Distribution of PER Protein, Pigment-Dispersing Hormone, Prothoracicotropic Hormone, and Eclosion Hormone in the Cephalic Nervous System of Insects

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Abstract Investigations performed on adult insects revealed that putative components of the central pacemaker, the protein Period (PER) and the pigment-dispersing hormone (PDH), are immunocytochemically detectable in discrete sets of brain neurons throughout the class of Insecta, represented by a bristletail, mayfly, damselfly, 2 locust species, stonefly, 2 bug species, goldsmith beetle, caddisfly, honeybee, and 2 blowfly species. The PER-positive cells are localized in the frontal protocerebrum and in most species also in the optic lobes, which are their only location in damselfly and goldsmith beetle. Additional PER-positive cells occur in a few species either in the deuto- and tritocerebrum or in the suboesophageal ganglion. The PER staining was always confined to the cytoplasm. The PDH immunoreactivity consistently occurs in a cluster of perikarya located frontoventrally at the proximal edge of the medulla. The mayfly and both locust species possess additional PDH neurons in 2 posterior cell clusters at the proximal edge of the medulla, and mayfly, waterstrider, and 1 of the blowfly species in the central brain. PDH-positive fibers form a fanlike arrangement over the frontal side of the medulla. Two or just 1 bundle of PDH-positive fibers run from the optic lobe to the protocerebrum, with collaterals passing over to the contralateral optic lobe. Antisera to the prothoracicotropic (PTTH) and the eclosion (EH) hormones, which in some insects regulate the molting and ecdysis rhythms, respectively, typically react with a few neurons in the frontal protocerebrum. However, the PTTH-positive neurons of the mayfly and the damselfly and the EH-positive neurons of the caddisfly are located in the suboesophageal ganglion. No PTTH-like antigen was detected in locusts, and no EH-like antigens were detected in the damselfly, stonefly, locusts, and the honeybee. There are no signs of co-localization of the PER-, PDH-, PTTH-, and EH-like antigens in identical neurons.

Key words insect brain clock, circadian rhythm, PER, PDH, PTTH, EH, insect neuroanatomy

Oscillations with a period close to 24 h are characteristic for many biological processes (Rosbash, 1995). Their pace in the various cells of metazoan organisms is controlled by endogenous clocks that are synchro-

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nized and adjusted to environmental oscillations by a master clock located in the central nervous system (Dunlap, 1999). The master clock includes an input pathway transmitting environmental signals to a core oscillator that generates circadian signals released to the rest of the body via an output pathway.

The nature of the clock remained elusive until genetic and molecular investigations in *Drosophila melanogaster* disclosed distinct diurnal oscillations in the expression of the genes *period* (*per*) and *timeless* (*tim*; reviewed in Hall, 1998). Their products PER and TIM form a heterodimer that is translocated into the nucleus, where it interferes with the activity of the constitutive transcription factors CLOCK and CYCLE and thereby inhibits the expression of *tim* and *per* (reviewed by Sauman and Hashimi, 1999). The rate of assembly of the TIM/PER dimer, which is regulated by a kinase called double-time (DBT), provides the circadian rhythm, and the degradation of TIM upon illumination synchronizes this system with the environmental alterations of light and darkness (reviewed by Williams and Sehgal, 2001).

The PER protein is widely distributed in the nuclei of neurons and glial cells in the *D. melanogaster* brain (Liu et al., 1988; Siwicki et al., 1998). By contrast, nuclear localization of PER was found only in some photoreceptor and midgut cells in the silkmoth *Antheraea pernyi* (Sassone-Corsi, 1996; Sauman and Reppert, 1996a). In this species, most distinct *per* expression occurred in 4 pairs of brain neurons that also produced *per*-antisense RNA, which was viewed as a regulator of PER production. The PER protein accumulated in the cytoplasm and appeared to be subject to axonal transport. Recent investigations on the hawkmoth *Manduca sexta* disclosed nuclear localization of PER in numerous brain cells similarly to *D. melanogaster* but distinct cytoplasmic accumulation of PER in 4 pairs of brain neurons (identified as Ia_1 neurosecretory cell group) like in *A. pernyi* (Wise et al., 2002).

The differences in the PER distribution in the 3 insect species examined so far are intriguing and provided impetus for this study. We chose species from all 5 insect cohorts and analyzed their brain-suboesophageal complexes immunocytochemically for the presence of PER at 3 to 6 hr zeitgeber time (ZT). A more detailed circadian study was performed with a damselfly representing the cohort Palaeoptera to complement our parallel investigations on the remaining cohorts (see the Discussion section). Consistent demonstration of PER exclusively in the cytoplasm of relatively small numbers of neurons in the representatives of all insect clades is an important point of this article.

The mosaic studies and transplantation experiments localized the control of behavioral rhythmicity in *D. melanogaster* to a relatively small number of lateral neurons (Hall, 1998). About half of them react with antibodies to the pigment-dispersing hormone (PDH) (Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995), an octadecapeptide that regulates circadian rhythm of pigment migration in certain crustaceans (cf. Rao, 2001). The PDH-positive cells in *D. melanogaster* are close to the accessory medulla neuropile that was described as a projection site of the extraocular photoreceptors (Hagberg, 1986) and later found to be innervated with PDH-immunopositive fibers (Homberg et al., 1991; Stengl and Homberg, 1994). Surgical experiments in cockroaches (reviewed by Page, 1984) and other evidence invited the conclusion that accessory medulla and allied perikarya represented the insect pacemaker (Helfrich-Förster et al., 1998). Disturbances in the eclosion rhythm in *D. melanogaster* deprived of PDH by surgery or mutagenesis (Renn et al., 1999), or with ectopically misexpressed PDH (Helfrich-Förster et al., 2000), proved that this compound is a component of the master clock. We decided to examine if the PDF-positive neurons occur in comparable positions in all insect cohorts.

The secretion of several neurohormones exhibits diurnal rhythms that are probably subject to regulation by a central clock. In the silkworm *Bombyx mori* and the kissing bug *Rhodnius prolixus*, oscillations were shown in the release of the prothoracicotropic hormone (PTTH), which is a pivotal regulator of the molting process and metamorphosis (Vafopoulou and Steel, 2001). PTTH was identified in several lepidopterans as a product of 2 pairs of brain neurons that are distinct from those expressing *per* and *tim* (Sauman and Reppert, 1996b; Wise et al., 2002). The secretion of PTTH initiates a relatively long multistep process of molting that is terminated by ecdysis, when the old cuticle is shed. Ecdysis behavior is controlled by several hormones, including the eclosion hormone (EH) that is typically secreted from 2 pairs of ventrolateral protocerebral neurons (Copenhaver and Truman, 1986). EH secretion is often gated to a certain time of the day (Truman, 1972). Since the EH-producing cells do not express *per* and *tim* (Sauman and Reppert, 1996a), the cyclic release of EH is apparently regulated by the central clock. One of the aims of our study was to find out whether PTTH- and EH-like proteins occur

