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Comparative anatomy of pigment-dispersing hormone-immunoreactive neurons in the brain of orthopteroid insects

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Summary. In a comparative study, the anatomy of neurons immunoreactive with an antiserum against the crustacean β-pigment-dispersing hormone was investigated in the brain of several orthopteroid insects including locusts, crickets, a cockroach, and a phasmid. In all species studied, three groups of neurons with somata in the optic lobes show pigment-dispersing hormone-like immunoreactivity. Additionally, in most species, the tritocerebrum exhibits weak immunoreactive staining originating from ascending fibers, tritocerebral cells, or neurons in the inferior protocerebrum. Two of the three cell groups in the optic lobe have somata at the dorsal and ventral posterior edge of the lamina. These neurons have dense ramifications in the lamina with processes extending into the first optic chiasma and into distal layers of the medulla. Pigment-dispersing hormone-immunoreactive neurons of the third group have somata near the anterior proximal margin of the medulla. These neurons were reconstructed in *Schistocerca gregaria, Locusta migratoria, Teleogryllus commodus, Periplaneta americana*, and *Extatosoma tiaratum*. The neurons have wide and divergent arborizations in the medulla, in the lamina, and in several regions of the midbrain, including the superior and inferior lateral protocerebrum and areas between the pedunculi and α-lobes of the mushroom bodies. Species-specific differences were found in this third cell group with regard to the number of immunoreactive cells, midbrain arborizations, and contralateral projections, which are especially prominent in the cockroach and virtually absent in crickets. The unusual branching patterns and the special neurochemical phenotype suggest a particular physiological role of these neurons. Their possible function as circadian pacemakers is discussed.

Key words: Pigment-dispersing hormone – Orthopteroid insects – Immunocytochemistry – Insect brain – *Periplaneta americana, Schistocerca gregaria, Teleogryllus commodus* (Insecta)

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Crustaceans display color changes by translocating pigment granules within epithelial chromatophores. These pigment migrations are under hormonal control and so far, members of two peptide families have been identified as chromatophorotropins (Rao and Riehm 1988a). Red-pigment concentrating hormone (RPCH), isolated from the prawn *Pandalus borealis* (Fernlund and Josefsson 1972), is a member of the arthropod adipokinetic family of peptides (Gäde 1990) and causes pigment concentration in erythrophores (Rao 1985; Rao and Riehm 1988a). Members of the pigment-dispersing hormone (PDH) family of peptides have been isolated from several crustacean species, including *P. borealis* (α-PDH), the crabs *Uca pugilator* and *Cancer magister* (β-PDH), and the crayfish *Procambarus clarkii* (review: Rao and Riehm 1989). Immunocytochemical studies on the eyestalks of decapod Crustacea revealed PDH-like immunoreactivity (PDHLI) in a number of interneurons and in neurosecretory terminals in the sinus gland (Dircksen et al. 1987; Mangerich et al. 1987). PDHs released from the sinus gland trigger chromatophoral pigment dispersion, evoke light adaptational migration of retinal screening pigment (Rao and Riehm 1988a), and apparently synchronize the effector systems with regard to circadian rhythms (Aréchiga and Mena 1975; Fingerman and Fingerman 1977; Larimer and Smith 1980).

Melanophore pigment dispersion in *U. pugilator* has also been demonstrated with head extracts from insects such as the honey bee *Apis mellifera*, the cockroach *Periplaneta americana* (Dores and Herman 1981), and the cricket *Acheta domesticus* (Mohrherr and Rao 1985). These factors have recently been isolated and sequenced from head extracts of two insect species, the lubber grasshopper *Romalea microptera* (Rao et al. 1987) and the cricket *A. domesticus* (Rao and Riehm 1988b, 1989). Like the crustacean PDHs, these pigment-dispersing factors (PDFs) are octadecapeptides and they show 78%–83% sequence similarity with β-PDH of the *Uca/Cancer* type. Whereas the PDFs cause pigment dispersion in crustaceans (Rao and Riehm 1989), their functional role in insects is not known. Using an antiserum against syn-

thetic β-PDH, Zahnow et al. (1987) reported immunoreactivity in the brain of *R. microptera* in three cell groups in the optic lobe and widely distributed arborizations throughout the supraoesophageal ganglion.

To reveal possible functions for PDH-like peptides in insects, we investigated the distribution of PDHLI in the brain of several orthopteroid insects including locusts, crickets, a cockroach, and a phasmid. The possible physiological role of some of the immunoreactive neurons is discussed in relation to anatomical criteria proposed for circadian pacemaker neurons (Page 1984; Chiba and Tomioka 1987). Parts of this study have been reported in an abstract form (Würden et al. 1990).

Materials and methods

Animals

The species examined were: four locusts (*Locusta migratoria, Schistocerca gregaria, Schistocerca americana, Melanoplus differentialis*), four crickets (*Acheta domesticus, Gryllus bimaculatus, Teleogryllus commodus, Teleogryllus oceanicus*), a phasmid (*Extatosoma tiaratum*), and a crockroach (*Periplaneta americana*). *Locusta migratoria* and *S. gregaria* were taken from crowded laboratory cultures at the University of Konstanz, FRG. They were kept in 12L:12D photoperiod, at about 60% relative humidity, and a temperature of 35° C during the day and 25° C during the night. *Teleogryllus commodus* were reared at the University of Konstanz under 12L:12D photoperiod at 30° C, and 55% relative humidity. *Schistocerca americana, M. differentialis*, and *P. americana* were provided by the ARL Division of Neurobiology at the University of Arizona, Tucson, Az., USA. *Schistocerca americana* and *M. differentialis* were reared under long-day photoperiod (16L:8D), 30° C and 27% relative humidity, and *P. americana* under 17L:7D photoperiod, at 29° C and about 40% relative humidity. Specimens of *G. bimaculatus* were obtained from a culture at the Zoological Institute of the University of Hamburg; *A. domesticus* from a colony at the University of Konstanz; *T. oceanicus* from the Max-Planck-Institut für Verhaltensphysiologie, Seewiesen, FRG; and *E. tiaratum* from Dr. R. Kittmann at the University of Konstanz.

Immunocytochemistry

Immunocytochemistry was performed on free-floating Vibratome sections by means of the indirect peroxidase-anti-peroxidase (PAP) technique (Sternberger 1979). After dissection, brains were fixed for 4 h or overnight in 4% paraformaldehyde/7.5% picric acid in phosphate buffer (0.1 M, pH 7.4). Brains were embedded in a gelatin/albumin mixture and cut at 30 µm with a Vibratome (Technical Products, St. Louis, Mo., USA). The immunocytochemical staining was performed as described by Homberg and Hildebrand (1989). The anti-PDH antiserum (3B3) was raised in rabbits against conjugates of synthetic *Uca pugilator/Cancer magister* β-PDH and bovine thyroglobulin as described by Dircksen et al. (1987). To reduce nonspecific background staining, sections were incbuated for 1 h in 0.1 M TRIS HCl-0.3 M NaCl (pH 7.4) containing 2% normal goat serum (Gibco Laboratories, Chagrin Falls, Ohio, USA) and 0.5% Triton X-100. The anti-PDH antiserum was diluted at 1:5000 to 1:100000 with optimal signal to noise ratio at around 1:50000. It was applied to the sections for 20 h at 15° C. The sections were incubated in secondary antiserum (goat anti-rabbit IgG, Sigma, Deisenhofen, FRG) for 1 h at a dilution of 1:40 and in rabbit PAP (Dako, Hamburg, FRG) for 1 h at 1:100. The diaminobenzidine reaction was carried out as described previously (Homberg and Hildebrand 1989). The sections were mounted on chrome alum/gelatin-coated glass slides, dehydrated, cleared in xylene, and mounted in Permount (Fisher Scientific, Fair Lawn, N.J., USA) or Entellan (Merck, Darmstadt, FRG) under glass coverslips. The immunoreactive neurons were reconstructed from serial frontal sections using a Zeiss microscope equipped with a camera lucida attachment. The nomenclature of brain structures largely follows the teminology used by Strausfeld (1976). The orientation of all figures is indicated relative to the body axis. Therefore, horizontal brain sections of the prognathous phasmid *E. tiaratum* are roughly equivalent to frontal brain sections of the hypognathous species.

Specificity controls

The anti-PDH antiserum has been well characterized by immunodot-blotting assays of HPLC-separated crude sinus-gland extracts, preadsorption controls on crustacean nervous tissue (Dircksen et al. 1987; Mangerich et al. 1987), and by a recently developed ELISA for PDH (Bonomelli et al. 1988). Preincubation of the diluted anti-PDH antiserum with 10 µM *Uca/Cancer* β-PDH for at least 5 h at room temperature abolished all immunoreactive staining on brain sections of *S. gregaria, T. commodus*, and *P. americana*.

