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REGULAR ARTICLE

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Immunocytochemical distribution of pigment-dispersing hormone in the cephalic ganglia of polyneopteran insects

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Abstract Material detectable with antisera to the pigment-dispersing hormone (PDH) is regarded as a component of the circadian clock residing in some insects in the optic lobe. This paper demonstrates that the position of the PDH-positive neurones and the course of their processes are similar in all representatives of the insect cohort Polyneoptera. A basic morphological pattern, which includes the proximal frontoventral (Pfv), distal posteriodorsal (Dpd) and posterioventral (Dpv) clusters of PDH-positive neurones, was found in the examined species of locusts, crickets, walking sticks, cockroaches, earwigs and termites. The Pfv cluster is located close to the accessory medulla and usually consists of a set of smaller and a set of larger perikarya. The Dpd and Dpv clusters occupy a dorsal and a ventral position, respectively, at the distal edge of the medulla. These clusters are lacking in stonefly and praying mantid species. The fan-like arrangement of PDH-positive fibres within the frontal medulla face (the locusts and the praying mantid have an additional, smaller fan on the posterior medulla face) is another characteristic feature of Polyneoptera. One (two in the locusts and the praying mantid) nerve bundle runs from the optic lobe to the lateral protocerebrum where it ramifies. One branch gives rise to a fibre network frontally encircling brain neuropile in the area of mushroom bodies. One thin fibre in the crickets and the earwig, and several thicker and anastomosing fibres in the other insects, connect the brain hemispheres. The arrangement of other PDH-positive structures specifies taxa within Polyneoptera. Specific features comprise the presence of PDH-positive perikarya in protocerebrum (walking stick and termite), deutocerebrum (cricket, walking stick, and one cockroach species), tritocerebrum (another cockroach species), and the suboesophageal ganglion (cricket, walking stick and termite). In the walking stick and the termite, PDH-positive fibres pass from the cephalic to the frontal ganglion and from there via the recurrent nerve to the corpora cardiaca where they make varicosities indicative of peptide release into the haemolymph.

Keywords PDF · PDH · Accessory medulla · Circadian clock · Neuroanatomy (Insecta, 13 species)

Introduction

In insects, most developmental and behavioural events occur with a periodicity that is adjusted to circadian and seasonal changes in their environment. Surgical experiments on cockroaches demonstrated the importance of optic lobes for the control of circadian periodicity (Nishiitsutsui-Uwo and Pittendrigh 1968a, 1968b). Bilateral lesions of a region between the medulla and the lobula were shown to cause arrhythmicity (Sokolove 1975), while transplantation of an optic lobe from another animal into the arrhythmic individual restored rhythmic behaviour, imposing the free-running period of the donor to the recipient (Page 1982). The regeneration of neural connections between the implant and the recipient midbrain was essential for the renewal of rhythmicity (Page 1983).

Extirpation of the optic lobes in crickets also brought about loss of rhythm in the locomotory and stridulatory activities (Sokolove and Loher 1975). Subsequently it was demonstrated both in cockroaches (Colwell and Page 1990) and crickets (Tomioka and Chiba 1992) that the optic lobes isolated in vitro were capable of generating a circadian neural activity. The periodic oscillations in frequency of generated impulses were self-sustained and independent of any humoral or neural connections with

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the rest of the organism. They apparently represented neural coupling between the optic lobe pacemaker and those parts of the central nervous system that execute control over the locomotor and stridulatory behaviour.

The location of circadian pacemaker at the base of optic lobes detected by the lesion experiments corresponded to a small neuropile region called the accessory medulla that was described as a termination site of larval and extraocular photoreceptors (Hagberg 1986). Immunocytochemical studies revealed fibres containing material similar to the pigment-dispersing hormone, PDH, in this region (Homberg et al. 1991a). This octadecapeptide was identified in crustaceans as a regulator of pigmentation changes, and insects contain structural orthologs sometimes called the pigment-dispersing factors, PDF (review in Rao and Riehm 1988). The occurrence of PDH-like material in fibres of the accessory medulla and in adjacent perikarya indicated that it represents a component of the pacemaker (Homberg et al. 1991a; Stengl and Homberg 1994). This assumption was verified by the finding that injection of PDH into the vicinity of the accessory medulla of cockroaches caused phase shifts in circadian locomotor activity (Petri and Stengl 1997). The shifts were different from those obtained with light pulses, suggesting that the PDH neurones are not part of the visual entrainment pathway but are either an integral component of the pacemaker or play a role in its non-photic input. The most important non-photic input obviously comes from the contralateral optic lobe pacemaker (Stengl and Homberg 1994). Coupling of the left and right pacemakers has repeatedly been proven. For example, it was demonstrated that integrity of just one compound eye allows entrainment of both pacemakers (Page et al. 1977) and that cooling of one optic lobe affected also the contralateral pacemaker (Page 1981).

The use of genetic and molecular techniques spurred investigations of the circadian system in *Drosophila*. The analysis of genetic mosaics and the transplantation experiments localized the control of behavioural rhythmicity to a small group of lateral brain neurones that express crucial components of the endogenous clock cascade, such as the Period protein (reviewed by Hall 1998). A ventral subgroup (LN_V) of these cells, located frontoventrally at the base of the optic lobe and sending fibres to the lamina region as well as to the protocerebrum, concurrently expresses PDH (Helfrich-Förster and Homberg 1993; Helfrich-Förster 1995). An ablation of LN_V or mutations of the *pdf* gene (Renn et al. 1999), and/or ectopic expression of PDH in certain other neurones (Helfrich-Förster et al. 2000), cause abnormalities in the free-running activity rhythm, proving that PDH is an important component of the *Drosophila* pacemaker. No circadian changes were detected in the content of *pdf* mRNA in the LN_V cells but their axon terminals exhibited a diurnal rhythm in the PDH immunoreactivity (Park et al. 2000). The data suggest that PDH is produced according to the entrained circadian pattern as a pacemaker output factor regulating rhythmic activities, such as oscillations in the axon calibre of certain interneurones in the housefly lamina (Meinertzhagen and Pyza 1996). PDH released from axon terminals of the LN_V neurones in the dorsolateral brain region of the fly modulates local neurone activity (Helfrich-Förster et al. 2000).

It is possible that PDH functions both as an internal pacemaker component (Petri and Stengl 1997) and as an output factor of the pacemaker (Jackson et al. 2001), and that the significance of the two functions is different in various insect groups. Diverse distribution of the PDH-positive neurones may indicate functional differences. Homberg et al. (1991a) showed in an excellent immunocytochemical study that several locust and cricket species, a phasmid, and a cockroach contained in each optic lobe a proximal frontoventral (Pfv), a distal posteriodorsal (Dpd), and a distal posterioventral (Dpv) cluster of PDH-positive cells. This finding was confirmed for cockroaches (Nässel et al. 1991; Stengl and Homberg 1994) and crickets (Okamoto et al. 2001) and extended to a beetle (Frisch et al. 1996). By contrast, two examined species of cyclorrhaphous flies possessed in each lobe only the LN_V neurone group (Nässel et al. 1991, 1993; Helfrich-Förster 1997), which is obviously homologous to the Pfv cluster of the other species. In the silkmoth, *Antheraea pernyi*, the number of PDH-positive neurones in the optic lobe is reduced to just two pairs of perikarya in the position of the Pfv cluster (Sauman and Reppert 1996). No PDH staining was observed in this region in the hawkmoth, *Manduca sexta* (Homberg et al. 1991b; Wise et al. 2002). In contrast to species possessing three groups of PDH-positive neurones in the optic lobe, the flies and moths contained distinct PDH-positive neurones in the central brain.