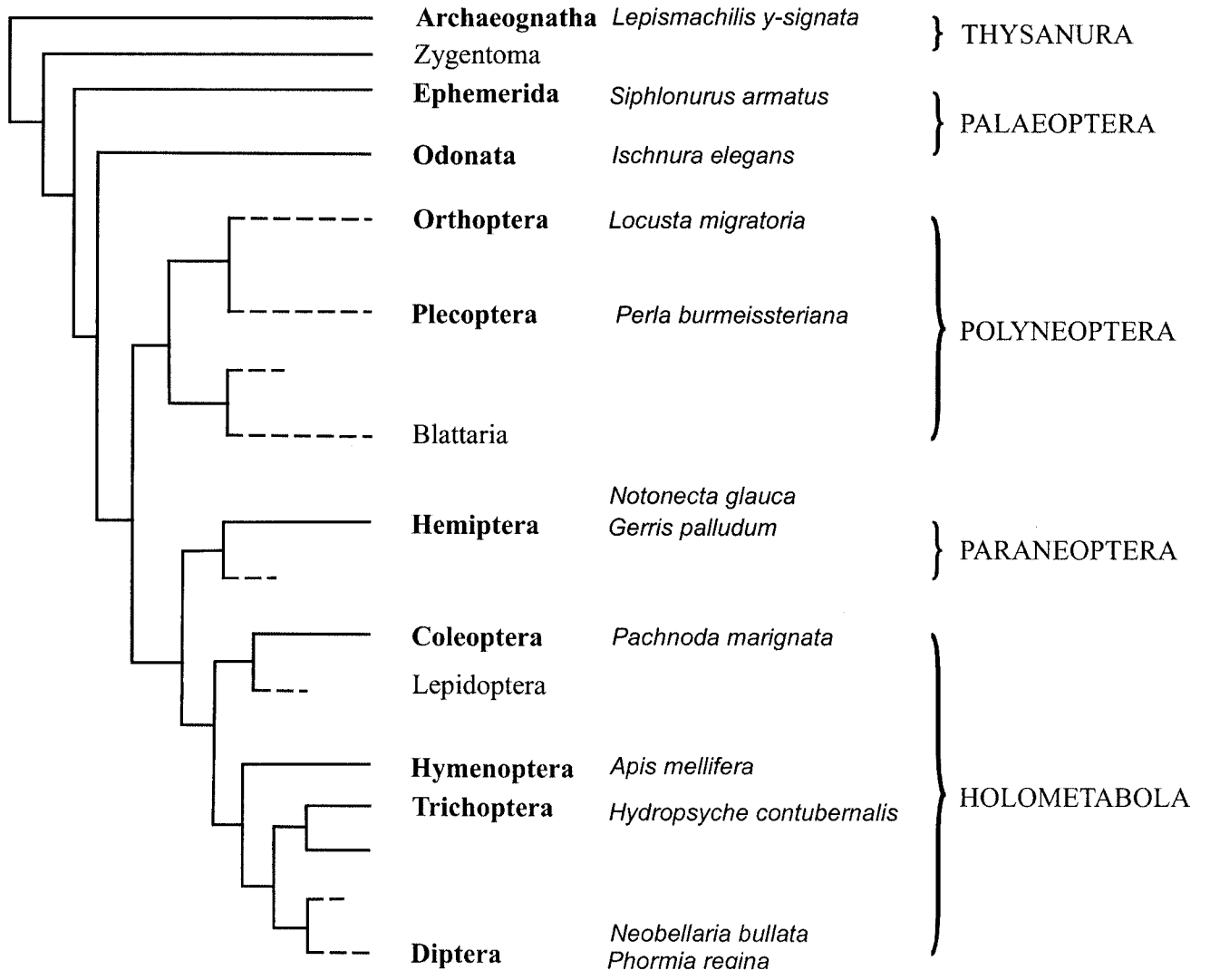

Figure 1. Schematic cladogram of Insecta. The phylogenic relationships of orders and their affiliation to cohorts (in capital letters) after Wheeler et al. (2001); evolutionary lineages that split to more than one order are indicated with interrupted lines. Only orders that have been examined in respect to the circadian clock are listed; those included in the present study are printed in bold, and the name of the representative species is given.

in all major insect taxa and how the patterns of their distribution in the brain relate to the neurons expressing PER- and PDH-like antigens.

MATERIALS AND METHODS

Animals

Most of the chosen species represent insect orders that have not yet been examined with respect to the brain clock (Fig. 1). For the sake of comparison, only adult insects were used. They were either collected outdoors in the summer months (natural light cycle with dawn at 0400-0500 and dusk at 2200-2100) or taken from the laboratory cultures maintained at species-specific optimal conditions. The bristletail *Lepismachilis y-signata* was found in the Křivoklát karst in central Bohemia. The mayfly *Siphlonurus armatus*, the damselfly *Ischnura elegans*, the stonefly *Perla burmeisteriana*, the backswimmer *Notonecta glauca*, the waterstrider *Gerris palludum* (the 2 latter species represent heteropteran infraorders Nepomorpha and Geromorpha, respectively), and the caddisfly *Hydropsyche contubernalis* were collected in the vicinity of České Budějovice. Locusts *Locusta migratoria* and

Table 1. Primary antibodies (all produced in rabbits), their dilutions in PBST, and references to their sources.

Code	*Antigen*	*Dilution*	*Source*
Anti-PER57	*Antheraea pernyi* PER	1:200	Antibody 57/10w of Sauman and Reppert (1996a)
Anti-PER58	*Antheraea pernyi* PER	1:200	Antibody 58/10w of Sauman and Reppert (1996a)
Anti-αPER	*Drosophila melanogaster* αPER[a]	1:1000	M. Young, Rockefeller University, USA
Anti-PTTH	*Antheraea pernyi* PTTH	1:500	Antibody 274/IV-A of Sauman and Reppert (1996b)
Anti-EH	*Manduca sexta* EH	1:2000	J. Truman, University of Seattle, USA
Anti-PDH	*Uca pugilator* PDH	1:10,000	H. Dircksen, University of Bonn, Germany

NOTE: PTTH = prothoracicotropic hormone; EH = eclosion hormone; PDH = pigment-dispersing factor.
a. Baculovirus recombinant protein was used as an antigen for immunization.

Schistocerca gregaria (orthopteran subfamilies Catantopinae and Oedipodinae, respectively), were taken from our standard colonies reared at 30 °C and 12:12 h photoperiod, blowflies *Neobullaria bullata* and *Phormia regina* from cultures maintained at 24 °C and 12:12 h photoperiod, and the goldsmith beetle *Pachnoda marignata* from a culture kept at 24 °C and 16:8 h photoperiod. Honeybee workers *Apis mellifera* came from a local beehive.

Three to 6 h after lights-on (ZT 3-6), selected insects were briefly anaesthetized with CO_2 and rapidly dissected in chilled, sterile insect saline. Just a dozen bristletails were available for the dissections, but at least 5 animals were used for each type of immunostaining in all other cases. The damselflies *Ischnura elegans*, which were field collected at natural LD cycle 17:7 h, were maintained at this light regime until dissection at ZT 6, ZT 12, ZT 18, and ZT 24.