Results

General staining pattern

In all species studied, three cell groups with somata in the optic lobe exhibit intense PDHLI. The location of these cells and their arborizations are similar in all species. Two of the cell groups lie at the posterior dorsal

Table 1. Number of PDFLa and PDFMe neurons[a] and major projection areas[b] of PDFMe-neurons in the brain of orthopteroid insects. +, Sparse; ++, medium; +++, dense innervation; αL/P, area between α-lobe and pedunculus; antCa, area anterior to the calyces of the mushroom body; Co, commissures; ILP, inferior lateral protocerebrum; Lo, lobula; PB, protocerebral bridge; POTu, posterior optic tubercle; SLP, superior lateral protocerebrum; SMP, superior median protocerebrum; VLP, ventro-lateral protocerebrum

Species	PDFLad	PDFLav	PDFMe	Lo	antCa	αL/P	SMP	SLP	ILP	VLP	POTu	PB	Co
Schistocerca gregaria	20–26	30–38	15–17	+	++	+++	++	+	++	+	++	+	++
Schistocerca americana	23–30	23–29	16–17	+	++	+++	++	+	++	+	++	+	++
Locusta migratoria	22–27	19	11–13		++	+++	++	+	++		++		+
Melanoplus differentialis	17–18	20–24	16		++	+++	++	+	++		++		+
Teleogryllus commodus	12–22	12–17	14	+	++	+++	++	++	+++	+			
Teleogryllus oceanicus	16–18	12–17	9–10	+	++	+++	++	++	+++	+			
Acheta domesticus	49–56	42–52	9–12	+	++	+++	++	++	+++	+			
Gryllus bimaculatus	43–70	46–53	13–15	+	++	+++	++	++	+++	+			
Extatosoma tiaratum	80–92	79–85	16	+	++	++	++	++	++	++			++
Periplaneta americana	56–59	56–62	14		++	+	++	+	+++	++	+		+++

[a] Counts per hemisphere from 2–4 cell clusters
[b] Peripheral projections in lamina and medulla not included

(PDFLad) and posterior ventral (PDFLav) edge of the lamina, and cells of the third group (PDFMe) are localized at the anterior edge of the medulla. Owing to the large number of immunoreactive profiles from these neurons, the projections of PDFLa and PDFMe neurons could not completely be separated. It appears that the PDFLa neurons arborize in the lamina, in the distal medulla, and in an ovoid neuropil at the anterior proximal edge of the medulla, which will be called the "accessory medulla" according to a similar structure in Trichoptera (Ehnbohm 1948; Hagberg 1986). The PDFMe neurons innervate the lamina and the accessory medulla and give rise to extensive ramifications in the midbrain. Interspecific differences in the immunoreactive staining pattern were especially observed with respect to the number of immunoreactive cells and the arborizations of PDH-immunoreactive neurons in the midbrain. In addition to the PDFLa and PDFMe cells, weak PDHLI is detected mainly in the trito- and deutocerebrum of some species, originating from ascending fibers, tritocerebral cells, or neurons in the inferior protocerebrum.

Locusts

In the locusts, the dorsal and ventral PDFLa clusters are, as in the other orthopteroids, of similar size and consist of 17–38 cells each with uniform diameters of about 10 µm (Table 1; Fig. 1). Immunoreactive arborizations in the lamina are largely restricted to the inner, most proximal layer of neuropil (Fig. 1A, B). In the first optic chiasma, numerous PDH-immunoreactive fibers connect the posterior face of the lamina and the anterior face of the medulla. Some fibers enter the medulla along that course and terminate in its outermost layer (Fig. 1). Others apparently enter the accessory medulla, which shows dense granular immunoreactive staining (Fig. 1C).

Whereas the immunoreactive staining pattern in the lamina and medulla is indistinguishable in all acridids, the lobula and midbrain arborizations, which appear to originate exclusively from the PDFMe cells, show some interspecific differences. In *S. gregaria* and *S. americana*, the PDFMe cluster consists of 15–17 cells of different sizes, ranging from 20–35 µm (Table 1, Fig. 1C). The somata are in the anterior median cortex of the optic lobe proximal to the accessory medulla and send primary neurites into this neuropil. Fibers projecting to the midbrain join two major tracts, one passing dorsally and the other, ventrally around the lobula complex.

Mostly large immunoreactive fibers run in the bundle along the dorsal face of the lobula into the median protocerebrum. Small fibers leave the tract and innervate

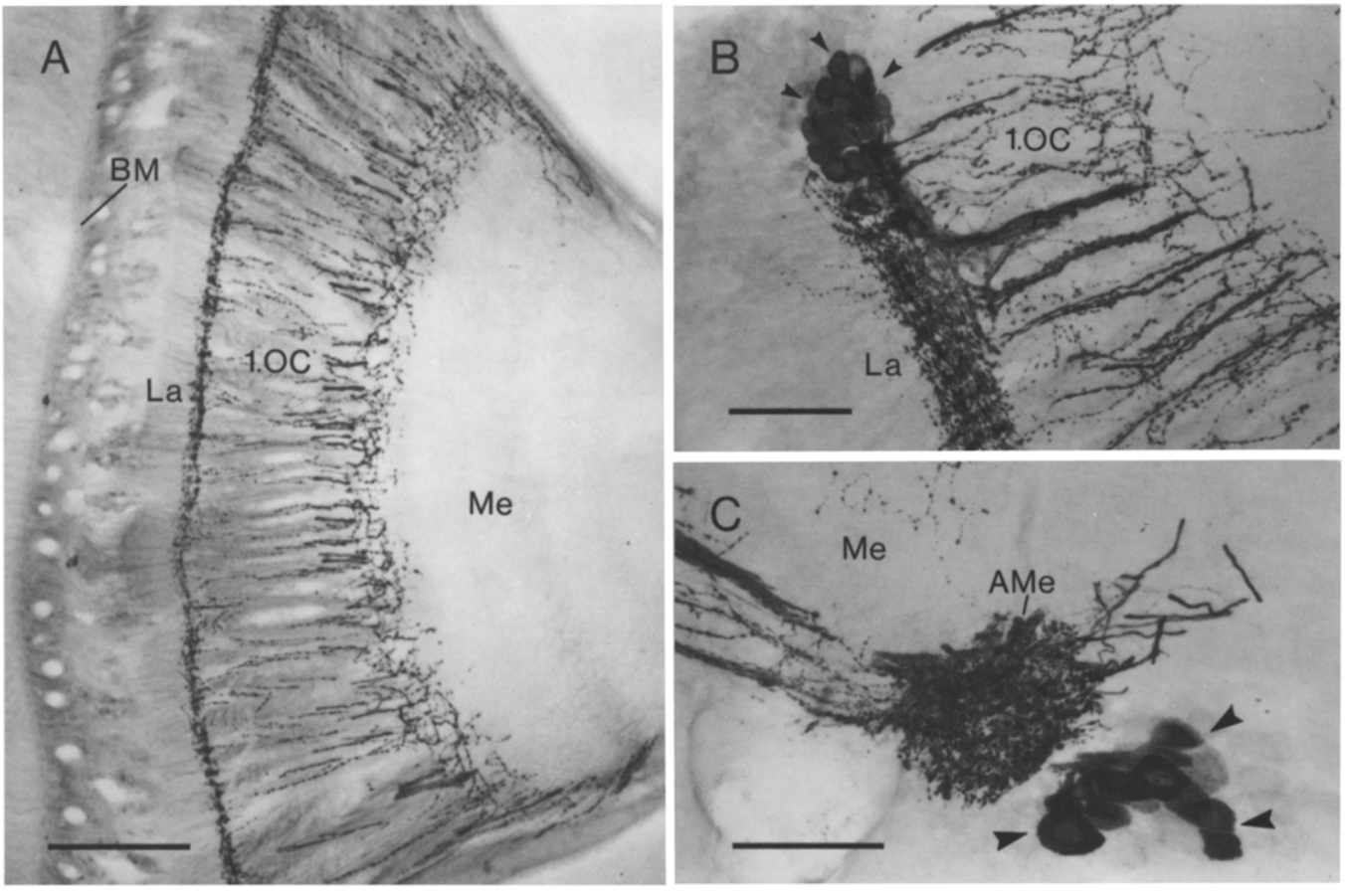


Fig. 1A–C. Pigment-dispersing hormone-like immunoreactivity (PDHLI) in the optic lobe of grasshoppers. **A** *Melanoplus differentialis*; **B, C** *Schistocerca gregaria*. **A** Frontal section showing PDHLI in the lamina (*La*), first optic chiasma (*1.OC*), and medulla (*Me*). Only a proximal layer of the lamina and the outermost layer of the medulla are innervated by PDH-immunoreactive processes. *BM* Basement membrane. **B** Somata of PDFLa neurons (*arrowheads*) and immunoreactive fibers at the posterior dorsal edge of the lamina (*La*) and in the first optic chiasma (*1.OC*). **C** Frontal section showing PDFMe somata (*arrowheads*) and dense immunoreactive terminals in the accessory medulla (*AMe*). *Me* Medulla. *Scale bars:* 200 µm (**A**), 100 µm (**B, C**)