Available data indicate that the orders of beetles, flies, and moths, respectively, which belong to the cohort Holometabola, differ from one another by the arrangement of PDH-positive neurones in the brain. This is in sharp contrast to the finding that locusts, crickets, phasmids, and cockroaches, which represent three orders of the cohort Polyneoptera, possess very similar systems of cells and fibres containing PDH-like material (Homberg et al. 1991a). We decided to verify whether the Pfv, Dpd, and Dpv clusters of the PDH-positive neurones epitomize a basic morphological pattern of Polyneoptera and to examine whether the distribution of PDH-like material in other parts of the cephalic nervous system is also uniform throughout this cohort. In the present paper we describe the location of perikarya and the course of fibres stained with an anti-PDH antiserum in 13 species of six polyneopteran orders.

Materials and methods

Animals

Immunocytochemical experiments were performed on adult insects. In the case of termites, pseudergates (immature termites representing the cast of workers) and soldiers were also included. Earwigs were collected outdoors, and walking sticks and praying mantids were purchased from a dealer. These insects were sacrificed in the

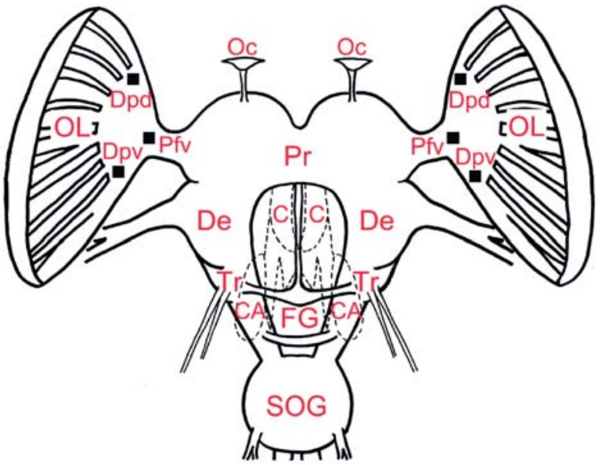


Fig. 1 Schematic diagram illustrating the cephalic neural complex of insects with abbreviations used in the photographs (*CA* corpora allata, *CC* corpora cardiaca, *De* deutocerebrum, *Dpd* distal posteriodorsal cell cluster, *Dpv* distal posterioventral cell cluster, *FG* frontal ganglion, *Oc* ocellus, *OL* optic lobe, *Pfv* proximal frontoventral cell cluster, *Pr* protocerebrum, *Tr* tritocerebrum, *SOG* suboesophageal ganglion)

morning of a natural light cycle 18:6 h (light:dark). The remaining species were taken from laboratory cultures that had been kept at 28°C and a 12:12 h photoperiod for at least 7 days. Dissections were carried out between 4 and 10 h after lights-on. The examined species represented six orders of the cohort Polyneoptera: locust *Schistocerca gregaria* Stål. (order Orthoptera, suborder Acridoidea); cricket *Acheta domestica* L. (order Orthoptera, suborder Grylloidea); walking stick *Sipyloidea sipylus* (Westwood) (order Phasmida); earwig *Forficula auricularia* L. (order Dermaptera); termite *Neotermes castaneus* (Burm.) (order Isoptera); praying mantid *Hierodula membranacea* Burm. (order Mantodea); blattoid cockroaches (order Blattaria, superfamily Blattoidea): *Blatta orientalis* L. and *Periplaneta americana* (L.) (family Blattidae, subfamily Blattinae); blaberoid cockroaches (order Blattaria, superfamily Blaberoidea): *Blattella germanica* (L.) of the family Blattellidae; *Blaptica dubia* Stål. and *Blabera craniifer* Burm. (family Blaberidae, subfamily Blaberinae); *Pycnoscelus surinamensis* (L.) (family Blaberidae, subfamily Pycnoscelinae); and *Nauphoeta cinerea* (Olivier) (family Blaberidae, subfamily Oxyhaloinae).