Immunocytochemistry on Paraplast Sections

The dissected brain-suboesophageal ganglion complex was immediately fixed in modified Bouin-Hollande solution (0.7% mercuric chloride, no acetic acid) at 4 °C overnight (Levine et al., 1995). Tissues were brought through an ethanol series to chloroform, embedded in paraplast, and sections 4 to 10 µm thick were attached to microscopic slides. After deparaffinization in xylene and the following rehydration, the sections were treated with Lugol's iodine followed by 7.5% sodium thiosulfate to remove residual heavy metal ions and then washed in distilled water and phosphate-buffered saline supplemented with 0.3% Tween-20 (PBST). Blocking with normal goat serum (10% in PBST, 30 min at room temperature [RT]) was followed by incubation (overnight at 4 °C in a humidified chamber) with a primary antibody diluted with PBST. The primary antibodies (all produced in rabbits) and their corresponding dilutions in PBST are listed in Table 1. Control slides were incubated with normal goat serum instead of the primary antiserum.

After rinsing with PBST (3 three times for 10 min at RT), the samples were incubated for 1 h at RT with goat antirabbit IgG-horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, 1:1000 in PBST). The slides were then washed in PBST (3 times for 10 min at RT) followed by a final wash in 0.05 M Tris-HCl (pH 7.4, for 10 min at RT). After the staining procedure with hydrogen peroxide (0.005%) substrate and 3, 3'-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M Tris-HCl, pH 7.4) chromogen, the sections were dehydrated, mounted in DPX mounting medium, and examined with the aid of the Zeiss Axioplane 2 microscope equipped with Nomarski (DIC) optics and CCD camera. The immunoreactivity was roughly quantified on the basis of staining intensity as absent (–), weak (+), moderate (++), considerable (+++), and strong (++++).

Whole Mount Immunocytochemistry

The dissected brains were fixed in 4% paraformaldehyde (in PBS, pH 7.4) at 4 °C overnight. After their transfer to RT and a rinse in PBST, they were treated with collagenase (0.5 mg/ml in PBS; Sigma) for 30 to 60 min, washed with methanol and PBST, and treated with normal goat serum (10% in PBST) for 2 h. Then they were incubated with the anti-PDH antibody at 4 °C for 48 h, washed thoroughly with PBST (3 times for 10 min at RT), and incubated with a secondary goat antirabbit antibody at 4 °C overnight. Secondary antibodies were conjugated either to horseradish peroxidase (HRP) or to Cy3-fluorophore (Jackson ImmunoResearch, 1:1000 dilution). In either case, incubation was terminated with washing the brain in PBST (3 times for 10-30 min at RT). The enzymatic activity of HRP was detected as described above; the brains were mounted in 80% glycerol and observed

Table 2. The intensity of immunostaining detected in the brain-suboesophageal ganglion complex of the examined insects.

Insect Species/Tested Antibody	*αPER*	*PER 58*	*PER 57*	*PDH*	*PTTH*	*EH*
Bristletail *Lepismachilis y-signata* (Archaeognatha)	–	+++	–	++++	+++	++
Mayfly *Siphlonurus armatus* (Ephemeroptera)	–	++++	–	++++	+++	++
Damselfly *Ischnura elegans* (Odonata)	++	–	–	++++	+++	–
Locust *Schistocerca gregaria*	++	–	–	+++	–	–
Locust *Locusta migratoria* (Orthoptera)	+++	–	–	++++	–	–
Stonefly *Perla burmeisteriana* (Plecoptera)	–	++++	–	++	++++	–
Backswimmer *Notonecta glauca*	++++	++++	–	++++	+++	+++
Waterstrider *Gerris palludum* (Hemiptera)	+++	++	–	++++	++	+++
Goldsmith beetle *Pachnoda marignata* (Coleoptera)	–	+++	–	+++	+++	+++
Honeybee *Apis mellifera* (Hymenoptera)	–	+++	++	+++	++	–
Caddisfly *Hydropsyche contubernalis* (Trichoptera)	–	+++	+++	++++	++	+
Blowfly *Neobellaria bullata*	++++	+++	++	++++	++++	+++
Blowfly *Phormia regina* (Diptera)	–	–	+++	++++	++	++

NOTE: PDH = pigment-dispersing factor; PTTH = prothoracicotropic hormone; EH = eclosion hormone. Quantified subjectively as absent (–), weak (+), moderate (++), considerable (+++), and strong (++++).

under a standard dissection microscope. Those labeled with the Cy3-fluorophore were soaked in Murray's clearing medium (benzylalcohol: benzylbenzoate, 1:2) for at least 1 h, mounted in fresh medium, and examined with the aid of the Zeiss LSM 410 laser scanning confocal microscope.

RESULTS

The Specificity and Cellular Localization of Immunostaining

The examined species differed considerably in number, location, and staining intensity of the immunoreactive neurons (Table 2). Males and females were often examined separately, but no sex-specific differences were found.

The different antisera to PER (cf. Table 1) reacted, to some degree, species specifically. Most species contained neurons recognized by anti-PER58, which was the only antiserum reacting in the bristletail, mayfly, stonefly, and the beetle. Immunoreactivity limited to anti-αPER occurred in the damselfly and both locust species, and reaction restricted to anti-PER57 was detected in one of the blowflies. Reactions with all 3 antisera to PER, known to occur in the silkmoth *Antheraea pernyi* (Sauman and Reppert, 1996a) and the firebrat *Thermobia domestica* (Závodská, unpublished), were seen in the fly *N. bullata*. It must be emphasized that when several PER antisera react in a species, all of them recognize the same set of neurons. In pilot experiments to the present study, we applied PER antibodies to the sections of *Drosophila* head. Consistent with published reports (Levine et al., 1995), a positive signal was detected in the nuclei of the photoreceptor cells in compound eyes and in the lateral neurons of the central brain. By contrast, the reaction in all other species we examined occurred exclusively in the cytoplasm of the immunopositive cells.

The anti-PDH antibody reacted with specific neurons in all examined insects. Very regular was the staining of a fronto-ventral cluster of neurons located proximally to medulla (Pfv cluster). The antisera to PTTH and EH usually recognized specific sets of neurons, but no PTTH reactivity was detected in locusts and no EH-reactivity in locusts, the damselfly, and the stonefly (Table 2).

The Bristletail *Lepismachilis y-signata* (Order Archaeognatha)

There were 2 distinct PER-positive neurons in the fronto-lateral region of the brain (Fig. 2A). Their axons extended ventrally, and their numerous fine branches could be traced into the deuto- and tritocerebrum and to the adjacent region of the suboesophageal ganglion. Some of the branches ran within the ipsilateral and others passed into the contralateral brain hemisphere. Two groups of somewhat smaller PER-positive neurons occurred in each optic lobe (Fig. 2 A,B). Two neurons were located dorsally and 8 to 10 fronto-ventrally of the medulla, and the 2 groups were connected with a meshwork of fine fibers. Laterally to the fronto-ventral cluster was the Pfv group of about 10 large neurons containing PDH-like antigen (Fig. 2 A,C). The Pfv group occupied about one third of the ventral circumference of the optic lobe. Major fibers of the PDH cells passed over the medulla, and their fine branches reached the lamina. Other axons ran into the

