon (Fig. 2), the terminal arbors of the OGT are divided approximately evenly between the medulla terminalis and hemiellipsoid body, with an extensive innervation of the latter. In contrast, the hemiellipsoid body is the principal target of the olfactory lobe output tract in the pleocyematan decapods *P. pugio* and *S. hispidus*, with the medulla terminalis receiving only minimal innervation. In these two species, therefore, the hemiellipsoid body appears to be the most important second-order neuropil in the olfactory pathway.

Although the observed OGT projection patterns suggest that the anatomy of this tract has progressively changed during the phylogeny of the Eumalacostraca, it will be necessary to examine additional basal taxa to understand fully how the anatomy of this tract has evolved. Although the Stomatopoda represents the most basal eumalacostracan taxon, this group diverged early in the evolution of the Eumalacostraca and thus possesses numerous anatomical characters that are not shared by the remaining eumalacostracan taxa (Richter and Scholtz, 2001). Han-

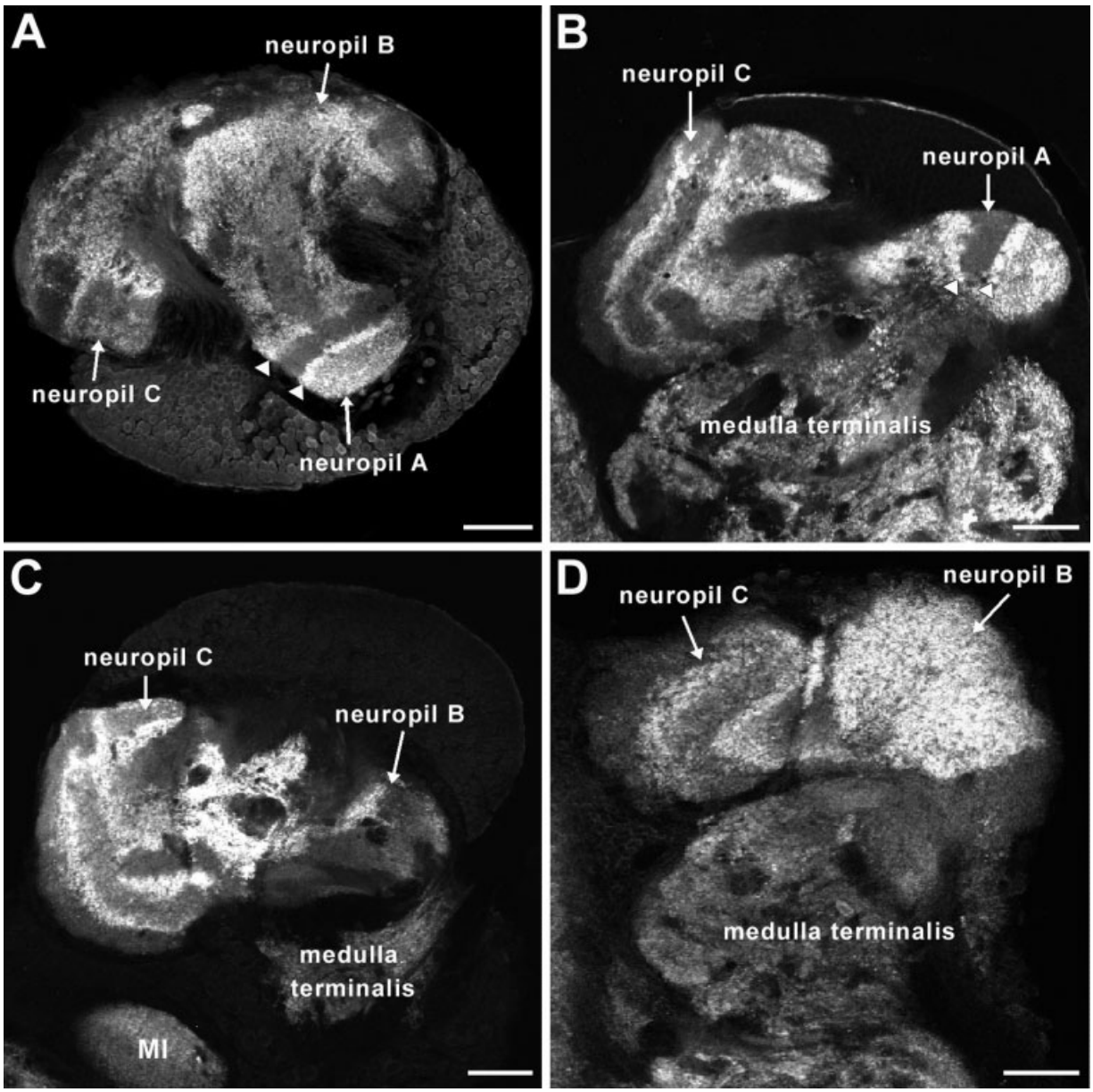