Immunocytochemistry

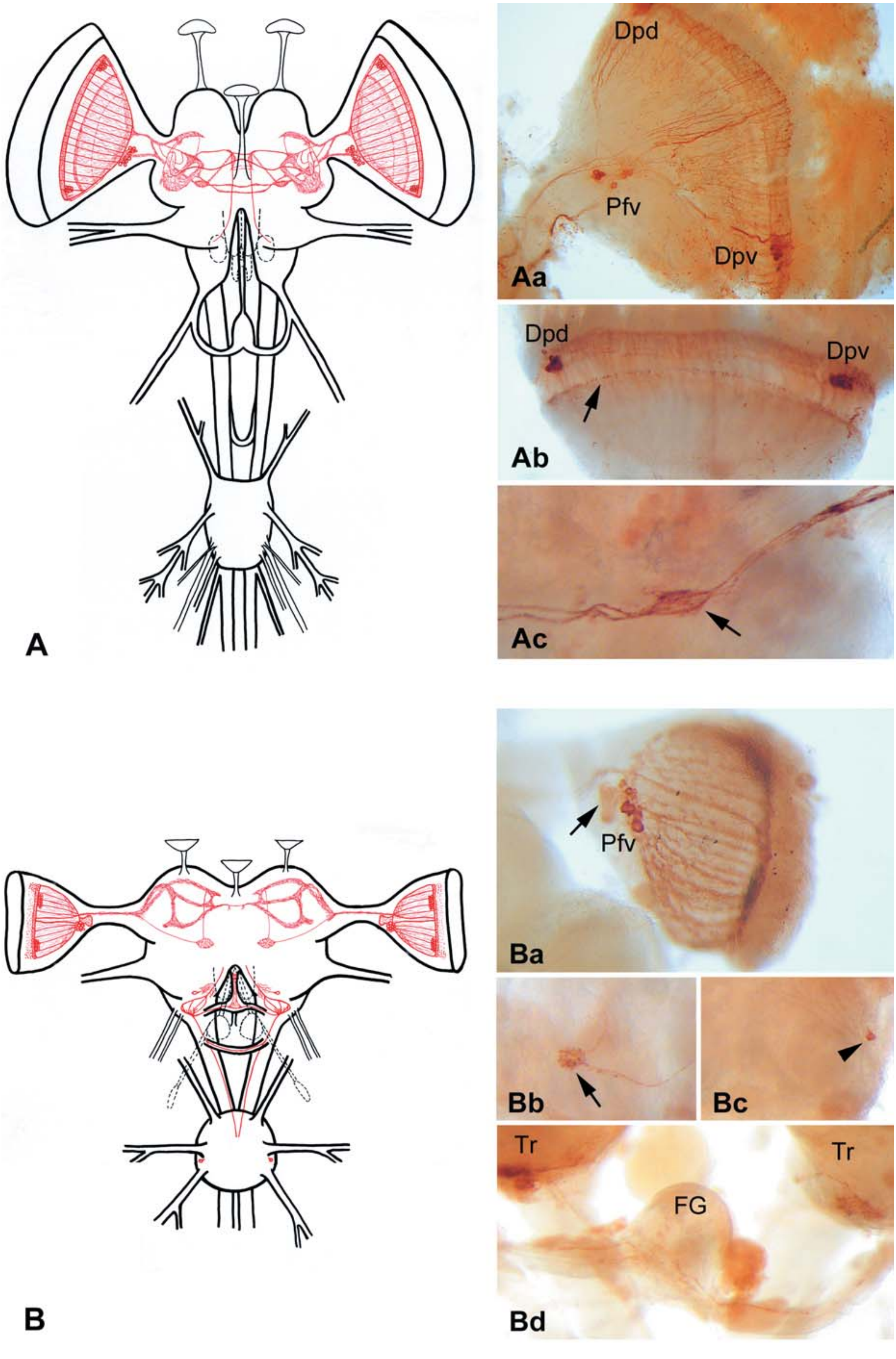
The brain (supraoesophageal ganglion), with attached suboesophageal ganglion, frontal ganglion, and the corpora cardiaca and corpora allata (Fig. 1), was dissected from water-anesthetized animals under sterile Ringer's solution. The preparation was immediately submersed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.5. After an overnight fixation at 4°C, the tissues were washed three times for 10 min at room temperature (r.t.) in PBS supplemented with 1–2% Triton X-100 (PBS-T) and then treated with collagenase (0.5 mg/ml PBS) for 1–6 h (depending on the brain size). Following washing in a methanol series (50%, 75%, 100%, 75%, and 50%, always for 10 min) and PBS-T (twice for 10 min), the preparations were blocked with 10% normal goat serum in PBS-T (2 h). Subsequently, they were incubated, for 2 days at 4°C, in an affinity-purified rabbit antiserum against the PDH of *Uca pugilator* (Crustacea) (Dircksen et al. 1987) diluted 1:10,000 in PBS-T. In the control experiments, primary antibody was replaced with normal goat serum. After a thorough rinsing with PBS-T (three times for 10 min at r.t.), the preparations were transferred to goat anti-rabbit IgG with conjugated horseradish peroxidase (HRP; Jackson ImmunoReseach) that was diluted 1:1000 in PBS-T. Following overnight incubation at 4°C, the preparations were rinsed with PBS-T (twice for 10 min at r.t.) and 0.05 M TRIS-HCl, pH 7.5 (three times 10 min), and the enzymatic activity of HRP was visualized with hydrogen peroxide (0.005%) and 3, 3'-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M TRIS-HCl, pH 7.5). The reaction was stopped by a 5-min wash in distilled water and the specimens were mounted in 80% glycerol. They were examined and photographed in frontal view plane under an Olympus SZX12 stereomicroscope.

Results

The anti-PDH antibody reacted with discrete perikarya and fibres in all examined species. Distinct staining always occurred in the optic lobes and in most cases revealed three cell clusters as described by Homberg et al. (1991a). The proximal cluster Pfv is located in a frontoventral position between the lobula and the medulla, and the two distal clusters, Dpd and Dpv, are placed posteriodorsally and posterioventrally, respectively, between the medulla and the lamina. A general feature of the optic lobe innervation is the arrangement of fibres into a semi-funnel that embraces the frontal side of the medulla. Upon reaching its distal edge, the fibres follow the lateral medulla surface to the vicinity of the Dpd and Dpv clusters on the rear of the lobe, where they turn backwards and run anteriorly over the inner lamina surface. There are only small modifications to this arrangement of fibres that is henceforth referred to as the optic fan. The course of fibres outside the optic lobes is specific for each of the examined insect orders.

Schistocerca gregaria

The Pfv cluster consists of 6–10 small and 3–4 distinctly larger neurones, and the clusters Dpd and Dpv each contain 10–15 small cells (Fig. 2Aa). In addition to the major optic fan, a much less distinct fan of fibres, which do not reach the distal medulla edge, spans the proximal region of the posterior medulla surface (Fig. 2Ab). Two bundles of PDH-positive fibres run from the optic lobe centripetally. One of them originates in the Pfv cell group area under the lobula complex and forms a small region of arborization in the posterior optic tubercle at the base of the protocerebrum (Fig. 2Ac). Some fibres then follow the tract linking the tubercle with the protocerebral bridge, but most continue through the ventral protocerebrum to the opposite brain hemisphere. The second centripetal fibre bundle begins in the optic lobe region, where the major anterior and the minor posterior optic fans merge. The bundle overpasses the lobula and in the lateral protocerebrum branches into a complex network that extends as a frontal semicircle from the dorsal to the ventral brain side. Three trajectories link this arborization to its counterpart in the other brain half. One trajectory runs close to the frontal brain surface, while the other two are more internal and pass through the central and the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basal parts of the protocerebrum, respectively. Some of the fibres of the central trajectory branch to other brain regions, including the deutocerebrum. No staining was found in the suboesophageal and frontal ganglia or in the corpora cardiaca and allata.

Acheta domestica

The three groups of PDH-positive cells in the optic lobes are similar to those of the locust except that the Dpd and Dpv clusters are closer to one another. The cricket is characterized by dense immunostaining of a relatively large accessory medulla (Fig. 2Ba). A single bundle of fibres runs from the optic fan base towards the midbrain, bypassing the Pfv cell group (Fig. 2Ba) and continuing over the posterior lobula surface to the lateral protocerebrum, where it breaks into three branches. A single fibre forms a small posterioventral arborization in the posterior optic tubercle (Fig. 2Bb), and continues to the frontal protocerebrum (Fig. 2B). Two branches of the original track form a network in the lateral protocerebrum and then continue centripetally close to the dorsal brain surface as three loose fibre bundles that terminate before reaching the midbrain. Only a single thin fibre provides a connection with the opposite hemisphere.

The cricket is distinguished from most other polyneopterans by PDH staining in the tritocerebrum. A symmetrical pair of relatively large immunopositive cells lie at its border with the deutocerebrum. The fibres, which are presumably associated with these cells, make a small arborization in the deutocerebrum, and another one in the tritocerebrum. In each brain half, one fibre extends for a short distance towards the protocerebrum and one runs into the frontal ganglion, where it ramifies (Fig. 2Bd) and continues to a group of four cells in the recurrent nerve. The third fibre leaves the tritocerebrum and via the circumoesophageal connective reaches the suboesophageal ganglion; the paired fibres of the left and right connectives are mutually linked via their side branches in the postoesophageal commissure. Within the suboesophageal ganglion, the fibres seem to terminate without making connections to a pair of ventrolateral PDH-positive perikarya (Fig. 2Bc).