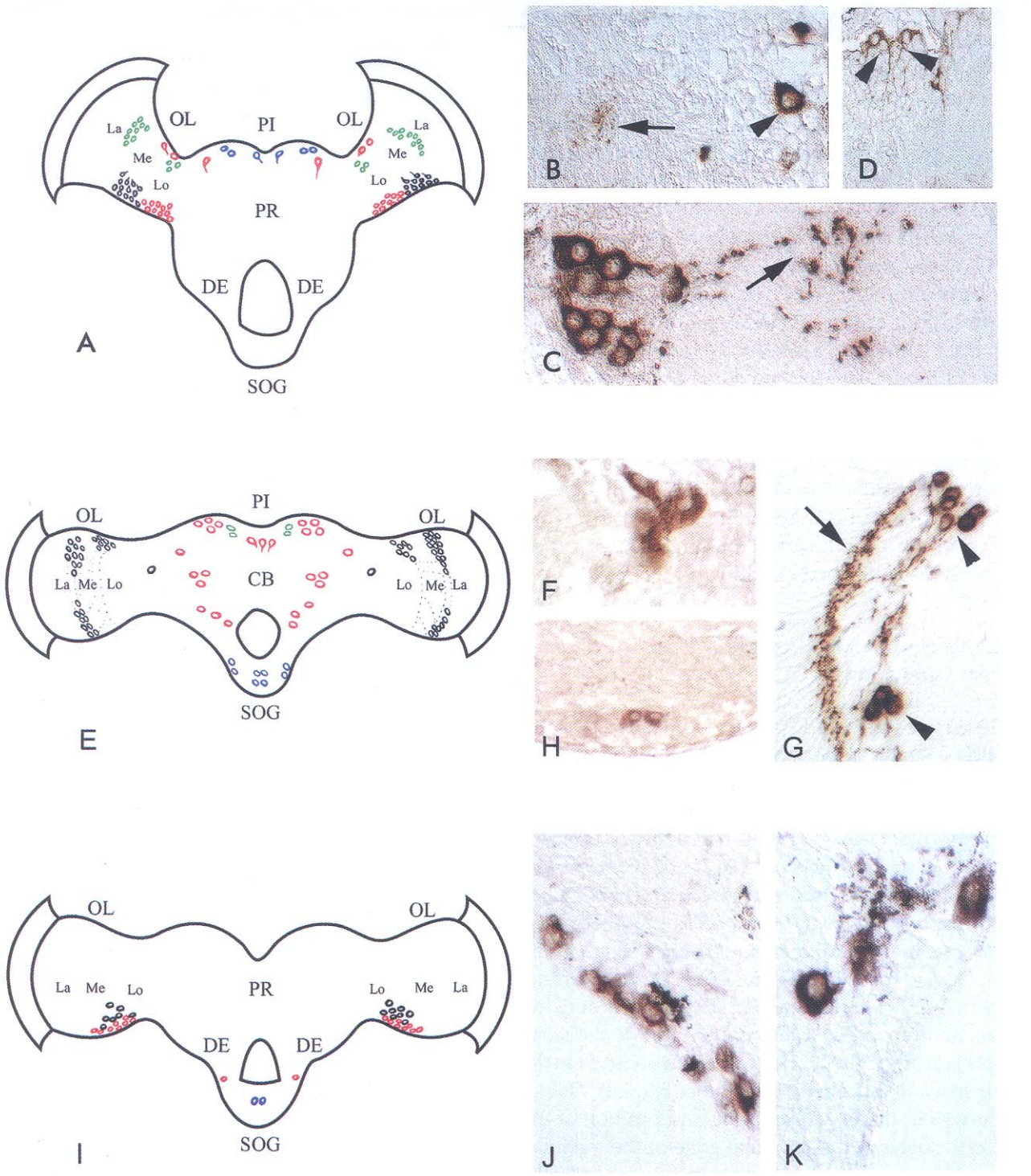

Figure 2. Immunoreactive cells in the brain and suboesophageal ganglion of Thysanura and Palaeoptera. Schematic diagrams illustrating the topography of neurons producing PER-like (red), PDH-like (black), PTTH-like (blue), and EH-like (green) antigens in (A) the bristletail *Lepismachilis y-signata*, (E) the mayfly *Siphlonurus armatus*, and (I) the damselfly *Ischnura elegans*. Photos of *L. y-signata*: (B) PER-positive cells and axon arborization (arrow) (note PER absence in the nucleus [arrowhead]); (C) PDH-positive cells with their axonal arborization (arrow) in optic lobe; (D) PTTH-positive cells (arrowheads) in pars intercerebralis. *S. armatus*: (F) PER-positive cells in pars intercerebralis. (G) Distal optic lobe PDH-positive cell clusters (arrowheads) with fiber arborization in lamina (arrow); (H) PTTH-positive cells in suboesophageal ganglion. *I. elegans*: Neurons in the ventral region of optic lobes reacting with (J) anti-PER and (K) anti-PDH antisera. Magnification: 210×. CB = central brain; DL = dorsolateral brain region; PI = pars intercerebralis; PR = protocerebrum; DE = deutocerebrum; TR = tritocerebrum; SOG = suboesophageal ganglion; OL = optic lobe; La = lamina; Me = medulla; Lo = lobula; PDH = pigment-dispersing hormone; PTTH = prothoracicotropic hormone; EH = eclosion hormone.

protocerebrum and continued to the suboesophageal ganglion.

The antiserum to PTTH reacted in each hemisphere with 1 large medial neuron located close to the dorsal-anterior brain surface and with 2 equally large lateral neurons deeper in the brain (Fig. 2 A,D). Processes of the medial neurons could be traced for a short distance only, but fine PTTH-positive fiber arborizations were found in the ventro-medial protocerebrum and apparently a separate meshwork in the deuto- and tritocerebrum. Reactions with the anti-EH antibody occurred in 2 groups of neurons in each optic lobe (Fig. 2A). A group of 3 small cells (comparable in size to the PER cells) was located dorso-posteriorly of the lobula, and a group of about 10 cells was located dorsally of the medulla/lamina boundary. No axons extending from the EH-positive perikarya could be found, but staining occurred in fine fibers around most of the medulla circumference and in a band extending into the central protocerebrum and then ventrally along the brain midline.

The Mayfly *Siphlonurus armatus* (Order Ephemerida)

Three strongly PER-positive cell somata were located dorsally in the pars interecerebralis and 4 in a region of the mushroom bodies (Fig. 2 E,F). Slightly less intensive but distinct staining occurred in 3 perikarya that were close to the frontal brain surface at the level of the central body, 3 in a more ventral position, and 1 in an extreme lateral position. The course of PER-positive fibers was indicated as slightly stained dots. It seemed that the fibers formed 3 layers in the optic lobes (possibly at the level of the lamina, medulla, and lobula), connected the right and left lobes, and ran through the central brain to the nervi corpori cardiaci. The PDH-like antigen occurred in the optic lobes in 3 clusters of neurons (Fig. 2E). One of them was in the usual fronto-ventral position of the Pfv cell cluster at the proximal edge of the medulla. Two other clusters were dorsally and ventrally, respectively, at the distal edge of the medulla (Fig. 2G). A single PDH-positive cell was detected in each optic lobe between the lobula and the lateral protocerebrum. No continuous axons were found, but dots of immunostaining indicated presence of fibers running on interfaces between the lobula and the medulla and between the medulla and the lamina. A few fibers connected these 2 meshworks, and other fibers traversed the brain providing bilateral linkage of the optic lobes.

Two ventro-medial pairs of the PTTH-positive cells laid roughly in the central part and 2 lateral pairs laid in the posterior part of the suboesophageal ganglion (Fig. 2 E,H). Short axons extending toward the brain were seen in the latter cells. Fine immunopositive dots continued to the central body and along the base of the pedunculi to the brain region above the mushroom bodies. Two neurons, which were located rather anteriorly in each medio-lateral brain region, expressed an EH-like antigen (Fig. 2E). Their axons ran toward the brain midline and then ventrally to the base of the protocerebrum.