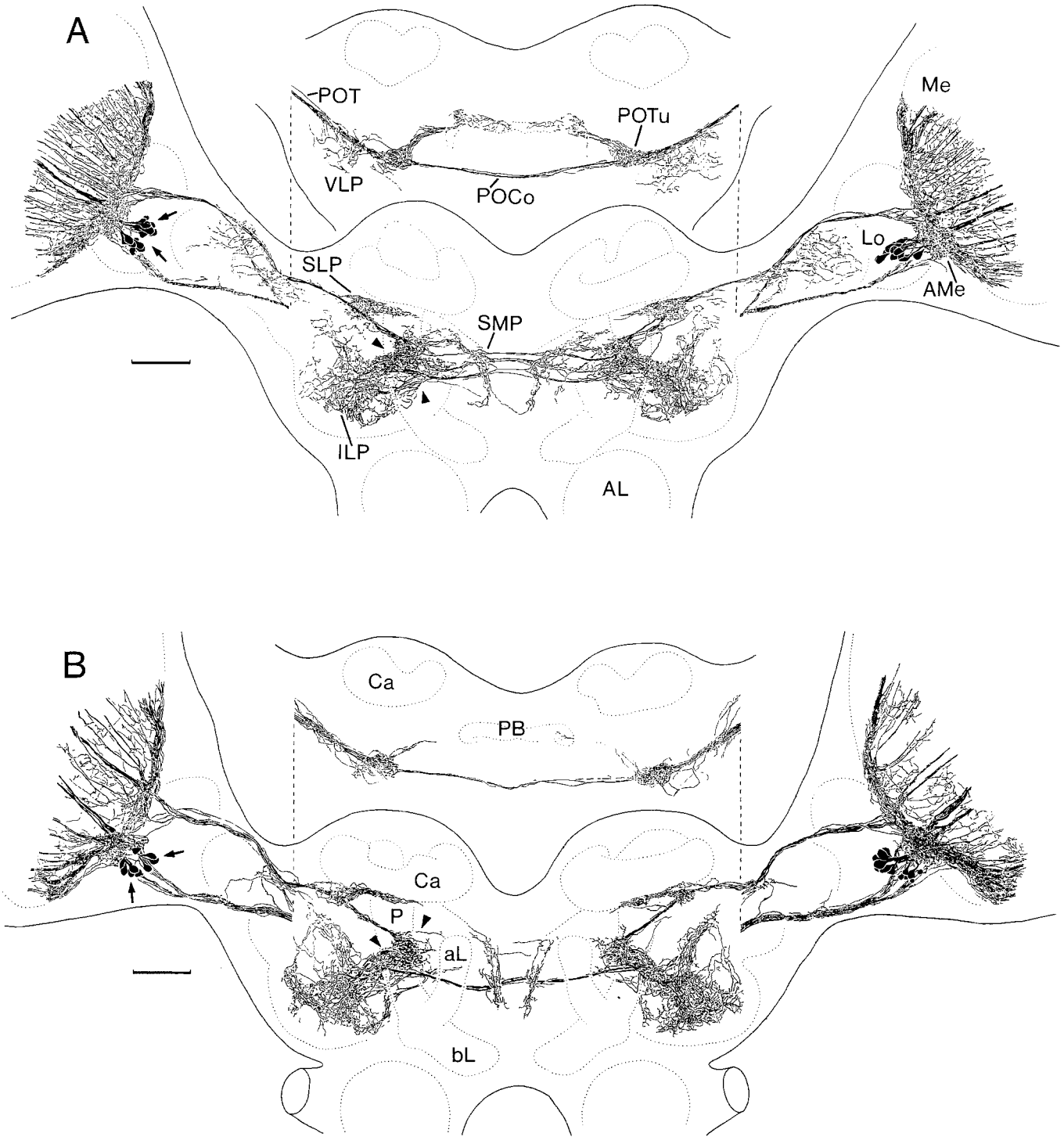


Fig. 2A, B. Frontal reconstructions of PDHLI in the midbrain, lobula (*Lo*), and parts of the medulla (*Me*) of *Schistocerca gregaria* (**A**) and *Locusta migratoria* (**B**). PDHLI in the lamina and distal parts of the first optic chiasma has been omitted in this and all other reconstructions. *Arrows* point to PDFMe somata. In both species, immunoreactive terminals in the midbrain are concentrated in the superior protocerebrum between α-lobe (*aL*) and pedunculus (*P*) of the mushroom bodies (*arrowheads*) and in the inferior lateral protocerebrum (*ILP*). Side branches innervate small areas in the superior lateral protocerebrum (*SLP*) anterior to the calyces (*Ca*) of the mushroom bodies and in the superior medium protocerebrum (*SMP*) near the median furrow of the two hemispheres. *Insets* show immunoreactive fibers that arborize in the posterior optic tubercles (*POTu*), protocerebral bridge (*PB*), and the ventro-lateral protocerebrum (*VLP*) via the posterior optic tract (*POT*) and posterior optic commissure (*POCo*). *AL* Antennal lobe; *bL* β-lobe of the mushroom body. *Scale bars:* 200 μm

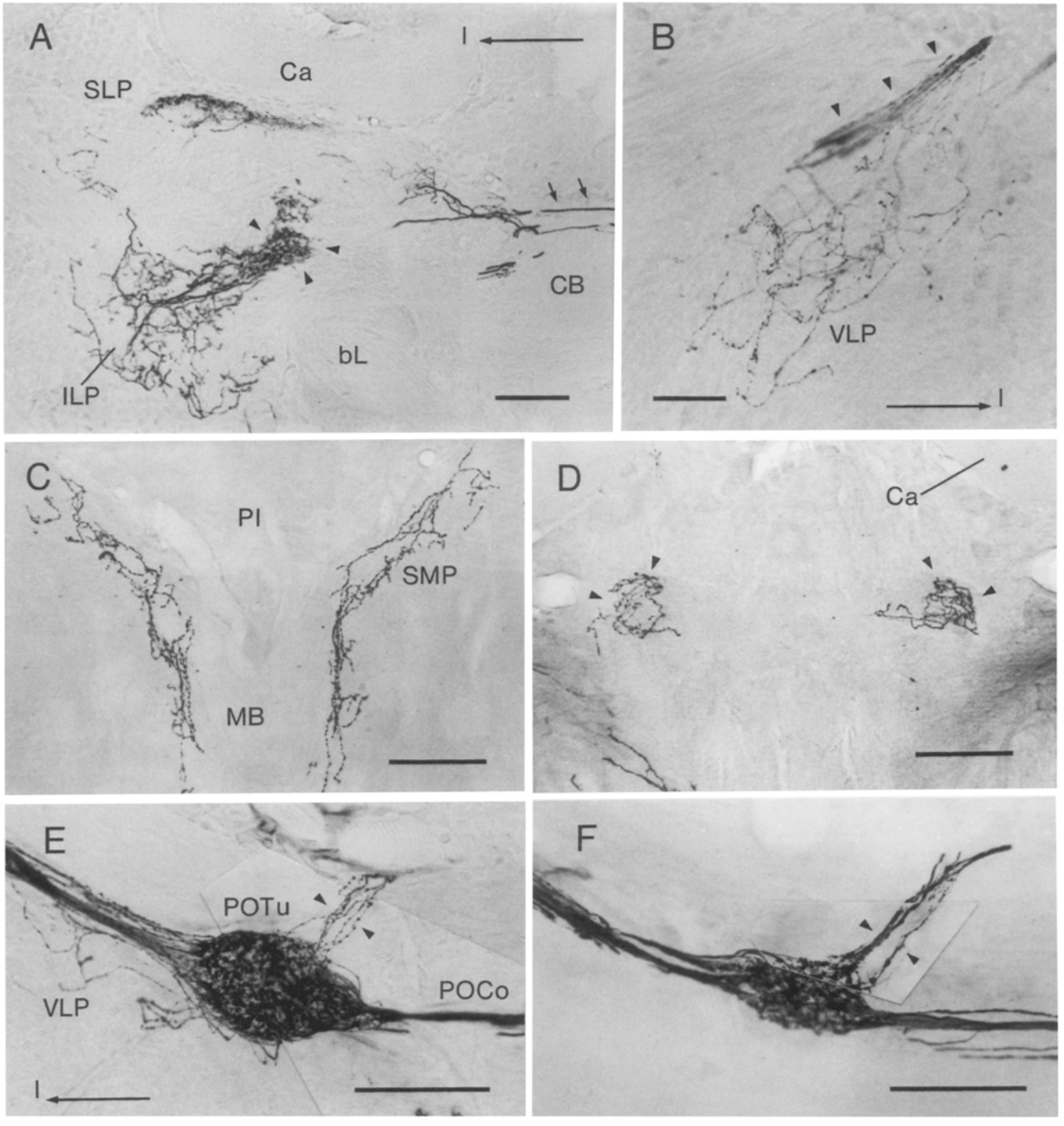


Fig. 3A–D. Frontal Vibratome sections showing PDHLI in the midbrain of *Schistocerca gregaria*. **A** Section through the left brain hemisphere. PDH-immunoreactive arborizations are concentrated in neuropil between α-lobe and pedunculus (*arrowheads*) and extend into the inferior lateral protocerebrum (*ILP*). Side branches innervate a small area in the superior lateral protocerebrum (*SLP*) ventro-lateral to the calyces (*Ca*) of the mushroom body. Several fibers in the superior protocerebrum run contralaterally (*arrows*). *bL* β-lobe of the mushroom body; *CB* central body; *l* lateral. **B** Sparse varicose immunoreactive processes in the ventro-lateral protocerebrum (*VLP*) originate from the fiber bundle (*arrowheads*) that bypasses the lobula ventrally. **C** PDH-immunoreactive fibers and terminals in the superior median protocerebrum (*SMP*). *PI* Pars intercerebralis; *MB* median bundle. **D** Immunoreactive processes in the lateral parts of the protocerebral bridge (*arrowheads*). *Ca* Calyces of the mushroom body. **E, F** PDHLI in the posterior optic tubercle (*POTu*) of *Locusta migratoria* (**E**) and *Schistocerca americana* (**F**). Immunoreactive fibers entering the protocerebral-bridge optic-tubercle tract (*arrowheads*) are sparse in *L. migratoria* but prominent in *S. americana*. Side branches into the ventro-lateral protocerebrum (*VLP*) shown in **E** are also present in *S. americana*, but are not depicted on the section shown in **F**. *POCo* Posterior optic commissure. *Scale bars:* 100 μm (**A, C–F**), 200 μm (**B**)

the proximal posterior lobula and the optic stalk or approach the immunoreactive fibers in the inferior tract (Fig. 2A, see below). In the superior protocerebrum, a superficial shell of neuropil ventro-lateral to the calyces is innervated by varicose ramifications (Figs. 2A, 3A). The main axons bypass the pedunculus anteriorly and arborize densely in an area between the α-lobe and the pedunculus of the mushroom body (Figs. 2A, 3A). Fibers continue in several superior commissures toward corresponding areas in the contralateral hemisphere or make a lateral turn anteriorly or posteriorly around the pedunculus and terminate in wide-field ramifications in the inferior lateral protocerebrum lateral to the pedunculus (Figs. 2A, 3A). Side branches of the commissural fibers innervate a superficial layer in the superior median protocerebrum near the midline furrow separating the two brain hemispheres (Figs. 2A, 3C).