Fig. 2 PDH immunoreactivity in the brain of adult locust *Schistocerca gregaria* (**A**) and cricket *Acheta domestica* (**B**). *S. gregaria*: **Aa** Frontal view of three clusters of perikarya and the fan of fibres in optic lobe, ×40; **Ab** posterior view of distal cell clusters and the major (in background) and minor (termination marked with *arrow*) fans of fibres over the frontal and rear medulla hemispheres, ×32; **Ac** fibre arborization (*arrow*) in posterior optic tubercle, ×90. *A. domestica*: **Ba** frontal view of the Pfv cell cluster, optic fan, and fibre arborization in accessory medulla (*arrow*), ×90; **Bb** small fibre arborization (*arrow*) in the posterioventral region of protocerebrum, ×90; **Bc** a ventrolateral cell in the suboesophageal ganglion, ×90; **Bd** fibres connecting tritocerebrum with the frontal ganglion, ×90. For abbreviations see Fig. 1

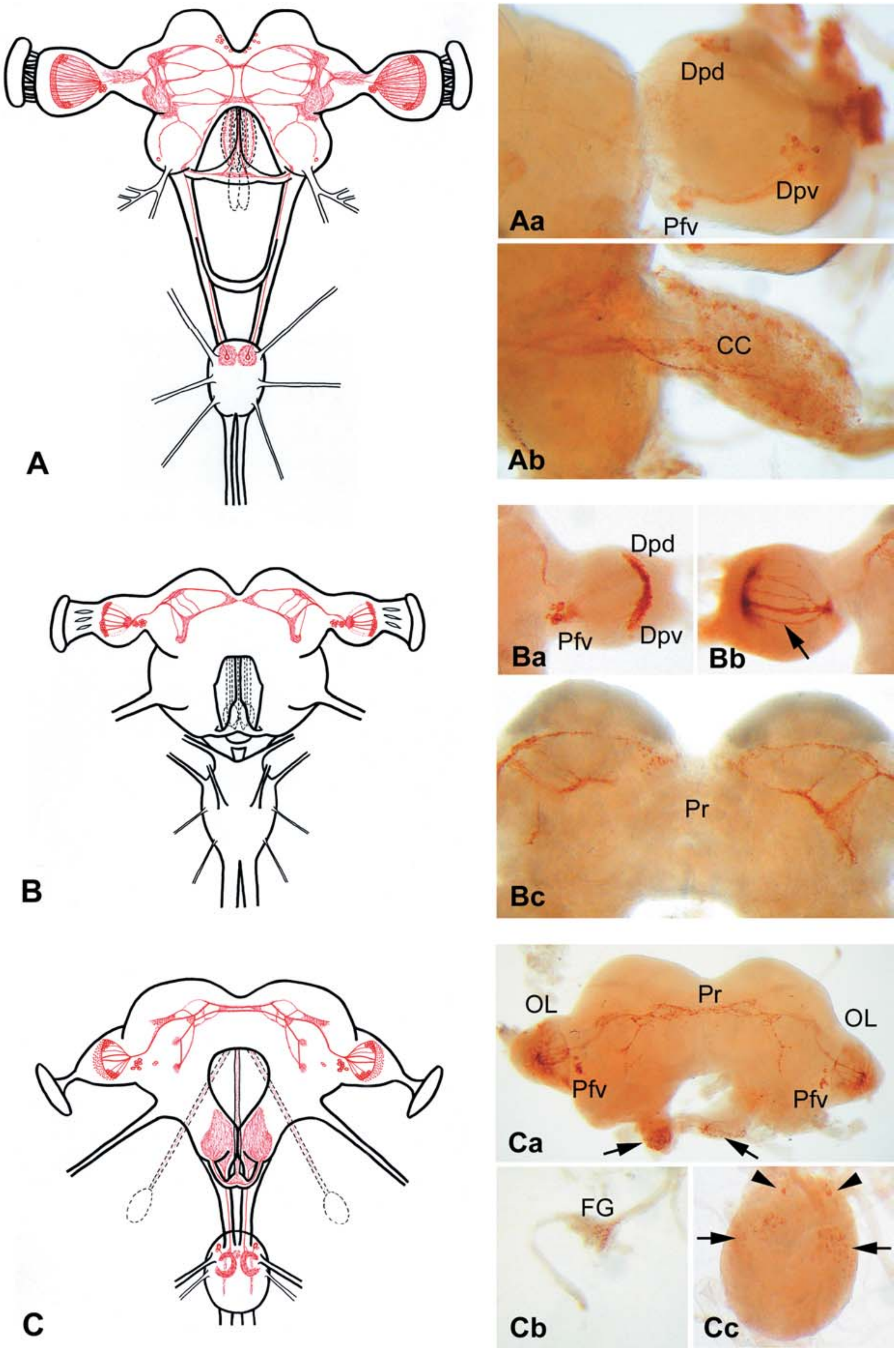
Sipyloidea sipylus

Immunoreactivity in the central nervous system of the walking stick is weaker than in most other insects. The Pfv cluster contains about 10 relatively large cell bodies, and the Dpd and Dpv clusters each comprise 10–15 smaller cells (Fig. 3Aa). The optic fan has a typical structure and gives rise to a single fibre bundle that runs over the posterior lobula side to branch in the lateral protocerebrum. A thick nerve tract continues centripetally to the opposite hemisphere. Some very fine neurites extend back to the optic lobe and innervate the posterior lobula. A third branch makes a large arborization that frontally circumvents the brain neuropile laterally to the mushroom bodies. At least four fibres originating in this arborization cross the brain midline dorsally to connect with their symmetrical counterparts. Two small ramifications, which occur in these connections in the central brain region, do not come close to about ten PDH-positive cell bodies located in the pars intercerebralis. No processes of these cells were found.

The major fibre arborization in the lateral protocerebrum and the small fibre network in the ventral part of the central brain are linked with the deutocerebrum (Fig. 3A). The fibres make no obvious connections with the pair of the PDH-positive deutocerebral cells but continue to the tritocerebrum and further on to the frontal and suboesophageal ganglia, respectively. The branches innervating the frontal ganglion can be traced in the recurrent nerve, the *nervi cardiostomatogastrici*, and the corpora cardiaca, where they ramify and form varicosities (Fig. 3Ab). The branches entering the suboesophageal ganglion make loose coils around the two PDH-positive perikarya that are on the ventroanterior side of this ganglion.

Forficula auricularia

The arrangement of PDH-positive perikarya and processes in the optic lobe is rather unusual. The Pfv cluster is divided into a subgroup of about ten small cells that are in the usual position and a subgroup of about five larger cells that are shifted to the posterior side of the optic lobe (Fig. 3Ba). The Dpd and Dpv clusters, each comprising at least 30 cells, are fused and take up the entire posterior edge of the distal medulla face (Fig. 3Ba). The optic fan consists of only a few fibres (Fig. 3Bb) that do not extend beyond the anterior medulla face. At the fan base, there is a small region of dense fibre arborization from where a single nerve bundle runs to the brain. It makes a posterior loop around the lobula and then returns to the frontal side of the lateral protocerebrum where it splits into two main branches running towards the brain midline. They give rise to three small arborizations interconnected with several fine fibres (Fig. 3Bc). The two hemispheres are mutually connected with a single and very fine PDH-positive fibre (Fig. 3B).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Neotermes castaneus

The pseudergates and soldiers have considerably smaller compound eyes and narrower optic lobes than the reproductives, but the pattern of PDH staining is identical in all three casts. About eight relatively large PDH cells constitute a loose Pfv cluster, and about ten cells are present in each of the more compact clusters Dpd and Dpv. The optic fan on the frontal medulla face is relatively simple (Fig. 3Ca) and consists of two distinct and several finer fibres. However, its distal part in the region of the first optical chiasma is very well developed and includes a dense network of fibres entering the lamina. A single nerve trajectory originating at the fan base runs centripetally, bypasses the lobula as a posterior loop and, upon returning to the frontal brain side, ramifies to several branches. Two of them aim at the base of the protocerebrum and the adjacent deutocerebral region, two others proceed to the contralateral brain hemisphere, and a few finer branches anastomose within the ipsilateral protocerebrum (Fig. 3Ca).