The Damselfly *Ischnura elegans* (Order Odonata)

About 8 small PER-positive cells were found in the ventro-posterior region of each optic lobe (Fig. 2 I,J), with fibers extending over the proximal and distal medulla surfaces. A pair of PER-positive cells, which were positioned anterio-laterally in the region of the brain fusion with the suboesophageal ganglion, apparently sent fine axons to adjacent brain areas and the mushroom bodies, as well as to the ventral part of the suboesophageal ganglion. The described arrangement of the PER-positive perikarya and fibers was found with all 3 anti-PER antisera in damselflies that were sacrificed at ZT 3-6. Only the αPER antibody reacted in specimens that were analyzed at ZT 6, 12, 18, and 24 (just before sunrise); the distribution of immunoreactivity was as described above. It was always confined to the cytoplasm and never occurred in the nuclei.

Just above the PER-positive cells in the optic lobe laid the Pfv group of 6 to 8 distinctly larger neurons containing PDH-like antigen (Fig. 2 F,K). Some of their fibers arborized around the medulla, while others ran to the dorsal, central, and ventral brain regions. The PTTH-like material was found in only 2 perikarya laying in the posterio-medial part of the suboesophageal ganglion (Fig. 2F). The antiserum to EH did not react at all.

The Locusts *Schistocerca gregaria* and *Locusta migratoria* (Order Orthoptera)

Six pairs of PER-positive neurons were identified in the brain of both species (Fig. 3 A,D). All of them were

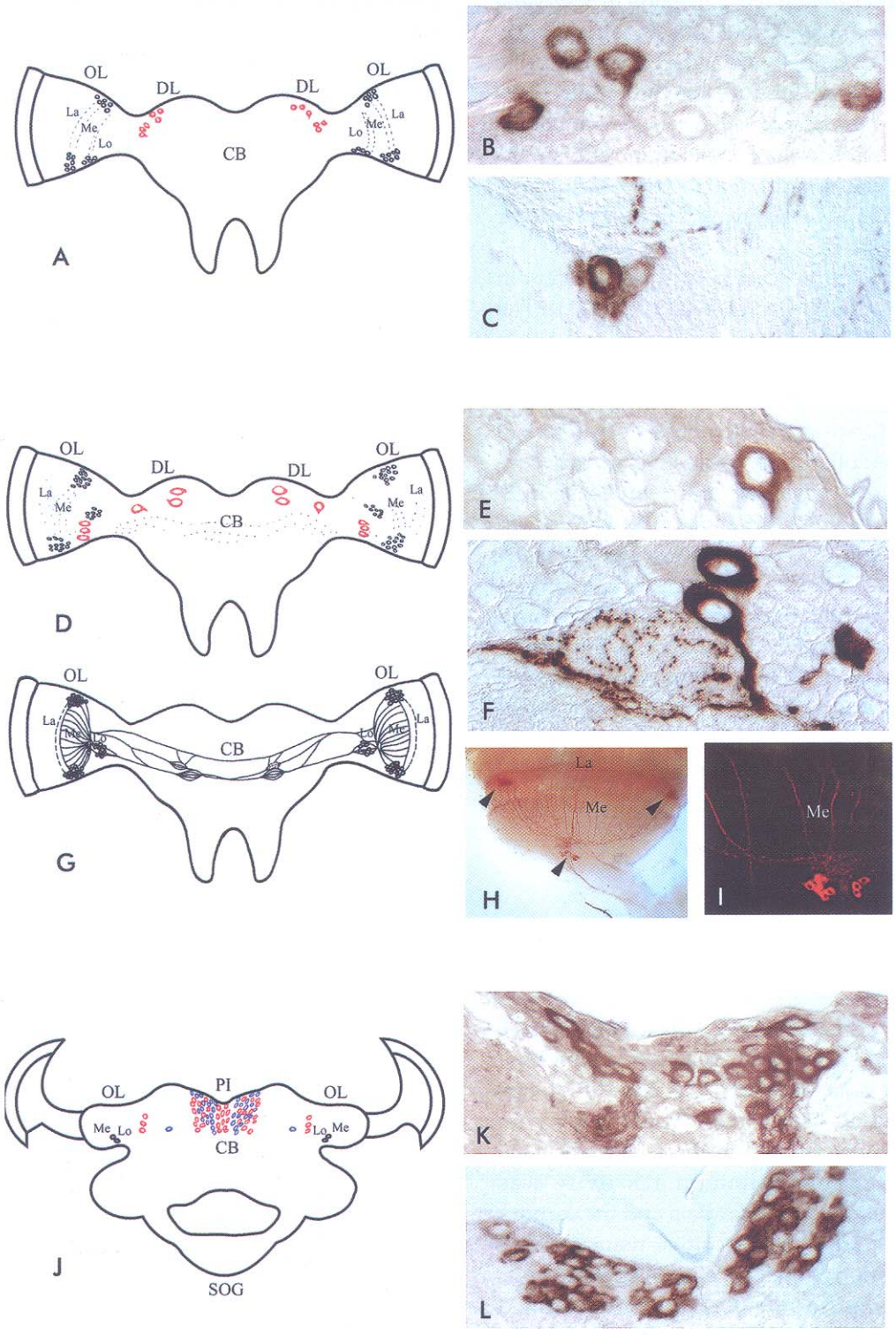

Figure 3. Immunoreactive cells in the brain of Polyneoptera. Relative topology of neurons containing PER-like (red), PDH-like (black), PTTH-like (blue), and EH-like (green) materials in the brain of locusts (A) *Schistocerca gregaria* and (D) *Locusta migratoria* and (J) the stonefly *Perla burmeisteriana*. Photos of *S. gregaria*: (B) PER-positive cells in the dorso-lateral central brain region; (C) the distal, ventro-posterior cluster of PDH-positive cells in optic lobe. *L. migratoria*: (E) 1 of the 2 PER-positive cells in dorso-lateral brain region; (F) PDH-positive perikarya (Pfv group) and axons in accessory medulla; (G) drawing of the brain distribution of the PDH-like antigen; (H) 3 PDH-positive cell clusters (arrowheads) in optic lobe (wholemount); (I) confocal image depicting PDH cells of the Pfv group with axons extending over the medulla (wholemount). *P. burmeisteriana*: (K) PER-positive and (L) PTTH-positive cells in central brain. Magnifications: B, C, E, F, K, L = 210×; H = 40×; I = 85×). For abbreviations, see the caption to Figure 2.

located in the anterio-lateral brain region in *S. gregaria* (Fig. 3B), while in *L. migratoria*, 3 cells were in a similar position (Fig. 3 D,E) and 3 were at the base of the optic lobe, ventrally to the lobula (Fig. 3D). Fine PER-positive fibers running across the pars intercerebralis were distinguishable in the dorsal region, and apparently unrelated fibers occurred in the ventral region of the protocerebrum in *L. migratoria*. No fibers could be detected in *S. gregaria*.