Immunoreactive fibers passing around the lobula in the inferior tract are smaller and bear varicosities along their whole course. They give rise to sparse terminals in the ventro-lateral protocerebrum (Figs. 2A, 3B) and densely innervate the posterior optic tubercles (Figs. 2A, 3F), which are, furthermore, connected by a tenuous bundle of immunoreactive fibers in the posterior optic commissure (Figs. 2A, 3F). Some fibers also enter the protocerebral bridge via the protocerebral-bridge optic-tubercle tract (Figs. 2A, 3F) and terminate in the lateral parts of the bridge (Fig. 3D). Occasionally, they innervate the bridge throughout and even enter the w-bundle toward the central body.

The projections of PDH-immunoreactive fibers are similar in *S. gregaria* and *S. americana*, but some differences were noticed when compared with the staining in *L. migratoria* and *M. differentialis*. In *L. migratoria*, only 11–13 PDFMe cells (diameters 10–35 μm) exhibit PDHLI. The PDH-immunoreactive midbrain projections in *L. migratoria* and *M. differentialis* are sparser than in the two *Schistocerca* species. Terminals in the lobula, protocerebral bridge, and ventro-lateral protocerebrum are rare or often completely absent (Figs. 2B, 3E). Areas in the superior and inferior protocerebrum show staining intensity comparable to that of *S. gregaria*, but usually only two (*M. differentialis*) or two to four (*L. migratoria*) commissural fibers are immunoreactive as compared to six in *S. gregaria* (Fig. 2).

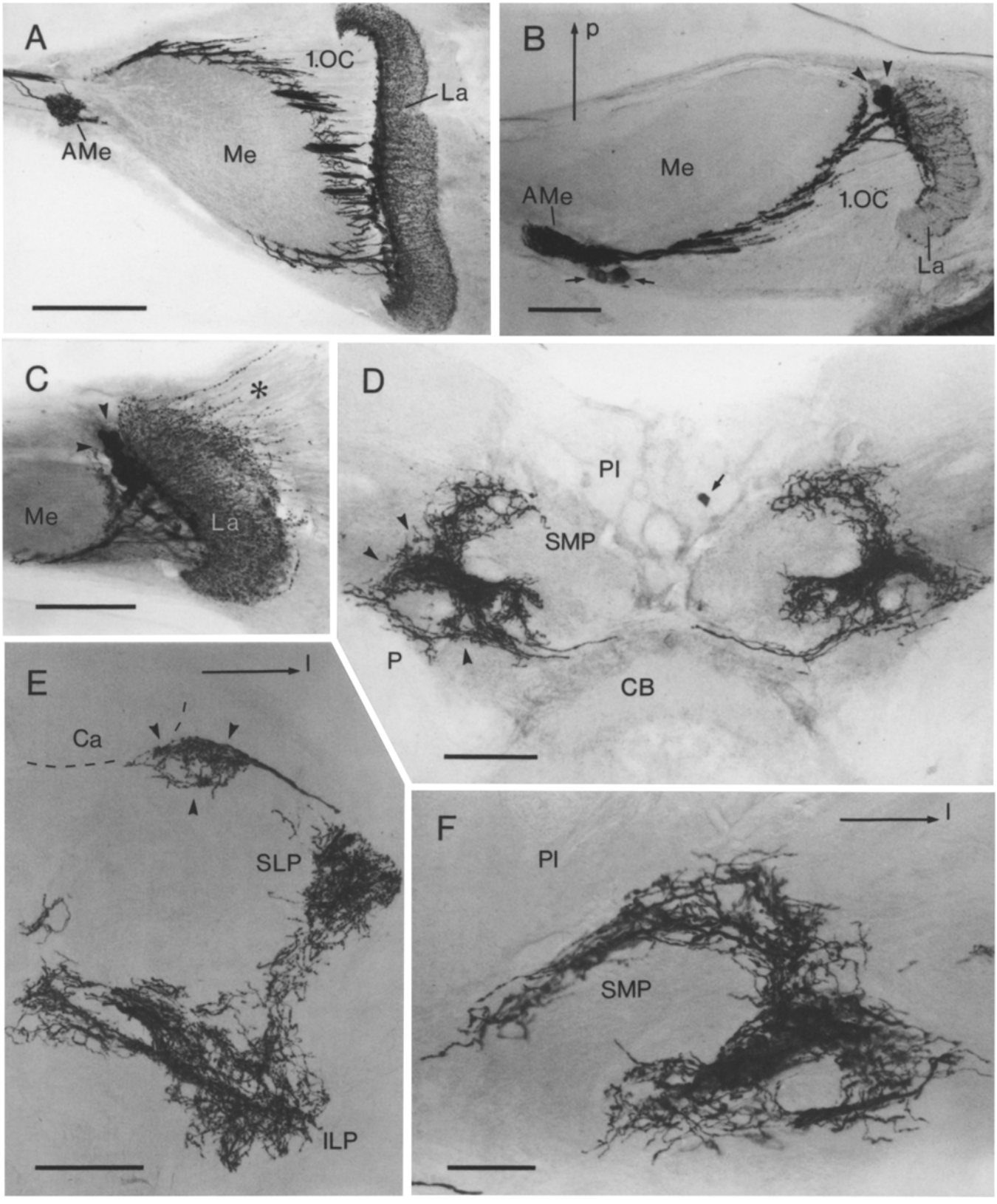
Crickets

While in *A. domesticus* and *G. bimaculatus* the PDFLa clusters consist of about 50 somata each, only about 15 PDFLa somata were counted in *T. oceanicus* and *T. commodus* (Table 1). This correlates with a much sparser innervation of the lamina by PDH-immunoreactive processes in the *Teleogryllus* species than in the two other crickets (Fig. 4A–C). Although immunostaining in all species is most intense along the inner face of the lamina, some immunoreactive processes usually project throughout the neuropil, and in *A. domesticus* some fibers even extend into the layer of the monopolar cell bodies (Fig. 4A–C.). Immunostaining in the medulla and accessory medulla closely resembles the staining pattern in acridids (Figs. 4A, B 5A). The PDFMe cluster consists of 9–15 cells with diameters of 10–20 μm (Table 1). The central projections of the PDFMe neurons in the lobula and midbrain are very similar in all crickets and have, therefore, only been reconstructed from one species, *T. commodus* (Fig. 5A). As in all orthopteroids studied, the primary neurits of the PDFMe cells project into the accessory medulla, which exhibits intense immunostaining (Fig. 4A, B). From the medulla, immunoreactive fibers run in a fiber tract along the posterior face of the lobula to the midbrain, giving off side branches into proximal areas of the lobula and into the optic stalk (Fig. 5A). In the superior lateral protocerebrum, the fibers pass anteriorly. As in locusts, small branches innervate an ovoid area anterior to the calyces of the mushroom body (Figs. 4E, 5A). The main fibers ramify extensively in superficial neuropil of the superior and inferior lateral protocerebrum, then turn medially and innervate most densely areas between the α-lobe and pedunculus of the mushroom body (Figs. 4D–F, 5A). This projection field extends to a neuropil area in the superior median protocerebrum, adjacent to the cell cortex of the pars intercerebralis (Figs. 4D, F, 5A). From the inferior lateral protocerebrum, some fibers also bypass the pedunculus posteriorly. Unlike in locusts, contralateral projections are virutally absent. Only one or two small varicose fibers cross the midline anterior or superior to the central body (Figs. 4D, 5A), but they apparently end without major ramification in the contralateral hemisphere.

Phasmid

In *E. tiaratum*, the PDFLa clusters are the largest among all species studied, each consisting of about 84 neurons

Fig. 4A–F. PDHLI in the optic lobe (**A–C**) and midbrain (**D–F**) of crickets. **A** Frontal section through the optic lobe of *Teleogryllus commodus*. **B** Oblique horizontal section through the optic lobe of *Gryllus bimaculatus*. **C** Oblique horizontal section through the lamina and distal part of the medulla of *Acheta domesticus*. In all species PDH-immunoreactive fibers innervate the lamina (*La*) and project through the first optic chiasma (*1.OC*) into the accessory medulla (*AMe*). The pattern of PDHLI in the lamina differs between the species, and in *Acheta*, varicose processes even extend into the distal cell body layer of the lamina (*asterisk* in **C**). In **B** and **C**, the clusters of PDFLav cells are shown at the posterior ventral edge of the lamina (*arrowheads*). *Arrows* in **B** point to PDFMe cells; *p* posterior. **D** Frontal section through the midbrain of *A. domesticus* showing dense PDHLI in an area between the pedunculus (*P*) and α-lobe of the mushroom bodies (*arrowheads*) and in the superior median protocerebrum (*SMP*). *Arrow* points to weakly immunoreactive soma in the pars intercerebralis (*PI*, see also Fig. 9C). *CB* Central body. **E** Frontal section showing PDHLI in the midbrain of *T. commodus* at a level anterior to that shown in **D**. Immunoreactive arborizations extend from the inferior lateral protocerebrum (*ILP*) to the superior lateral protocerebrum (*SLP*). In addition, an ovoid area ventro-lateral to the calyces (*Ca*) of the mushroom body is innervated by an immunoreactive plexus (*arrowheads*). **F** Frontal section showing PDHLI in the superior median protocerebrum (*SMP*) of *T. commodus*. The plane of the section and the pattern of immunoreactive arborizations are similar to that shown for *A. domesticus* (**D**). *Scale bars:* 200 μm (**A**), 100 μm (**B–E**), 50 μm (**F**)



with a uniform small size (diameter about 11 µm; Table 1). Immunoreactive arborizations in the lamina are, as in locusts, largely restricted to the proximal layer of the neuropil (Fig. 6A). *Extatosoma tiaratum* has 16 PDFMe cells per optic lobe (diameter 12–25 µm, Figs. 5B, 6B; Table 1). From the medulla, most immunoreactive fibers project in one bundle into the midbrain, giving off side branches into the proximal posterior lobula (Figs. 5B, 6C). Most of the fibers enter the midbrain through the superior protocerebrum. Side