Apparently separate from the PDH-positive neurones described above are two faintly stained perikarya in the lateral protocerebrum and a complex network of fine PDH fibres arborizing in the tritocerebrum and connecting it with the frontal and the suboesophageal ganglia. In the frontal ganglion the fibres disperse into a fine network (Fig. 3Cb) and then continue through the recurrent nerve and the cardiostomatogastric nerves to the corpora cardiaca. The fibres that reach the suboesophageal ganglion bilaterally form a small dorsal and a much larger ring-shaped ventral arborization, and connect with a pair of distinctly stained proximal perikarya (Fig. 3Cc). No connections were found to five weakly stained perikarya located in the ganglion medially and to two symmetrical pairs of cell bodies placed laterally.

Hierodula membranacea

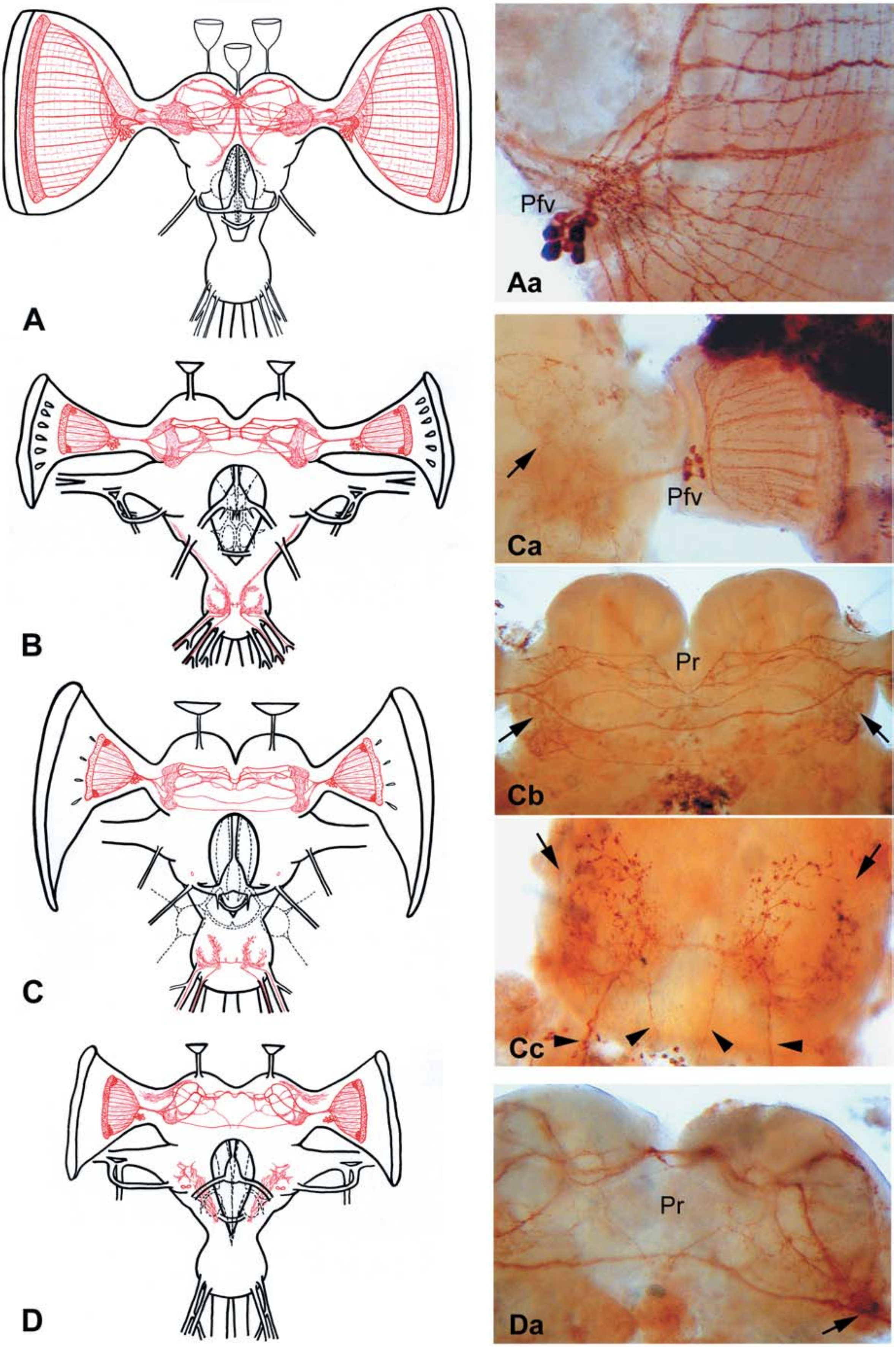
The praying mantid is distinguished from all other species examined in this study by the absence of the Dpd and Dpv cell clusters. The Pfv cluster contains about four large and four to five small cells that are associated with a horseshoe-shaped neuropile of the accessory medulla and linked to the base of the very distinct anterior optic fan (Fig. 4Aa). Another, albeit much smaller, fan composed of fine and densely arborizing fibres occurs in the basal part of the posterior medulla face. The two fans, which are characterized by numerous anastomoses splicing secondary centrifugal fibres, are interconnected by a nerve tract running along the anteriodorsal margin of the optic lobe, and by a bundle of fibres linking basal parts of the fans. A complex nerve trajectory rooted in both fans includes two ventrally running tracts to the opposite brain hemisphere, and a number of fibres that give rise to arborization in the lateral protocerebrum. The arborization reaches from the dorsal to the frontal brain region and medially condenses into four nerve tracts connecting to the opposite hemisphere, and a thick bundle formed frontally of the mushroom bodies. The bundle splits and arborizes in the pars intercerebralis, apparently crossing the brain midline, and sends a cord of fibres to the deutocerebral region that is also connected with the extensive arborization in the dorsolateral protocerebrum (Fig. 4A). No immunoreactivity was detected outside of the brain.

Blattaria

All examined cockroaches possess the typical arrangement of the PDH-positive perikarya and fibres in the optic lobes (Figs. 4Ca, 5Ca). The cell cluster Pfv includes 3–4 large and 6–10 small perikarya, and the clusters Dpd and Dpv each contain 10–15 small cells. The optical fan is well developed and a single centripetal nerve tract begins at its base. The tract runs to the posterior part of the lateral protocerebrum where it splits into several branches. Typically, a posterior branch runs through the ventral brain regions to the opposite hemisphere, an anterior branch gives rise to an arborization that frontally circumvents the lateral protocerebrum, and a medial branch follows the posterior optic commissure across the central brain. The arborization in the lateral protocerebrum sends off several centripetal processes that fuse to a few (in the suprafamily Blattoidea) or to just one (in the suprafamily Blaberoidea) fibres that traverse to the opposite brain hemisphere. Other differences between the two suprafamilies are as follows.