The anti-PDH serum reacted in 3 cell clusters in each optic lobe of both species (Fig. 3 A,D,G,H). The Pfv cluster included 8 cells in *L. migratoria* and 4 cells in *S. gregaria* and was associated with fiber arborization in the accessory medulla (Fig. 3F). Two clusters placed dorso- and ventro-posteriorly (Fig. 3C) at the distal medulla edge, respectively, each contained 12 cells in *L. migratoria* and 6 to 8 cells in *S. gregaria*. The course of PDH fibers was verified on wholemount preparations of the *L. migratoria* brain. A fan of fibers ran over the anterior medulla surface (Fig. 3 G,H,I). Two fiber bundles that each split into a frontal and a rear branch arborized in the protocerebrum. The bilateral PDH systems were connected with an anterior and a posterior thin fiber traversing the central brain (Fig. 3G). No immunoreactivity was detected with antisera to PTTH and EH.

The Stonefly *Perla burmeisteriana* (Order Plecoptera)

A large group of more than 25 PER-positive cells occupied the dorso-medial brain region (Fig. 3 J,K); the most lateral cells of this cluster showed short processes pointing centrally. Three other PER-positive perikarya occurred at the base of each optic lobe. Tiny immunopositive dots revealed presence of PER fibers between the medulla and lamina, toward and within the central brain region, and in the suboesophageal ganglion. Profound PER-immunoreactivity was detected in the nervi corpori cardiaci and the corpora cardiaca. Only 2 large PDH-positive neurons were found in the position of the Pfv cell cluster in the optic lobes (Fig. 3J); diffuse immunoreactivity indicated extension of their fibers into the distal parts of the optic lobes and, in the opposite direction, into the protocerebrum. PTTH-positive cells occurred in 2 clusters of about 25 cells each close to the brain midline (Fig. 3 J,L). Most of them were posterior to the assembly of the PER-positive cells. A single PTTH-positive neuron was found more laterally, roughly in the center of each protocerebral hemisphere. Fine speckles of immunostaining suggested connections of the medial cell cluster with the singly placed lateral cells and with the optic lobes. Some fibers seemed to run ventrally to join the nervi corpori cardiaci; a strong PTTH-signal was found in axon terminals within the corpora cardiaca. Dot-like PTTH-staining further occurred at the base of the olfactory lobes and in the suboesophageal ganglion (not shown). No staining was obtained with the anti-EH antiserum.

The Bugs *Notonecta glauca* and *Gerris palludum* (Order Heteroptera)

Both bugs were characterized by the absence of immunostaining in the suboesophageal ganglion and by large numbers of the PER-positive perikarya scattered through most of the brain, including the optic lobes (Fig. 4 A,L). In *N. glauca*, a prominent group of 4 to 6 cells with axons pointing toward the brain midline occurred somewhat laterally to the pars intercerebralis (Fig. 4B). Large numbers of PER cells were present in the dorsal as well as ventral protocerebrum, 2 groups of 6 cells each at the base of the optic lobes, and a few cells ventrally to the olfactory lobe. *G. palludum* contained at least 40 PER cells in each optic lobe and about 20 cells at the border between the optic lobe and the central brain (Fig. 4H). Some of the latter cells were situated in the pars intercerebralis squeezed between the very large optic lobes (Fig. 4L).

N. glauca contained a Pfv cluster of 8 PDH-positive cells and *G. palludum* of about 20 cells forming 2 subgroups (Fig. 4 D,M). An additional PDH-positive neuron was found in *G. palludum* in the central brain region and 2 at the base of the protocerebrum (Fig. 4 M,N). The PDH cells of the optic lobes sent a fan of fibers over the frontal medulla surface and 2 fiber bundles into the protocerebrum. *G. palludum* was distinguished from *N. glauca* by the reduced number of fibers running over the medulla and by their extensive arborization in the lamina region (Fig. 4 G,O). The course of fibers in the protocerebrum was different in the 2 species, but certain similarities could be detected (Fig. 4 D,M). Both species had a posterior PDH tract connecting the hemispheres and a fronto-dorsal arborization that was close to mushroom bodies. A small arborization around the transverse nerve in *G. palludum* might correspond to the more lateral arborization formed by a separate fiber bundle in *N. glauca*.

Two PTTH-positive cells occurred in the dorsal protocerebrum in *N. glauca* (Fig. 5 A,E), and 5 cells