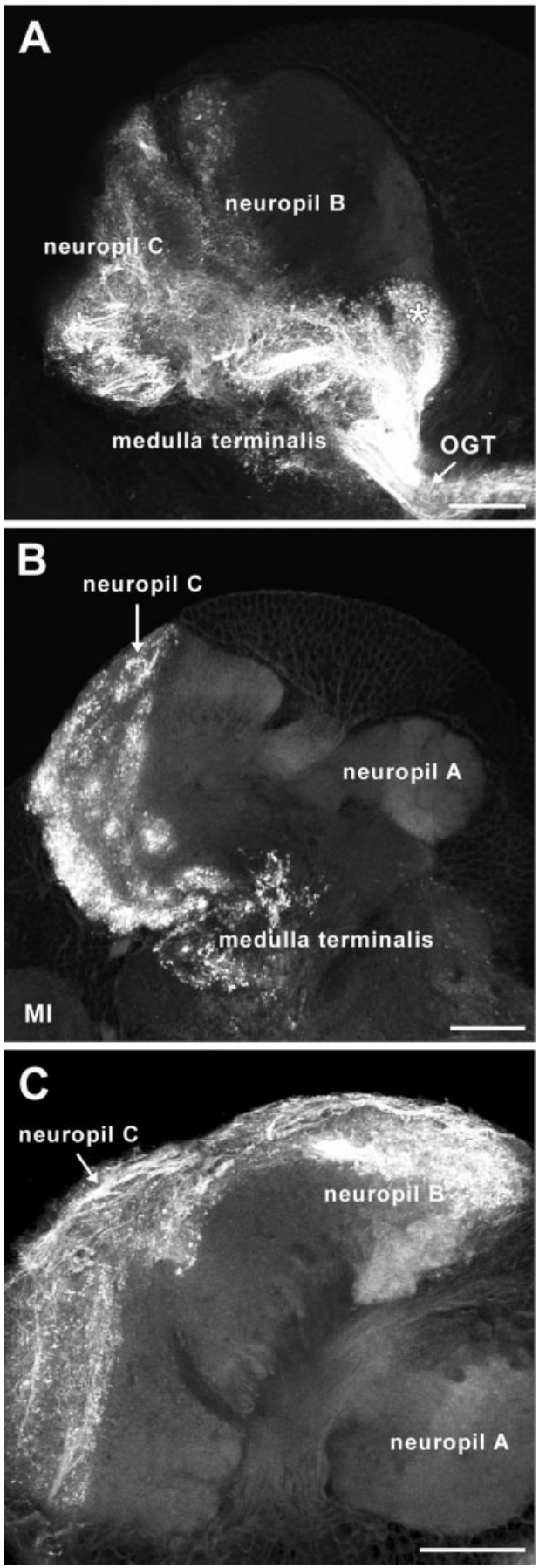


Fig. 8. Neuroanatomy of the lateral protocerebrum of the grass shrimp *Palaemonetes pugio* (Decapoda, Pleocyemata, Caridea). **A–D:** Stacked confocal images of longitudinal (A) and horizontal (B–D) sections through the lateral protocerebrum of *P. pugio* stained by using an antibody against *Drosophila* synapsin. The images show the relative positions of the three neuropil regions of the hemiellipsoid body: neuropils A, B, and C. The arrowheads in A and B indicate the prominent bands of intensely synapsin-immunoreactive neuropil within neuropil A. Rostral is to the left. Ventral is at the top in A. Abbreviation: MI, medulla interna. Scale bars = 100 μm in A–D.

ström (1947) identified the OGT in a representative of the Leptostraca (*Nebalia bipes*), the sister group to the Eumalacostraca (Fig. 2). The projection pattern of the OGT within the lateral protocerebrum of these animals, however, is not known. It remains unclear, therefore, whether the projection pattern of the OGT present in *G. bredini* represents a primitive (plesiomorphic) or derived (apomorphic) feature.