In Blattoidea, the bilateral protocerebral arborizations are connected with two very distinct tracts running along the frontal brain side (Fig. 4Cb). Other fibres originating in the lateral arborizations seem to terminate in, but may actually cross, the central brain region. An additional, thin connection of the two hemispheres occurs at the basal part of the protocerebrum in *Periplaneta americana*, which possesses a more complex arrangement of PDH-positive fibres in the brain than any other species. In Blaberoidea, the medial nerve tract, which obviously represents the posterior optic commissure, and the three to four nerve bundles originating in the protocerebral arborization (Fig. 5Cb, 5Da), fuse close to the median brain region into a single nerve bundle that connects the hemispheres.

Fig. 3 PDH immunoreactivity in the brain of adult walking stick *Sipyloidea sipylus* (**A**), earwig *Forficula auricularia* (**B**), and termite *Neotermes castaneus* (**C**). *S. sipylus*: **Aa** Posterior view of the optic lobe, ×90; **Ab** fibres with varicosities in the corpus cardiacum, ×90. *F. auricularia*: **Ba** Posterior view of the optic lobe showing posteriorly shifted subgroup of Pfv cells and the fused distal cell clusters, ×90; **Bb** simplified fan of fibres (*arrow*) on the frontal medulla face, ×90; **Bc** fibre projections in the protocerebrum (frontal view, ×90). *N. castaneus*: **Ca** Frontal overview of the PDH-positive cells and fibres in the brain with fibre arborizations in the tritocerebrum (*arrows*), ×63; **Cb** fibres with varicosities in the frontal ganglion, ×90; **Cc** ventral view of the suboesophageal ganglion (×90) with perikarya (*arrowheads*) and fibre arborizations with varicosities (*arrows*). For abbreviations see Fig. 1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The protocerebral arborization also sends numerous ramifying fibres to the lobula (lobula innervation by PDH-positive fibres is negligible in Blattoidea). The area of protocerebral arborization is reduced in *Blatella germanica* (Fig. 4Da), which represents the family Blatellidae. Blaberinae, one of the three subfamilies of the family Blaberidae, are distinguished from all other cockroaches examined by reduced staining of the Dpd and Dpv perikarya.

Fine extensions of the protocerebral PDH-positive fibres in the deutocerebrum were found only in *Nauphoeta cinerea*. The deuto- and tritocerebrum of cockroaches typically receive PDH-positive fibres from the suboesophageal ganglion (see below). One pair of weakly stained intrinsic cells is present in the tritocerebrum in *P. americana,* and extensive arborization in the deuto- and tritocerebrum, plus two pairs of PDH-positive perikarya in the deutocerebrum, occurs in *B. germanica.*

No PDH-like material is detectable in the suboesophageal ganglion of *B. germanica*. The pattern of immunoreactivity in other cockroaches depends on their taxonomic position. Both examined blattoids, *Blatta orientalis* and *P. americana*, contain two mutually connected semicircular arborizations of PDH-positive fibres with varicosities on the ventral side of the suboesophageal ganglion (Fig. 4Cc). Processes extending from these arborizations run into the gnathal nerves, and in *B. orientalis* also into the tritocerebrum. The representatives of Blaberidae are characterized by nerve tracts connecting the deuto- and tritocerebrum with the basal part of the suboesophageal ganglion, where they ramify (Fig. 5Aa, Cc) before they enter the gnathal nerves and the ventral nerve cord.

Discussion

Basic morphological pattern of the PDH-immunopositive system

Our data on 13 species, which represent six out of ten polyneopteran orders (Fig. 6), are consistent with the results of early studies on cockroaches (order Blattaria), locusts and crickets (Orthoptera), and a phasmid (Phasmida) (Homberg et al. 1991a; Nässel et al. 1991; Stengl and Homberg 1994). They are also in accord with the recent description of PDH staining in a stonefly (Závodská et al. 2003). It can be concluded that Polyneoptera possess a general morphological pattern in respect to the distribution of the PDH-like material. The optic lobe contains a proximal frontoventral (Pfv), a distal posteriodorsal (Dpd), and a posterioventral (Dpv) cluster of perikarya, and a fan of fibres which spread over the frontal and then over the distal faces of the medulla, eventually terminating in the first optic chiasma or in the lamina. The total number of PDH-positive perikarya in the optic lobes varies from just two in a stonefly (Závodská et al. 2003) to about 100 in the earwig (cf. "Results"). The cell numbers we recorded from the total preparations are generally lower than those reported by Homberg et al. (1991a), who reconstructed cell counts from the sections. The reasons for this discrepancy are unclear.