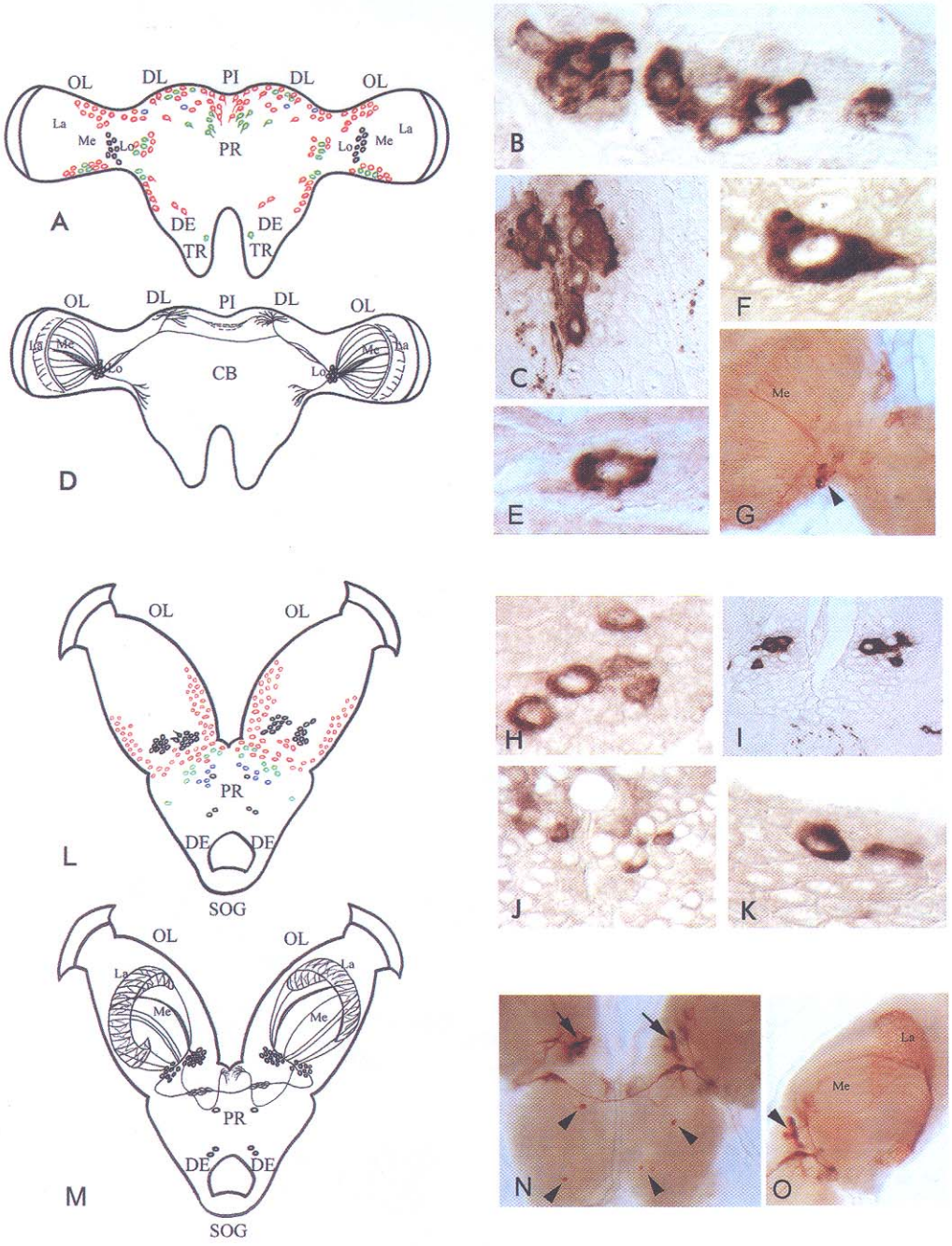

Figure 4. Immunoreactive cells in the brain of Paraneoptera. Schematic diagrams illustrating the topography of neurons recognized by the antisera to PER (red), PDH (black), PTTH (blue), and EH (green) in the brain of (A) the backswimmer *Notonecta glauca* and (L) the waterstrider *Gerris palludum*. Photos of *N. glauca*: (B) PER-positive cells in the pars intercerebralis; (C) Pvf cluster of the PDH-positive cells in optic lobe; (E) PTTH-positive cell in the dorso-lateral and (F) EH-positive cells in the central brain regions; (G) Pvf cluster of the PDH cells (arrowhead) and their fibers on the frontal face of medulla (wholemount). Drawings of the PDH-positive cells and their axonal projections in the brain of *N. glauca* (D) and *G. palludum* (M). Photos of *G. palludum*: (H) PER-positive cells at the border between protocerebrum and optic lobe; (I) PDH-positive cells of the anterior subclusters of both Pfv cell groups; (J) PTTH-positive neurons in central brain; (K) EH-positive cells in dorso-lateral brain region; (N) PDH-positive cells and fibers in optic lobes (Pfv clusters marked with arrows) and protocerebrum (perikarya indicated with arrowheads); (O) Pfv cell cluster (arrowhead) and PDH-positive fibers over medulla and lamina. Magnifications: B, C, E, I, and J = 210×; F, H, and K = 420×; G = 60×; N and O = 90×). For abbreviations, see the caption to Figure 2.

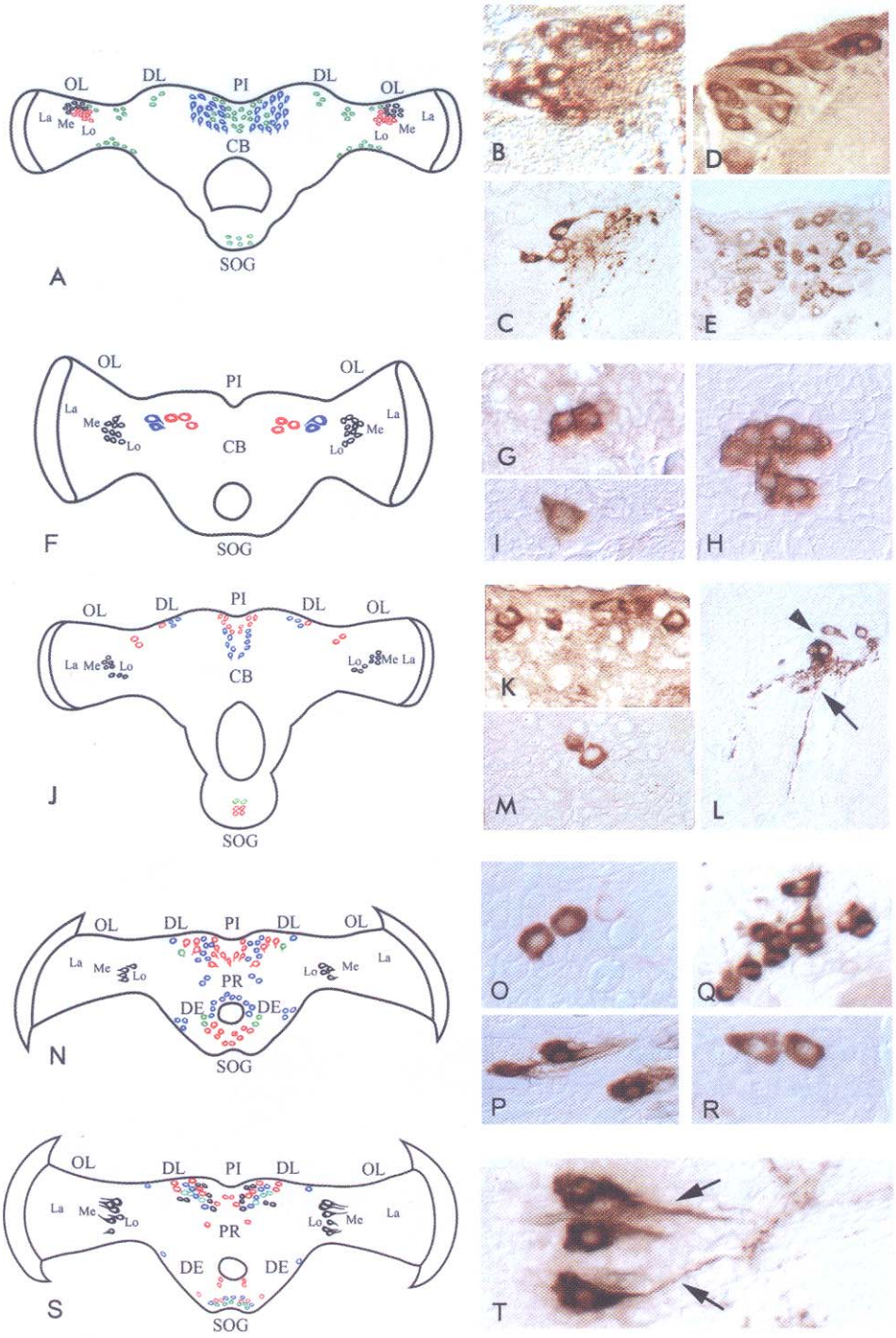

Figure 5. Immunoreactive cells in the brain and suboesophageal ganglion of Holometabola. Topology of neurons recognized with the antisera to PER (red), PDH (black), PTTH (blue), and EH (green) in (A) the goldsmith beetle *Pachnoda marignata*, (F) honeybee (*Apis mellifera*) worker, (J) the caddisfly *Hydropsyche contubernalis*, and the blowflies (N) *Neobellaria bullata* and (S) *Phormia regina*. Photos of *P. marignata*: (B) PER-positive cells and (C) PDH-positive cells with their axonal arborization in the optic lobe; (D) PTTH-positive neurons in the dorso-lateral brain region; (E) EH-positive neurons in the pars intercerebralis. Honeybee neurons: (G) PER-immunoreactive cells in the lateral brain region; (H) PDH-positive cells in optic lobe; (I) PTTH-positive neuron in the dorso-lateral brain region. *H. contubernalis*: (K) PER-positive cells in dorso-lateral protocerebrum; (L) PDH-positive cells (arrowhead) with their axonal projections (arrow) in optic lobe; (M) EH-positive cells in suboesophageal ganglion. *N. bullata*: (O) PER-like and (R) EH-like immunoreactivity in suboesophageal ganglion; (P) PDH-like antigen in optic lobe; (Q) PTTH-like immunoreactivity in ventro-posterior protocerebrum. *P. regina*: (T) PDH-positive cells with distally oriented projections (arrows) in optic lobe. Magnification in C = 420×, all others = 210×). For definitions, see the caption to Figure 2.

occurred in a more central and posterior position in *G. palludum* (Fig. 4 L,J). A medial and a more lateral cluster, each of 3 to 4 EH-positive cells, were found in the dorsal protocerebrum in both species (Fig. 4 A,F,K,L). *N. glauca* contained additional EH-positive cells: 5 in the most lateral protocerebral region, 3 at the base of the optic lobe ventrally to the medulla, and 1 at the border between the deuto- and the tritocerebrum.