Although we currently have only limited information about the anatomy of the olfactory pathway in basal eumalacostracan taxa, this paper presents data on the projection patterns of this tract in representatives of each of the three most basal decapod taxa (Fig. 2). An examination of the projection patterns of the OGT in these animals should therefore provide insights into the evolution of the olfactory pathway in the Decapoda. In each of the decapod species examined in the present study, the olfactory lobe output tract extensively innervates the hemiellipsoid body. Innervation of the medulla terminalis by the OGT is most prominent in the dendrobranchiatan *P. duorarum* but is minimal in the representatives of the higher decapod taxa. Taken together, these observations suggest that although in the common ancestor of all decapods the projections of the OGT may have targeted the



medulla terminalis and hemiellipsoid body to approximately the same extent, the hemiellipsoid body subsequently became the most important second-order olfactory neuropil.

The evolutionary appearance of the accessory lobe in the eureptantian decapods represents perhaps the most marked change in the olfactory pathway that has occurred during the phylogeny of the Eumalacostraca (Fig. 2; Sandeman et al., 1993). Although accessory lobes have not been described in the basal decapod taxa examined in the present study, or in the Polychelida (Fig. 2; Sandeman et al., 1993), they are one of the most prominent neuropils in the brains of representatives of the basal eureptantian taxa (Achelata, Homarida, Astacida, Thalassinida) before becoming secondarily reduced in the higher taxa (Anomala, Brachyura; Sandeman et al., 1993; Scholtz and Richter, 1995). Anatomical and electrophysiological studies have shown that in eureptantian decapods the OGT functions as the output pathway from both the olfactory and the accessory lobes (Mellon et al., 1992; Wachowiak and Ache, 1994; Schmidt and Ache, 1996; Wachowiak et al., 1996; Mellon and Wheeler, 1999; Mellon, 2000; Sullivan et al., 2000; Sullivan and Beltz, 2001a). In lobsters and crayfish, which possess prominent accessory lobes, the output pathway from the olfactory lobe has been shown to target the medulla terminalis primarily, with little or no innervation of the hemiellipsoid body (Fig. 12; Sullivan and Beltz, 2001a, b). In contrast, projection neurons innervating the accessory lobes project exclusively to the hemiellipsoid body (Sullivan and Beltz, 2001a, b). As the results of the present study demonstrate that in the basal decapod taxa information from the olfactory lobe is transmitted either principally (Caridea and Stenopodidea) or to a substantial extent (Dendrobranchiata) to the hemiellipsoid body, these observations indicate that the evolutionary appearance of the accessory lobe was accompanied by marked changes in the organization of the olfactory pathway. Principal among these changes appear to have been an increased focus on the role played by the medulla terminalis in the olfactory pathway and extensive changes in the connectivity and presumably, therefore, the function of the hemiellipsoid body.

Evolution of the lateral protocerebral neuropils

It has been hypothesized that the accessory lobe, which receives higher order olfactory, visual, and mechanosensory inputs (Sandeman et al., 1995), represents an adaptation to benthic life (Sandeman et al., 1993). Although other eumalacostracan taxa contain representatives that inhabit the benthos, reptantian crustaceans form the only group to have completely colonized the benthos, and many of the anatomical features that distinguish reptantians from other crustaceans can be interpreted as adaptations

Fig. 9. Dye injections into the olfactory lobe of *Palaemonetes pugio* indicate that the projection neuron pathway from the lobe primarily innervates the hemiellipsoid body. **A–C:** Stacked confocal images of horizontal (A, B) and transverse (C) sections through the lateral protocerebrum of *P. pugio* in which the ipsilateral olfactory lobe was injected with DiA. The asterisk in A indicates labeled branches within the medulla terminalis. Rostral is to the left. Ventral is at the top in C. Abbreviations: MI, medulla interna; OGT, olfactory globular tract. Scale bars = 100 μm in A–C.