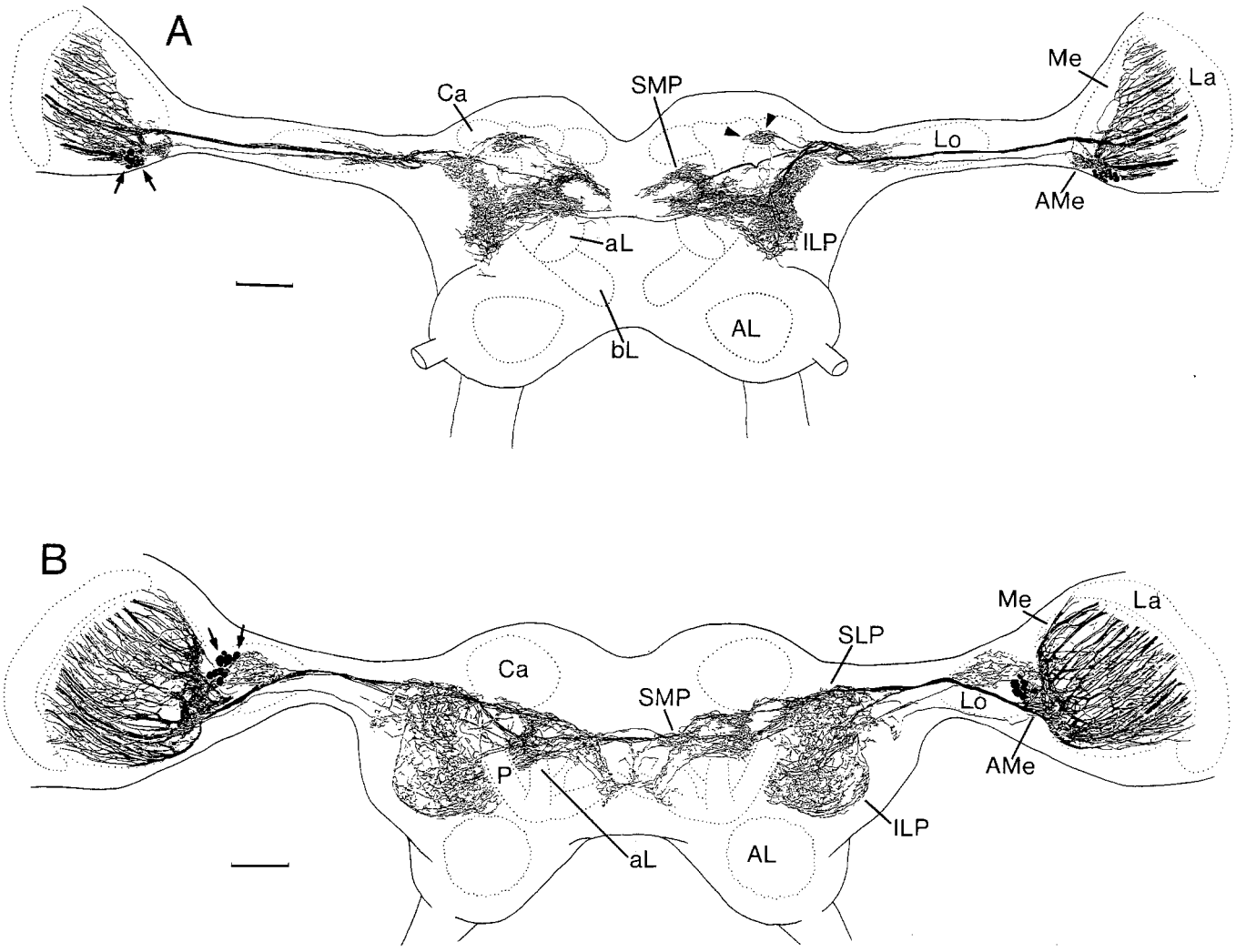


Fig. 5. **A** Frontal reconstruction of PDHLI in the brain, lobula (*Lo*) and medulla (*Me*) of the cricket *Teleogryllus commodus*. *Arrows* point to PDFMe cells. Immunoreactive processes in the optic lobe are concentrated in the first optic chiasma between medulla and lamina (*La*), and in the accessory medulla (*AMe*). PDFLa somata and immunoreactive arborizations in the lamina have been omitted. In the midbrain, immunoreactive ramifications are in the inferior lateral protocerebrum (*ILP*). They extend to neuropil between the α-lobe (*aL*) and pedunculus of the mushroom body and to a small field of terminals in the superior median protocerebrum (*SMP*). Some side branches innervate an ovoid area in the superior lateral protocerebrum (*arrowheads*). *AL* Antennal lobe; *bL* β-lobe and *Ca* calyces of the mushroom body. **B** Oblique horizontal reconstruction of PDHLI in the brain of the phasmid *Extatosoma tiaratum*. Immunoreactive fibers from the PDFMe neurons (*arrows*) and PDFLa neurons (arborizations in the lamina and somata not shown) connect the posterior face of the lamina (*La*) and the anterior face of the medulla (*Me*). Immunoreactive processes innervate the accessory medulla (*AMe*) and some varicose side branches enter the posterior lobula (*Lo*). In the midbrain, immunoreactive fibers invade the superior and inferior lateral protocerebrum (*SLP, ILP*), neuropil between α-lobe (*aL*) and pedunculus (*P*) of the mushroom body, and the superior median protocerebrum (*SMP*) adjacent to the median bundle. *Scale bars:* 200 μm

branches innervate a superficial shell of neuropil anterior to the calyces of the mushroom body (Fig. 6D). Most immunoreactive processes arborize in the superior and inferior lateral protocerebrum and extend into a superficial neuropil in the superior median protocerebrum. Compared with the other species, the midbrain projections are more diffuse and extend over larger volumes of neuropil (Figs. 5B, 6D, E). From the common bundle in the optic stalk, some fibers project directly into the ventro-lateral protocerebrum posterior to the pedunculus of the mushroom body (Fig. 6E). Similar to locusts, at least four major and several smaller fibers cross the midline of the brain in superior commissures (Fig. 5B).

Cockroach

In *P. americana*, as in the other species, clusters of PDH-immunoreactive cells lie at the posterior dorsal and ventral edge of the lamina (Fig. 7A), each consisting of about 58 somata (diameter about 13 μm). Immunoreactive processes in the lamina are concentrated along the inner face of the neuropil. Varicose processes invade a proximal layer of the lamina and some fibers even extend into the distal cell body layer (Fig. 7B). The PDFMe cluster consists of 14 cells per hemisphere with diameters of 10–25 μm (Table 1; Fig. 7C). Immunoreactive arborizations in the midbrain resemble the pattern seen in the