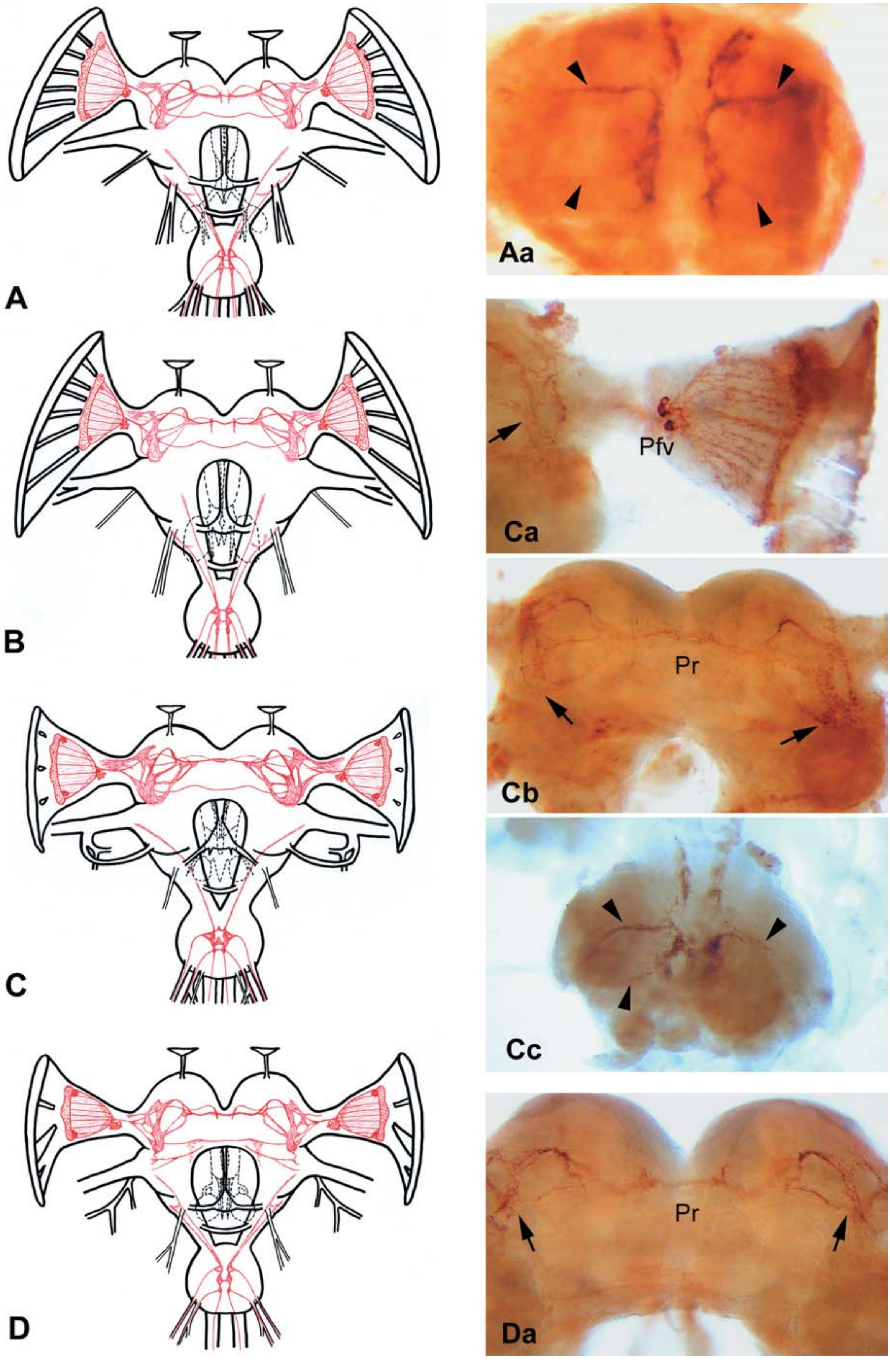
The Pfv neurone cluster is close to the accessory medulla that was studied in detail in cockroaches and found to include aminergic and peptidergic fibres (Petri et al. 1995). In our total preparations, the accessory medulla usually appeared as a small smear of PDH-positive fibres close to the Pfv cell cluster. In the cricket *Acheta domestica*, similarly to the previously examined crickets (Homberg et al. 1991a; Okamoto et al. 2001), the accessory medulla was identified as a relatively large and distinct globule of arborizing PDH-positive fibres. The horseshoe-shaped fascicle of fibres near the Pfv cluster in the praying mantid probably corresponds to the accessory medulla of this species.

Common to all Polyneoptera is the connection between the bilaterally symmetrical PDH systems. Typically, one fibre fascicle emanates from the region of the Pfv cell cluster and the firth of the optic fan, runs in a posterior loop around the lobula, and splits into several branches in the dorsal region of the lateral protocerebrum. Some form a fibre network that extends on the frontal face of the protocerebrum approximately at the level of the mushroom bodies. Several nerve bundles continue to the midbrain where they are interconnected by their side branches. One frontodorsal fibre in the crickets and the earwig, and several fibres in the other species, cross the brain midline.

Only a few exceptions from this distribution pattern of the PDH-positive cell bodies and processes were detected. The distal cell clusters Dpd and Dpv are fused in the earwig and absent in the praying mantid and a stonefly (Závodská et al. 2003). The optic fan on the anterior medulla face is supplemented with a smaller fan on the posterior side of the medulla in the locusts (cf. also Závodská et al. 2003) and the praying mantid. These species possess two, rather than one, nerve trajectories running from the optic lobe to the lateral protocerebrum. The area of fibre arborization in the lateral protocerebrum is reduced in the cricket, the earwig, and the cockroach *B. germanica*, and is nearly absent in the termite.

The occurrence of additional PDH-positive neurones in the central nervous system of Polyneoptera is not

Fig. 4 PDH immunoreactivity in the brains of adult praying mantid *Hierodula membranacea* **(A)** and cockroaches *Blatta orientalis* **(B)**, *Periplaneta americana* **(C)**, and *Blattella germanica* **(D)**. *H. membranacea*: **Aa** Pfv cell cluster with fibres linking the optic fan (*right*) with the protocerebrum (*to the left*), ×90. *P. americana*: **Ca** Pfv cell cluster, optic fan (*to the right*) and fibre arborization (*arrow*) frontally circumventing the lateral protocerebrum, ×90; **Cb** posterior view of the brain with fibres passing through the midline and arborizations in the dorsolateral protocerebrum (*arrows*), ×40; **Cc** ventral view of the suboesophageal ganglion showing fibre arborizations with varicosities (*arrows*) and fibres passing to gnathal nerves (*arrowheads*), ×90. *B. germanica*: **Da** Branching of fibres in the most lateral protocerebrum (*arrow*) and the course of fibres in the median brain region, ×90. For abbreviations see Fig. 1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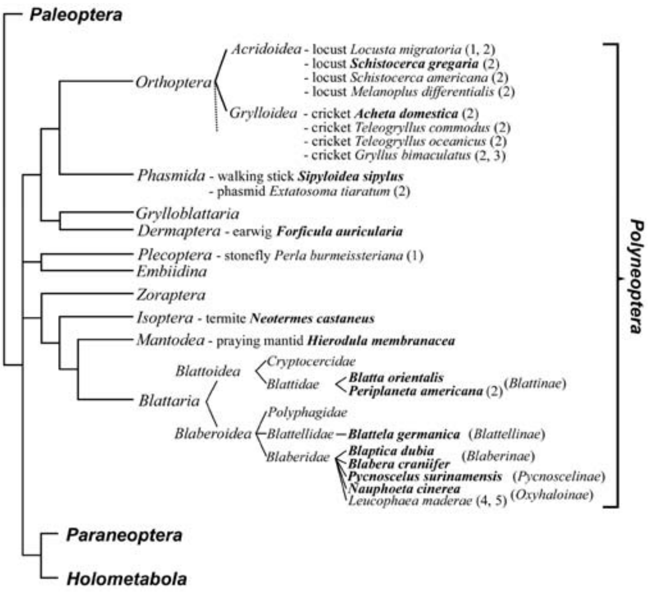

Fig. 6 Diagram showing the four evolutionary lineages (cohorts) of the extant pterygote insects and the relationships among the examined species of Polyneoptera (modified after Wheeler et al. 2001). Species investigated in the present study are shown in *bold*. Authors of the remaining data are: (1) Závodská et al. (2003), (2) Homberg et al. (1991a), (3) Okamoto et al. (2001), (4) Nässel et al. (1991), (5) Stengel and Homberg (1994)

uniform. We found a few perikarya in the protocerebrum of the walking stick and the termite, in the deutocerebrum of the cricket, the walking stick, and the cockroach *B. germanica*, and in the tritocerebrum of the cockroach *P. americana*. We failed to detect the pair of protocerebral neurones described in *A. domestica* by Homberg et al. (1991b). One or two pairs of the PDH-positive perikarya occur in the suboesophageal ganglion of the cricket, the stick insect, and the termite. The relationship of these somata and identified fibres is uncertain. Distinct fibre networks occur in the deuto- and tritocerebrum of *B. germanica* and ventrally in the suboesophageal ganglion of blattoid cockroaches; the termite has fibre ramifications in all three locations. The cricket, stick insect, and termite examined contain PDH-like material in the frontal ganglion and the recurrent nerve, and the latter two species also in the corpora cardiaca.