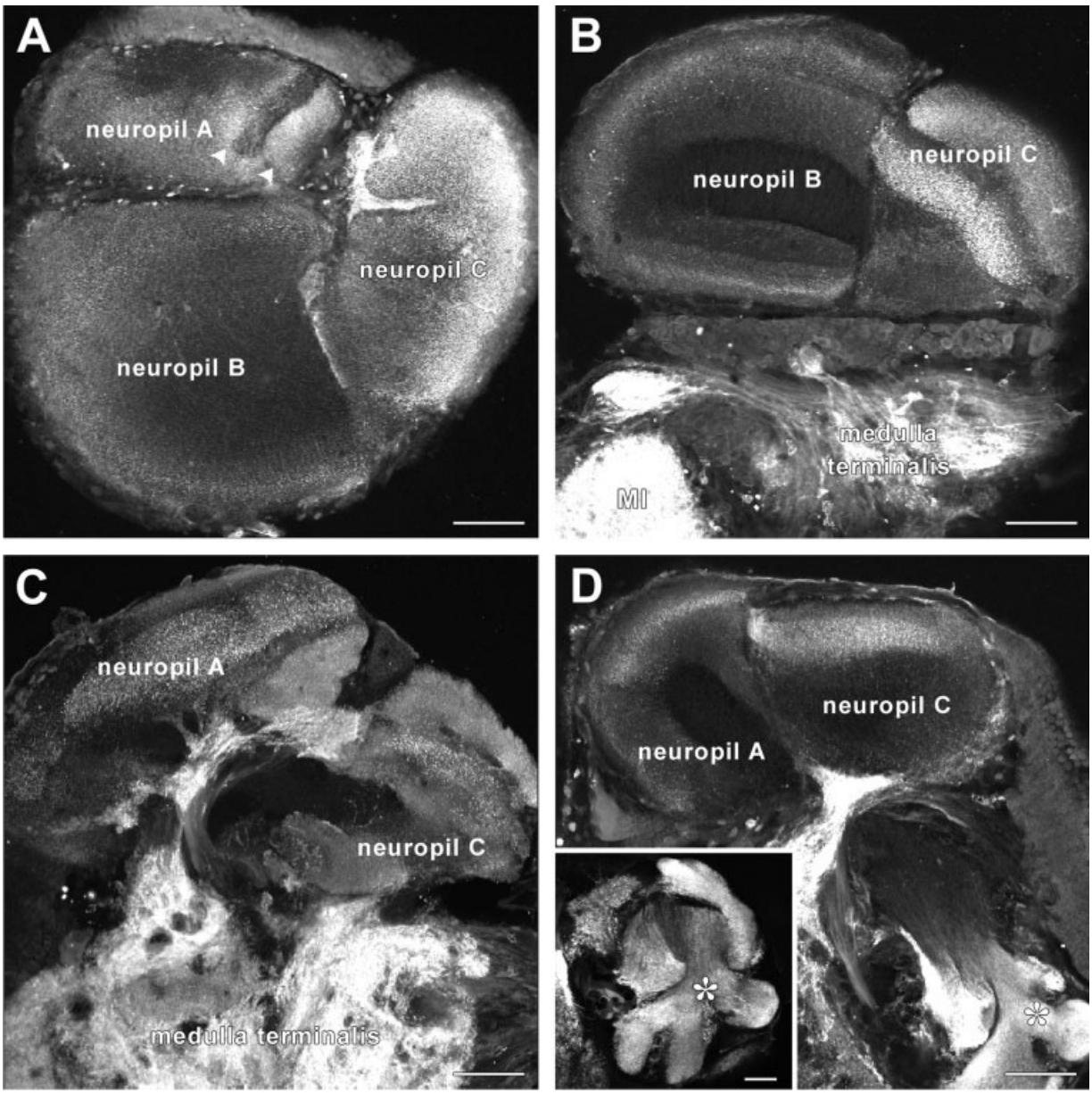


Fig. 10. Neuroanatomy of the lateral protocerebrum of the banded coral shrimp *Stenopus hispidus* (Decapoda, Pleocyemata, Stenopodidea) showing the relative positions of the medulla terminalis and the hemiellipsoid body neuropils A, B, and C. **A–D:** Stacked confocal images of longitudinal (A) and horizontal (B–D) sections through the lateral protocerebrum of *S. hispidus* stained by using an antibody against *Drosophila* synapsin. The images show the relative positions of the three hemiellipsoid body neuropils. The arrowheads in A indicate the prominent bands of intensely synapsin-immunoreactive neuropil within neuropil A. **Inset, D:** The lateral protocerebral complex, a complex of neuropils situated at the caudal margin of the lateral protocerebrum. The asterisks show the largest of these neuropils, an elongated neuropil with several arms. Rostral is to the left. Ventral is at the top in A. Abbreviation: MI, medulla interna. Scale bars = 100 μm in A–D.

to life on the benthos (Sandeman et al., 1993; Scholtz and Richter, 1995). It is of interest to note that among the species examined in the present study the two species that have benthic lifestyles, *G. bredini* and *S. hispidus*, also possess neuropil regions situated adjacent to neuropils in the olfactory pathway that have not been observed in other taxa. Like the accessory lobe of the Eureptantia, both the *corpo allungato* of the Stomatopoda and the lateral protocerebral complex of *S. hispidus* have no obvious homologues in other taxa and appear to have arisen de novo during the evolution of these animals. Both stomatopods and stenopodids have extensively colonized the benthos and, like most eureptantians, are territorial and possess prominent claws (Serène, 1954; Limbaugh et al., 1961; Dingle and Caldwell, 1969; Johnson, 1977). It is likely, therefore, that the brains of these animals, like those of the eureptantians, exhibit functional and anatomical adaptations to their benthic lifestyles. At present, however, nothing is known about the connectivity or possible functions of either the *corpo allungato* or the lateral