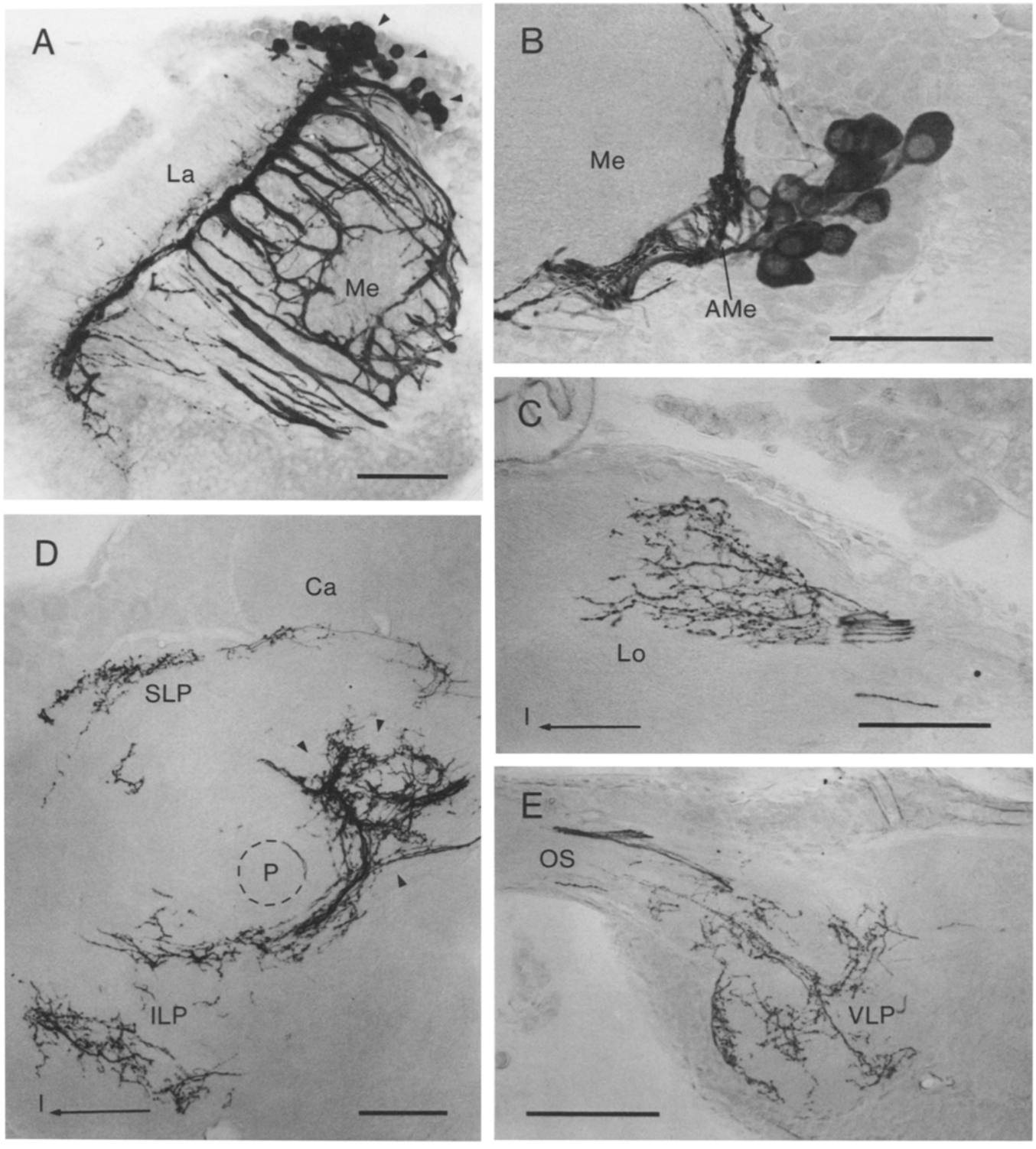


Fig. 6A–E. PDHLI in the brain of *Extatosoma tiaratum*. **A** Horizontal section through the lamina (*La*) and dorsal face of the medulla (*Me*). Immunoreactive processes, mostly from PDFLa cells (*arrowheads*) are concentrated at the inner surface of the lamina and extend via the first optic chiasma dorsally around the medulla. **B** Frontal section showing PDFMe somata and immunoreactive arborizations in the accessory medulla (*AMe*). **C** Frontal section showing PDH-immunoreactive terminals that innervate the posterior lobula (*Lo*). *l* Lateral. **D** Horizontal section through the left hemisphere of the midbrain. Immunoreactive arborizations are concentrated between the pedunculus (*P*) and the α-lobe of the mushroom body (*arrowheads*) and extend into the inferior lateral protocerebrum (*ILP*). Side branches also innervate neuropil in the superior lateral protocerebrum (*SLP*) antero-lateral to the calyces (*Ca*) of the mushroom body. **E** Frontal section showing varicose PDH-immunoreactive fibers and terminals in the ventro-lateral protocerebrum (*VLP*). *OS* Optic stalk. *Scale bars:* 100 μm (**A–D**), 200 μm (**E**)

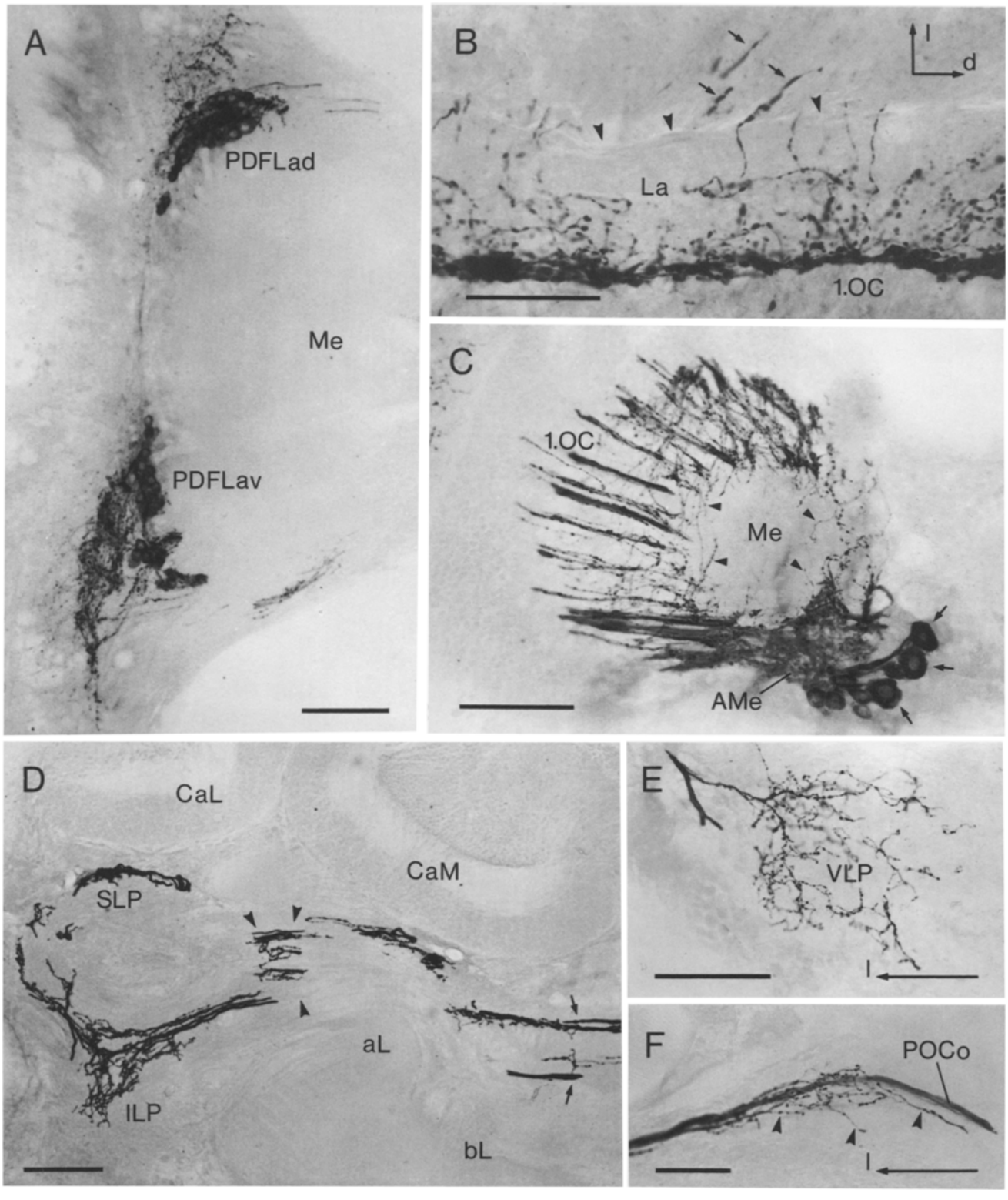


Fig. 7A–F. PDHLI in the brain of *Periplaneta americana*. **A** Frontal section through the posterior edge of the lamina showing the dorsal (*PDFLad*) and ventral (*PDFLav*) clusters of PDH-immunoreactive cells. *Me* Medulla. **B** Frontal section through the lamina (*La*). Varicose immunoreactive fibers are concentrated at the inner face and in the proximal layer of the lamina but occasionally extend into the distal cell-body layer (*arrows*). *Arrowheads* indicate distal boundary of lamina neuropil. *d* Dorsal; *l* lateral; *1.0C* first optic chiasma. **C** Frontal section through the anterior face of the medulla (*Me*) and first optic chiasma (*1.0C*). A mesh of fine varicose fibers extends over large parts of the outer surface of the medulla (*arrowheads*). The accessory medulla (*AMe*) is densely innervated by PDH-immunoreactive processes. *Arrows* point to PDFMe cells. **D–F** Frontal sections through the midbrain of the cockroach. **D** PDH-immunoreactive fibers arborize in the superior and inferior lateral protocerebrum (*SLP, ILP*) and in an area between the α-lobe (*aL*) and pedunculus of the mushroom body (*arrowheads*). Several fibers project via superior commissures contralaterally (*arrows*). *bL* β-lobe of the mushroom body; *CaL, CaM* lateral and medial calyx of the mushroom body. **E** Varicose PDH-immunoreactive fibers and terminals in the ventro-lateral protocerebrum (*VLP*). **F** PDH-immunoreactive fibers in the posterior optic commissure (*POCo*). Varicose processes (*arrowheads*) sparsely innervate the posterior optic tubercle. *Scale bars:* 100 μm (**A, C–E**), 50 μm (**B, F**)