Fig. 5 PDH immunoreactivity in adult cockroaches *Blaptica dubia* (**A**), *Blabera craniifer* (**B**), *Pycnoscelus surnamensis* (**C**), and *Nauphoeta cinerea* (**D**). *B. dubia*: **Aa** Ventral view of the suboesophageal ganglion with small regions of dense fibre arborizations and connections (*arrowheads*) to the gnathal nerves, ×90. *P. surnamensis*: **Ca** Pfv cell cluster and the fan of fibres in the optic lobe (*to the right*), and fibre arborization (*arrow*) frontally encircling the lateral protocerebrum, ×90; **Cb** branching of fibres (*arrow*) in the most lateral protocerebrum and their distribution in the brain, ×63; **Cc** ventral view of the suboesophageal ganglion with fibres passing to the gnathal nerves (*arrowheads*), ×90. *N. cinerea*: **Da** Lateral branching (*arrows*) and the central course of fibres in the brain, ×63. For abbreviations see Fig. 1

Characteristic features of individual polyneopteran orders

The cockroaches (order Blattaria), which are regarded as the most primitive Polyneoptera, display the typical morphological pattern of PDH-positive perikarya and fibres. They are distinguished from the remaining Polyneoptera by the presence of fibre arborizations either in the deuto- and tritocerebrum (*B. germanica*) or in the suboesophageal ganglion (other species), from where the fibres pass to the gnathal nerves. The cockroach suprafamilies Blattoidea and Blaberoidea differ primarily by number and course of fibres in the central brain and the suboesophageal ganglion.

Praying mantids (Mantodea) and termites (Isoptera) may be the closest relatives of cockroaches but exhibit a number of unique features in the distribution of PDH-like material. The praying mantid lacks the Dpd and Dpv cell clusters, possesses the posterior optic fan, exhibits an unusual fibre arrangement in the accessory medulla, and its optic lobe is linked with the protocerebrum by two fibre tracts. The termite is characterized by a simple fibre organization in the central brain, the presence of PDH perikarya in the lateral protocerebrum and suboesophageal ganglion, and especially by extensive deuto- and tritocerebral fibre arborization.

Dermaptera (earwigs) and Phasmida (walking sticks) are closer to Orthoptera than to Blattaria. Similar to crickets, the earwig examined has a typical arrangement of PDH-positive structures with a single fibre crossing the brain midline. The PDH system of the walking stick resembles that of orthopterans by the presence of multiple bilateral connections, and that of the crickets by the connection between the lower brain region, the suboesophageal ganglion and the stomatogastric ganglia. Unique features of the walking stick include PDH-positive perikarya in the pars intercerebralis, projection of the protocerebral arborization to the lobula (similar to the blaberoid cockroaches), and the suboesophageal system of perikarya and fibres resembling the situation in the termite and the blattoid cockroaches.

Our data demonstrate, in agreement with other reports (Homberg et al. 1991a; Okamoto et al. 2001; Závodská et al. 2003), that crickets (suborder Grylloidea) and locusts (suborder Acridoidea), which are regarded as separate orders by some entomologists, possess a standard arrangement of PDH-positive structures. The two taxa differ from one another by the number of optic lobe trajectories (one in crickets, two in locusts) and the number of bilateral hemisphere connections (one thin fibre in crickets, several thick ones in locusts). PDH-positive perikarya and fibres in the deuto- and tritocerebrum, the suboesophageal ganglion, and the adjacent stomatogastric system seem to occur only in crickets.

Diversity of the PDH-immunopositive system in Insecta: functional considerations

The loss of rhythmicity in cockroach locomotion after removal of the Pfv neurones proved that they form a crucial component of the circadian pacemaker and confirmed pacemaker localization in the optic lobe (Stengl and Homberg 1994). The involvement of PDH was demonstrated by changes induced by PDH injection in the activity rhythm of neurones that innervate the optic lobe lamina (Chen et al. 1999). The importance of PDH for transduction of clock signals to effector neurones was stressed by the finding that the rhythmic behaviour is disturbed in *Drosophila* displaying ectopic expression of the *pdf* gene in various neurones projecting to the dorsal protocerebrum (Helfrich-Förster et al. 2000). It is unknown, however, which of the PDH-expressing neurones participate in these and other possible functions of the circadian clock.

In the ground beetle (Frisch et al. 1996) and the mayfly (Závodská et al. 2003), which belong to distant insect cohorts Holometabola and Palaeoptera, PDH-positive structures are arranged in a pattern similar to that in Polyneoptera. In most non-polyneopteran insects, however, the Dpd and Dpv clusters are missing. Homberg et al. (1991b) did not find them in the hawkmoth *Manduca sexta*, Nässel et al. (1993) and Helfrich-Förster (1997) in two fly species, and Sauman and Reppert (1996) in the silkmoth *Antheraea pernyi* (all these insects belong to Holometabola). Závodská et al. (2003) failed to detect the Dpd and Dpv cell clusters in damselfly (Palaeoptera), backswimmer bug (cohort Paraneoptera), caddisfly, beetle, honeybee, and blowflies (representatives of different orders of Holometabola). It is not clear if the loss of the Dpd and Dpv neurones indicates that the Pfv cluster alone is sufficient for the clock function or that the clock has shifted from the optic lobe to another brain region.

Some insects seem to possess their main circadian pacemaker in the central brain (Helfrich-Förster et al. 1998). PDH-positive cells occurring in the protocerebrum of some species, notably those of Holometabola (Homberg et al. 1991b; Nässel et al. 1993; Frisch et al. 1996; Sauman and Reppert 1996; Helfrich-Förster 1997; Wise et al. 2002; Závodská et al. 2003), are possibly associated with this pacemaker. The existence of a protocerebral pacemaker is particularly likely in species such as *M. sexta* that have no PDH-expressing cells in the optic lobes but contain PDH-like material in certain protocerebral neurosecretory cells (Homberg et al. 1991b; Wise et al. 2002). Such a localization suggests that the material is transported via nervi corpori cardiaci I+II to the corpora cardiaca, where it may be released into the haemolymph. We found morphological signs of such a release in the corpora cardiaca of the walking stick and the termite. In these species, however, the PDH-like material reaches the corpora cardiaca via the recurrent nerve and the nervi cardiostomatogastrici and may originate in the suboesophageal ganglion. The function of this material is not known. PDH-like material, which is generated in the abdominal ganglia of a locust and also appears to be released into haemolymph, modulates neuronal circuits in the terminal ganglion (Persson et al. 2001).

In most cockroaches, PDH-positive fibres pass from the suboesophageal ganglion to the gnathal nerves that control the mouth organs. In the cricket, walking stick and termite, the fibres traverse the frontal ganglion to the recurrent nerve that branches to the corpora cardiaca but for the most part continues as the stomatogastric nervous system innervating the foregut. This resembles the situation in a blowfly, in which PDH-positive neurones from the abdominal ganglia innervate the hindgut (Shiga et al. 1993). The function of the PDH-like material in the digestive tract is unknown.

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