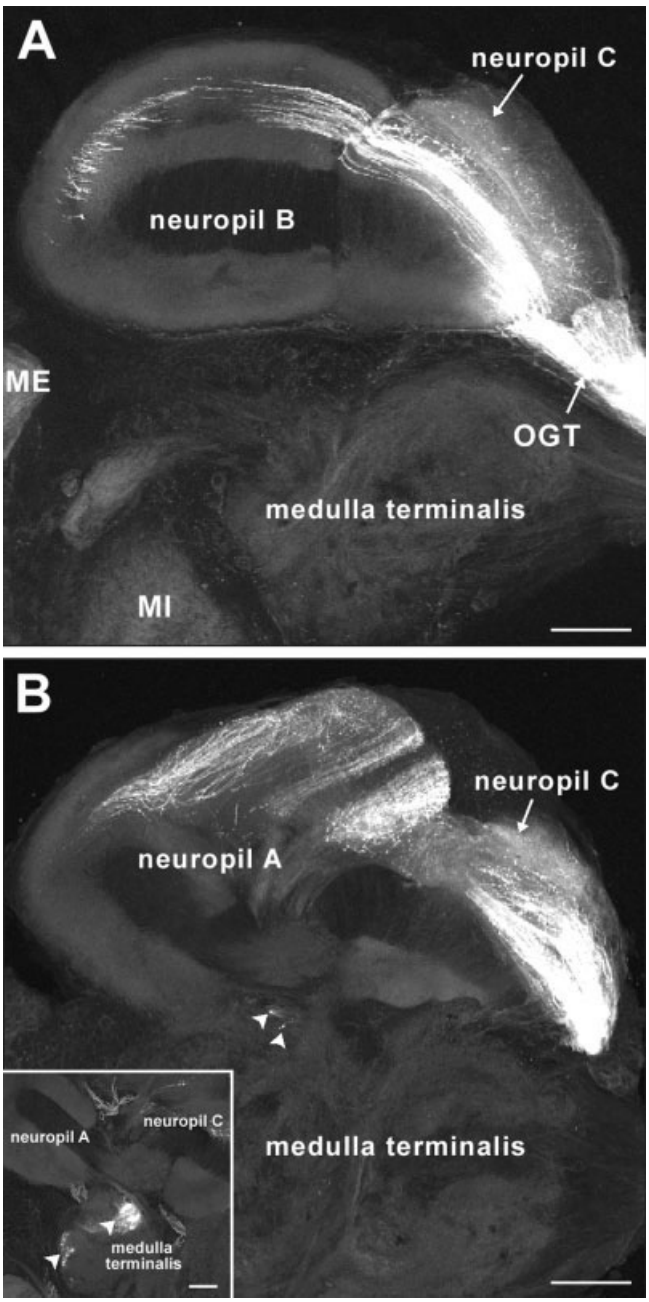


Fig. 11. Dye injections into the olfactory lobe of *Stenopus hispidus* show that the projection neuron pathway from this lobe projects primarily to the hemiellipsoid body. **A,B:** Stacked confocal images of horizontal sections through the lateral protocerebrum of *S. hispidus* in which the ipsilateral olfactory lobe was injected with DiA. The section shown in A is dorsal to that shown in B. The arrowheads in B show labeled projections within the medulla terminalis. **Inset, B:** Preparation in which labeled projection neurons more extensively innervate this region of the medulla terminalis. Rostral is to the left. Abbreviations: ME, medulla externa; MI, medulla interna; OGT, olfactory globular tract. Scale bars = 100 μm in A,B.