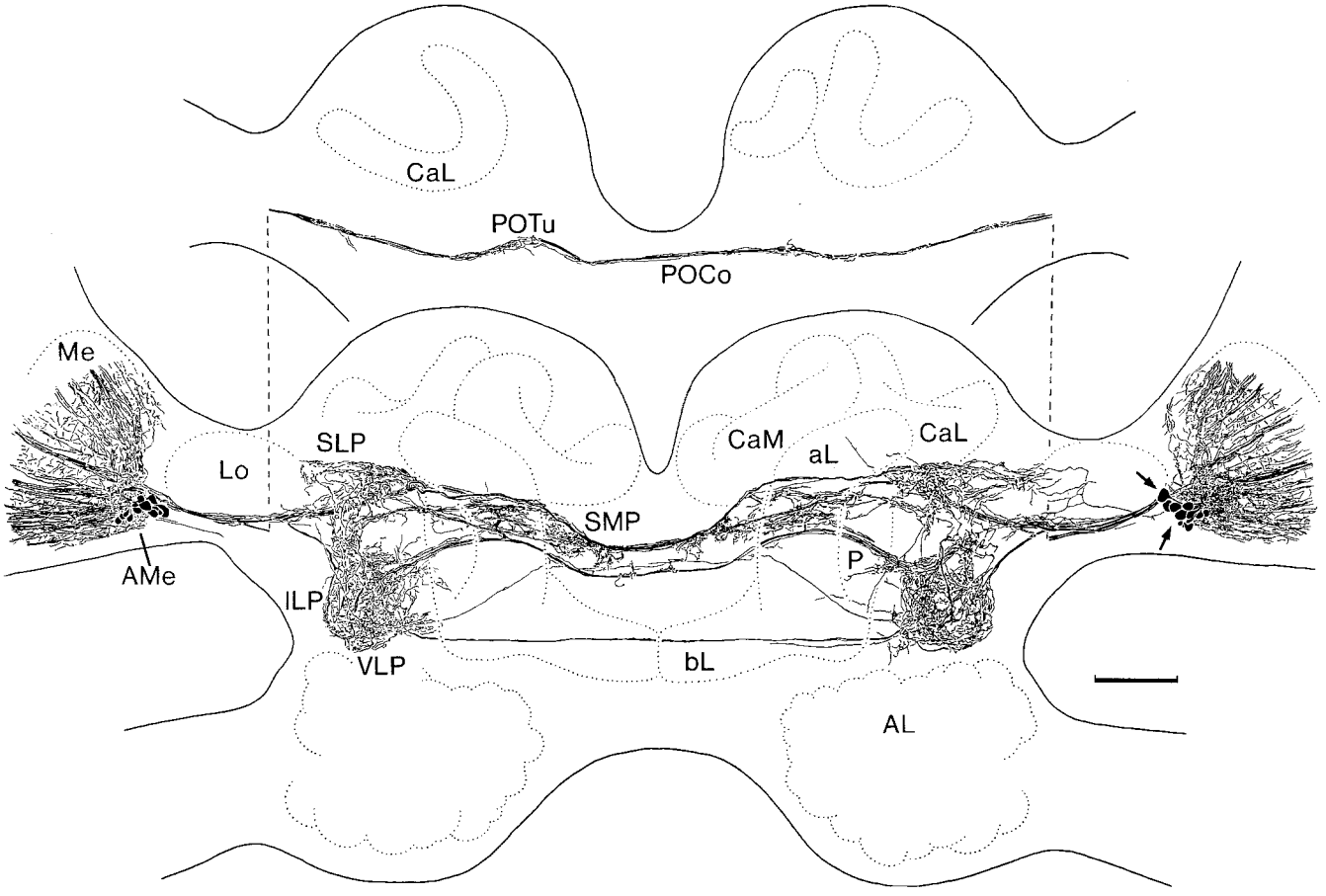


Fig. 8. Frontal reconstruction of PDHLI in the brain of *Periplaneta americana*. In the optic lobe, numerous immunoreactive fibers from the PDFMe cells (*arrows*) and PDFLa cells extend from the posterior edge of the lamina to the anterior edge of the medulla (*Me*) and innervate the accessory medulla (*AMe*). Immunoreactive cells and processes in the lamina have been omitted. In the midbrain, dense PDH-immunoreactive processes innervate the inferior lateral and ventro-lateral protocerebrum (*ILP, VLP*). Additional terminals are in an area in the superior lateral protocerebrum (*SLP*) anterior to the lateral calyx (*CaL*) of the mushroom body, in neuropil between α-lobe (*aL*) and pedunculus (*P*) of the mushroom body, and in superficial areas in the superior median protocerebrum (*SMP*). Several commisssures in the inferior and superior protocerebrum contain immunoreactive fibers. *Inset* shows PDH-immunoreactive fibers in the posterior optic commissure (*POCo*) with a few terminals in the posterior optic tubercles (*POTu*). *AL* Antennal lobe; *bL* β-lobe and *CaM* medial calyx of the mushroom body. *Scale bar:* 200 μm

other species. Ramifications in the lobula are sparse or completely absent, and the area between α-lobe and pedunculus is also less densely innervated than in the other species (Fig. 7D, 8). On their way from the optic lobe to the superior median protocerebrum, some immunoreactive fibers bypass the α-lobes anteriorly, which was never observed in the other species. The inferior lateral protocerebrum and the ventro-lateral protocerebrum exhibit especially dense PDHLI. Commissural fibers originating in these areas cross the midline in the inferior and superior protocerebrum (Figs. 7E, 8). In *P. americana*, more immunoreactive fibers connect both hemispheres than in any other species investigated. Two superior commissures contain six major and several smaller processes, and an inferior commissure posterior to the β-lobes of the mushroom bodies contains two immunoreactive fibers (Figs. 7D, 8). As in locusts, the posterior optic commissure contains a small bundle of immunoreactive fibers that extends from one optic lobe to its contralateral counterpart (Fig. 8). At the level of the posterior optic tubercles, the bundle of immunoreactive fibers becomes less fasciculated and some varicose processes enter the tubercles (Figs. 7F, 8).

Additional PDH-immunoreactive brain neurons

In addition to the PDFLa and PDFMe cells, small sets of neurons exhibit faint PDHLI in the midbrain of some species. In locusts, two pairs of immunoreactive neurons with somata in the inferior median protocerebrum and weakly immunoreactive arborizations in the superior protocerebrum have varicose ramifications in the tritocerebrum (Fig. 9A). The tritocerebrum also exhibits PDHLI in crickets. Unlike in locusts, it is innervated by one pair of PDH-immunoreactive neurons with somata in the frontal tritocerebrum and, in addition, by ascending fibers from the ventral nerve cord. In *A. domesticus*, but not in the other crickets, cells in the pars intercerebralis with fibers in the median bundle exhibit

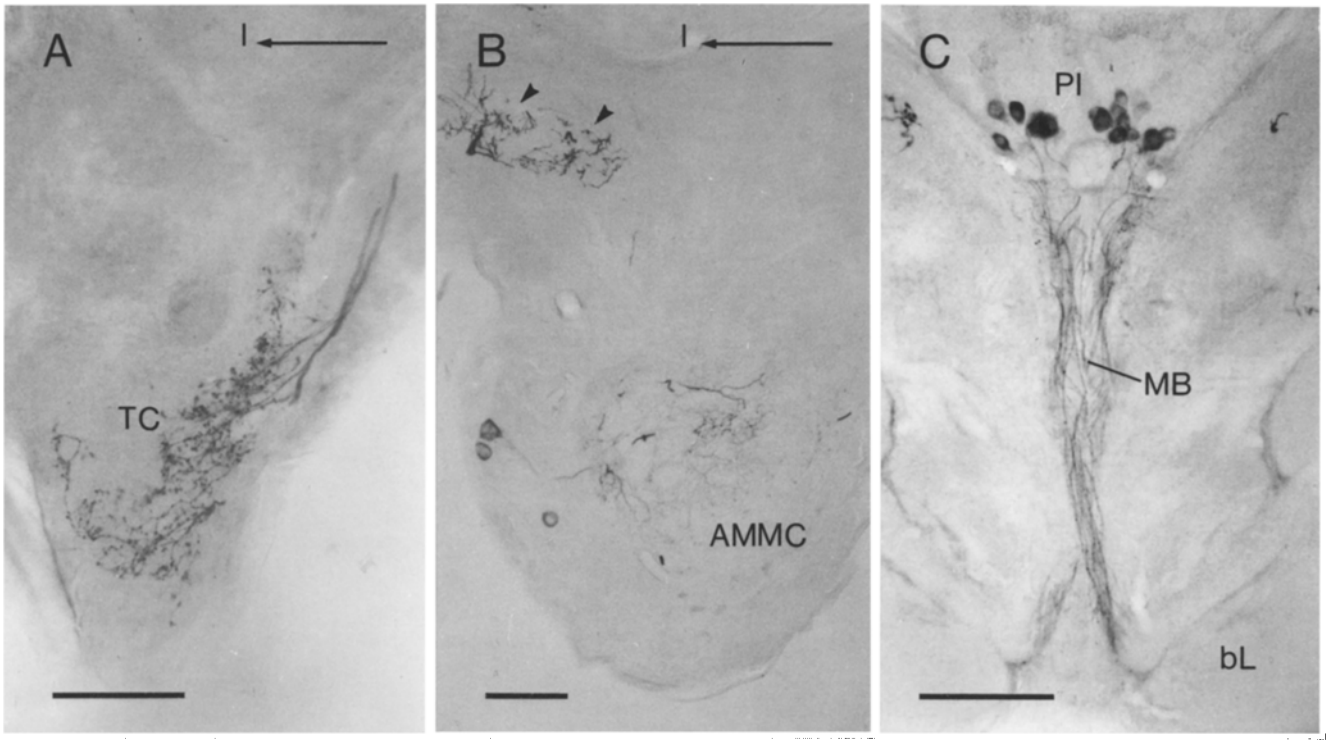


Fig. 9. A Frontal section through the tritocerebrum (*TC*) of the locust *Schistocerca gregaria*, showing varicose projections from a pair of weakly PDH-immunoreactive neurons with somata in the inferior medial protocerebrum. *l* Lateral. **B** Frontal section through the deutocerebrum of the phasmid *E. tiaratum*. Weakly PDH-immunoreactive neurons with somata in the lateral deutocerebrum innervate the antennal mechanosensory and motor center (*AMMC*). *Arrowheads* point to PDH-immunoreactive projections from PDFMe cells in the ventro-lateral protocerebrum. **C** Frontal section through the midbrain of the cricket. *A. domesticus* showing PDH-immunoreactive somata in the pars intercerebralis (*PI*) with immunoreactive fibers in the median bundle (*MB*). *bL* β-lobe of the mushroom body. *Scale bars:* 100 μm

PDHLI (Figs. 4D, 9C). In the phasmid *E. tiaratum*, 2–4 cells in the lateral antennal lobe with arborizations in the antennal mechanosensory and motor center are PDH-immunoreactive (Fig. 9B). All of these neurons in the midbrain show considerably weaker immunostaining than the optic lobe neurons, and, at higher dilutions of the primary antiserum, staining in these cells is often completely absent.