protocerebral complex. Further study of these neuropils will be necessary, therefore, to determine whether the connectivity and functions of these neuropils possess elements that parallel those of the accessory lobe.

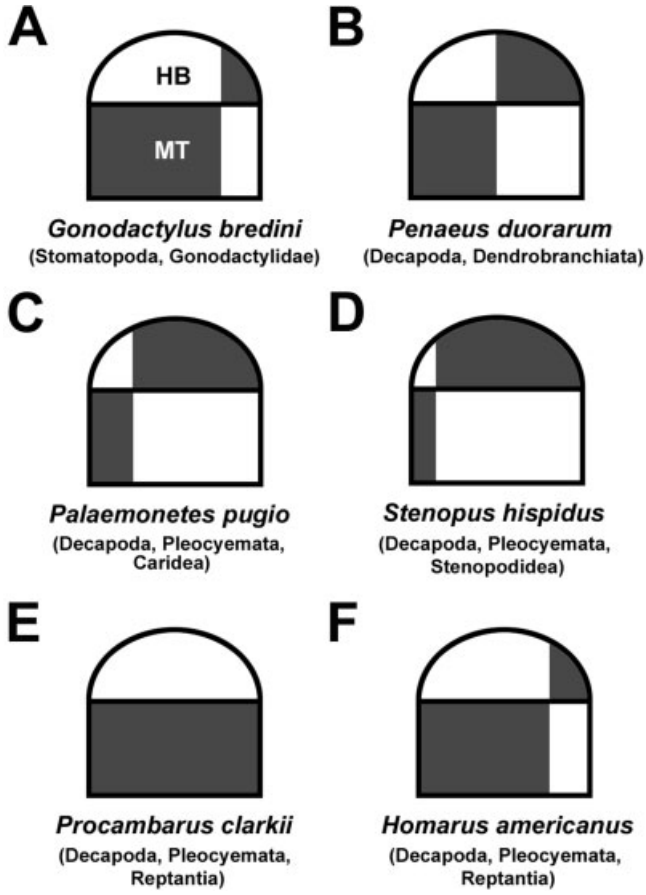


Fig. 12. Schematic diagrams illustrating the relative extent to which the olfactory lobe output pathway (dark shading) arborizes within the hemiellipsoid body (HB) and medulla terminalis (MT) of *Gonodactylus bredini* (**A**), *Penaeus duorarum* (**B**), *Palaemonetes pugio* (**C**), *Stenopus hispidus* (**D**), and the eureptantians *Procambarus clarkii* (**E**) and *Homarus americanus* (**F**). In both *P. clarkii* and *H. americanus*, the output pathway from the accessory lobe projects exclusively to the hemiellipsoid body. The diagrams are intended as descriptions of the innervation patterns and are not based on quantitative measurements.

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