Discussion

In the optic lobes of orthopteroid insects, small and highly conserved sets of interneurons exhibit intense PDHLI. Previous studies in the optic lobes of Crustacea have demonstrated PDHLI in neurosecretory cells with terminals in the sinus gland as well as in interneurons (Dircksen et al. 1987; Mangerich et al. 1987; Rao and Riehm 1989) suggesting a dual role of this peptide as a neurohormone and neurotransmitter/neuromodulator. The identification of peptides related to *Uca/Cancer* β-PDH in the brain of two orthopteroids, *A. domesticus* and *Romalea microptera* (Rao and Riehm 1989) strongly suggests that the PDFLa and PDFMe neurons described here contain peptides of the PDH family. In addition, different types of neurons in the midbrain of some orthopteroids show faint immunoreactive staining. These neurons might contain only small amounts of PDH-like peptides or molecules with lower affinity to the antiserum.

A comparison of the immunocytochemical staining patterns suggests that the PDFLa neurons as well as the PDFMe neurons are homologous sets of cells in orthopteroid insects (Table 1). In all species studied, the neurons show striking similarities in their branching pattern, have somata at corresponding positions in the optic lobe, and apparently contain closely related peptides. The PDFLa cells invariably lie at the posterior dorsal and ventral edge of the lamina, but their numbers differ considerably between the species (Table 1). This might be accounted for by different proliferation rates of the PDFLa neuroblasts or differences in the rate of cell death during differentiation of the PDFLa cells. If the PDFLa neuroblasts, in addition to PDFLa cells, give rise to non-PDH-immunoreactive neurons, different ratios of these progeny could also account for species-specific differences in the number of PDFLa cells. The PDFLa cells are of uniform size and appear to have similar ramifications in the lamina, distal medulla, and accessory medulla. Because no morphological polarity was observed in the branching pattern of the PDFLa neurons, they might be anaxonal cells of the lamina/medulla. Extensive horizontal fiber meshworks in the proximal layer of the lamina similar to the PDH-immunoreactive staining pattern have been described in the locust *S. gregaria* (Nowel and Shelton 1981), but projec-

tions into the medulla and the position of cell bodies were not reported. Lamina amacrines of flies usually have, unlike the PDFLa cells, somata in the first optic chiasma and processes restricted to the lamina (Campos-Ortega and Strausfeld 1973; Strausfeld 1976; Fischbach and Dittrich 1989). A neuron that closely resembles the PDFLa cells has, to our knowledge, only been described in the butterfly *Pieris brassicae* (Strausfeld and Blest 1970). Like the PDFLa cells, it innervates the lamina and has processes extending to the surface of the medulla and a soma at the edge of the lamina.

The accessory medulla

In all species, both the PDFLa and PDFMe cells appear to contribute to dense immunoreactive staining in the accessory medulla, an anterior appendage of the medulla. Similar structures have been described in a number of insects including Plecoptera (Hanström 1940), Trichoptera (Ehnbohm 1948; Hagberg 1986), and some Heteroptera (Pflugfelder 1936/37). In Trichoptera, Aphididae, and some Coleoptera (Schulz et al. 1984), reduced larval eyes are still present in the imago and reside near the posterior edge of the adult compound eye. As has been shown in Trichoptera (Hagberg 1986), these surviving larval photoreceptors project into an accessory lamina, and some have fibers extending via the first optic chiasma into the accessory medulla. In the developing optic lobe of Diptera, the larval stemmatal nerve terminates in an "ocellar neuropil" in a position corresponding to the accessory medulla (Pflugfelder 1937; Meinertzhagen 1973). Recently, extraretinal cells projecting from the rear edge of the compound eye to the anterior base of the medulla have been described in adult flies (Nässel et al. 1988; Hofbauer and Buchner 1989) and were suggested to derive from larval stemmata. Thus, in these insects, the accessory lamina and accessory medulla appear to be the remnants of the larval optic center subserving the stemmata. Observations on a lepidopteran species, *Manduca sexta* (U. Homberg, unpublished), and this study show that an accessory medulla is also present in insects with stemmata that do not survive metamorphosis (*M. sexta*) or in insects without larval stemmata. Therefore, the accessory medulla might be a common visual neuropil in many insects, irrespective of the survival or presence of larval stemmata and appears to be part of a visual pathway that bypasses the retinotopically organized portion of the opic lobe subserving the compound eye.

PDFMe cells

Unlike the PDFLa cells, the PDFMe neurons appear to be morphologically heterogeneous. The size of their somata shows substantial variations, and individual PDFMe neurons innervate only some of the immunoreactive areas in the midbrain. The number of commissural fibers in locusts, the cockroach, and the phasmid show that only a fraction of the neurons have contralateral processes in the superior protocerebrum. Thus, the PDFMe neurons are a group of neurons with perhaps similar arborizations in the optic lobe, but highly individual branching patterns in the midbrain. Besides ramifications in the lamina and the accessory medulla, several areas are innervated in the median protocerebrum. These include in all species (1) the superior median protocerebrum, especially near the median furrow of the two hemispheres, (2) a small area ventro-lateral or anterior to the calyces of the mushroom bodies, (3) neuropil between α-lobe and pedunculus of the mushroom bodies, (4) a region in the inferior lateral protocerebrum, and (5) parts of the superior lateral protocerebrum (Table 1). In addition, only in some species do the PDFMe cells innervate the lobula, the ventro-lateral protocerebrum, the posterior optic tubercles, and the protocerebral bridge (Table 1). These divergent projections suggest that the PDFMe cells directly communicate with several central circuits of the brain, but possibly also with efferent and descending neurons. The superior median protocerebrum is densely innervated by neurosecretory cells with axons to the retrocerebral complex (Pipa 1978; Koontz and Edwards 1980; Thompson et al. 1987; U. Homberg, personal observations). Although no colocalization experiments have been performed yet, parts of the dendritic trees of these neurosecretory cells seem to overlap with PDH-immunoreactive projections, thus allowing direct synaptic contact to neurosecretory cells. Connections with descending neurons might occur in the inferior lateral and ventro-lateral protocerebrum. Most descending interneurons from orthopteroid brains that are involved in flight stabilization have dendrites in posterior areas of the brain that are not innervated by PDH-immunoreactive neurons (Williams 1975; Griss and Rowell 1986; Hedwig 1986; Hensler 1988). Others, however, like the LG1–3 neurons of locusts (Williams 1975), which have not been characterized physiologically, have dendritic fields in the inferior lateral and ventro-lateral protocerebrum where they potentially overlap with PDH-immunoreactive arborizations.

The immunoreactive terminals in the posterior optic tubercles and continuing projections into the protocerebral bridge in acridids indicate a connection of the PDFMe cells with the central complex. Because the posterior optic tubercles are connected with the protocerebral bridge by prominent serotonin-immunoreactive fiber bundles (Williams 1975; Homberg 1991), the lack of significant PDH-immunoreactive projections in *L. migratoria* and *M. differentialis* into the protocerebral bridge might have only minor physiological consequences. In the cockroach, a similar serotonin-immunoreactive pathway exists from small posterior optic tubercles into the protocerebral bridge (Klemm et al. 1984; U. Binkle, U. Homberg, unpublished), suggesting again in this species an indirect connection between PDFMe neurons and the central complex. Although the posterior optic tract in crickets does not exhibit PDHLI, single immunoreactive fibers occasionally extend from the superior lateral protocerebrum in variable pathways into the posterior optic tubercle (data not shown), demonstrating its importance as a target of outgrowing PDFMe fibers.

Possible physiological role of the PDFMe neurons

The varicose appearance of all midbrain arborizations and the location of somata in the optic lobe suggest that the PDFMe neurons are centripetal neurons delivering visual information to the midbrain. Some immunoreactive processes in the accessory medulla and in the first optic chiasma that appear to originate from the PDFMe cells are less intensely immunolabeled than the midbrain arborizations and might therefore serve as the dendritic regions of the PDFMe neurons. With possible inputs in the nonretinotopically organized accessory medulla and the lamina, the PDFMe neurons might respond to simple visual signals like overall background illumination or large-field on/off stimuli and are probably not involved in color-coding mechanisms, motion or image perception, since these features require integration at higher stages of the visual system in the medulla and lobula complex (reviewed by Strausfeld 1984; Järvilehto 1985; Hertel and Maronde 1987).

With somata near the accessory medulla and arborizations extending from the lamina to many midbrain areas, the PDFMe neurons fullfill several important anatomical criteria that have been proposed for circadian pacemakers in crickets and cockroaches. Lesion experiments and extracellular recordings in these species have demonstrated that the optic lobe contains a self-sustained circadian oscillator, entrained by photoreceptors in the compound eye, that controls circadian changes in the electroretinogram, as well as motor activity (Page 1984; Chiba and Tomioka 1987; Colwell and Page 1990). These experiments have further shown that the circadian pacemaker resides close to the second optic chiasma, an area that includes the PDFMe somata and the accessory medulla (Sokolove 1975; Wills et al. 1985; Chiba and Tomioka 1987). Regarding their divergent projections, the PDFMe neurons are well suited to simultaneously control several physiological processes that are under circadian control such as the adaptational state of the visual system (Fleissner 1982; Wills et al. 1985) behaviors like stridulation (Loher 1972) or walking (Sokolove 1975; Page 1978), and the release of neurohormones (reviewed by Page 1985).

Both optic-lobe pacemakers are mutually coupled, but can be easily uncoupled in crickets by cooling the animals, while this has been difficult in cockroaches (Wiedenmann 1980, 1983). This observation correlates well with the differences in the PDH-immunoreactive staining pattern between the cockroach and the crickets. Whereas contralateral PDH-immunoreactive projections are virtually absent in crickets, prominent immunoreactive commissures and possibly direct connections between both optic lobes are found in the cockroach. This might enable synaptic contacts and thus a strong coupling between the right and left PDFMe neurons in the cockroach but not in crickets.

Although the anatomical features of the PDFMe neurons are consistent with a role as circadian pacemakers, other functions cannot be ruled out. Future experiments in our laboratory will address this question and investigate the role of this unique and putatively peptidergic system of neurons in the optic lobe of orthopteroid insects.

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