The Goldsmith Beetle *Pachnoda marignata* (Order Coleoptera)

Both the PER-positive and the PDH-positive cells occurred exclusively in the optic lobes. A group of 8 to 10 PER cells was localized anterio-dorsally to the lobula (Fig. 5 A,B), just beneath and in front of a cluster (apparently corresponding to the Pfv cell cluster but shifted to a dorsal position) of 8 to 10 PDH-positive cells (Fig. 5 A,C). Dispersed staining indicated that the PER and the PDH fibers ran through the dorsal protocerebrum to the brain midline. Other PER fibers continued in ventral direction up to the suboesophageal ganglion, and still other fibers circumvented the optic lobe at the medulla level. The PTTH antiserum reacted in each brain hemisphere with 16 to 20 large cells in the dorso-medial protocerebrum (Fig. 5A,D). There were no indications of cell processes. A large number of brain neurons reacted with the EH antiserum (Fig. 5 A,E). About 10 such cells were localized in the medial part, 3 in the lateral part, and 4 in the most lateral part of each protocerebral hemisphere. Two cells occurred in the optic lobe dorsally to the lobula, and 6 cells occurred ventrally at the very base of the optic lobe. A group of 6 cells was found in the suboesophageal ganglion.

The Honeybee *Apis mellifera* (Order Hymenoptera)

There were only 3 PER-positive cells located dorsally and rather posteriorly in the lateral region of each brain hemisphere (Fig. 5 F,G). Weekly stained dots indicated that the PER-positive fibers ran from these cells toward the optic lobes. Dispersed immunoreactivity, without obvious relation to the stained perikarya, was present in the olfactory lobes. The Pfv group of about 10 PDH-positive cells was localized in a typical position at the base of each optic lobe (Fig. 5 F,H). PDH-positive fibers could be traced to the protocerebrum, deutocerebrum, and the suboesophageal ganglion. The PTTH-like material occurred in 2 large and well-stained perikarya located anterio-dorsally in the lateral protocerebrum (Fig. 5 F,I). The antiserum to EH did not react.

The Caddisfly *Hydropsyche contubernalis* (Order Trichoptera)

Four to 5 pairs of PER-positive cells occurred in the pars intercerebralis, 1 pair in the dorso-lateral brain regions, and 2 pairs dorsally to the lobula in the optic lobes (Fig. 5 J,K). Diffuse staining indicated a wide distribution of PER-positive fibers in the brain. Four PER-positive cells laid ventro-medially in the suboesophageal ganglion (Fig. 5J). Slight staining in the neuropile occurred dorsally and laterally of these cells (not shown). The PDH antiserum reacted in about 8 cells of the Pfv group (Fig. 5 J,L). Some of their fibers reached the lamina, while other fibers ran into the opposite direction toward the brain center. A weak PTTH reactivity occurred in each hemisphere in 5 cells in the pars intercerebralis and in 3 cells located dorso-laterally (Fig. 5J). Weak staining in the neuropile suggested presence of PTTH fibers in the protocerebrum and the suboesophageal ganglion. The protocerebral fibers seemed to run toward the optic lobes; the orientation of fibers in the suboesophageal ganglion could not be discerned. The EH-like antigen occurred in just 2 anterio-medial cells of the suboesophageal ganglion (Fig. 5 J,M).

The Blowflies *Neobellaria bullata* and *Phormia regina* (Order Diptera)

The PER antiserum recognized 3 groups of cells in the dorsal part of each protocerebral hemisphere: 3 to 5 cells in the pars intercerebralis, 2 to 3 well-stained cells in the pars lateralis, and a single cell located somewhat posteriorly (Fig. 5 N,O,S). Fiber staining indicated a connection of the protocerebral PER-positive perikarya with optic lobes in *P. regina*. Five to 6 pairs of PER-cells occurred in the suboesophageal ganglion; all except 2 cells in *N. bullata* were in its anterior part. Four PDH-positive neurons in *N. bullata* and 6 in *P. regina* formed the Pfv cell cluster (Fig. 5 N,P,S,T). The processes of these cells pointed distally, consistent with the presence of dispersed PDH immunostaining in the distal part of the optic lobes. There was a mediolateral group of about 7 PDH-positive perikarya in each protocerebral hemisphere of *P. regina* (Fig. 5S). Their axons were not visible, but diffuse staining suggested that they ran beneath the lateral brain surface

Table 3. Pairs of perikarya stained in brain and suboesophageal ganglion (SOG) with antisera to PER, PDH, PTTH, and EH, respectively.

	PER						PDH			PTTH						EH					
Insect Species	*PI*	*DL*	*OP*	*DT*	*OL*	*SOG*	*DL*	*OP*	*OL*	*PI*	*DL*	*OP*	*DT*	*OL*	*SOG*	*PI*	*DL*	*OP*	*DT*	*OL*	*SOG*
Lepismachilis y-signata		1			12				10	1	2									13	
Thermobia domestica	4	3	7	5	3	[2]			10		2	4	3	7§			2				
Siphlonurus armatus	2	4	7					1§	8 + 8 + 14						4		2				
Ischnura elegans				1	8				6						1						
Scistocerca gregaria		6							4 + 8 + 8												
Locusta migratoria		3			3				8 + 12 + 12												
Perla burmeisteriana	14			3§					2		13	1									
Notonecta glauca	6	16	8	4	18				8		2						8	5	1	3	
Gerris palludum		18			> 40			3	20		5						7	1			
Pachnoda marignata					10				8		16					12	3	4		4 + 4§	3
Apis mellifera		3							10		2										
Hydropsyche contubernalis	5	1			2§	2			8	5	3										1
Neobellaria bullata	6	4				5			4	8	1	5	3				1				2
Phormia regina	4	3				5	7		6	4	1		1		3		3				3

NOTE: PI = Pars intercerebralis; DL = dorso-lateral protocerebrum; OP = other protocerebral regions; DT = deuto- and tritocerebrum (DT); OL = optic lobes. Three separate numbers indicate existence of 3 distinct clusters of PDH cells in OL, § marks cells that were at the border between central brain and OL, and brackets mark cells that could be detected only occasionally. A blank cell indicates that the antibody did not react with cells in the respective region in any species. Unpublished data on *Thermobia domestica* are included for comparison.

to the base of the protocerebrum. No corresponding cells were found in *N. bullata*.

The pars intercerebralis contained 9 PTTH-positive cell pairs in *N. bullata* and 4 in *P. regina* (Fig. 5 N,S). One cell in the lateral brain region occurred in both species, while a distinct group of about 12 PTTH cells in the anterio-ventral part of the protocerebrum was specific for *N. bullata* (Fig. 5Q). One cell in *P. regina* and 3 cells in *N. bullata* were present in the lateral part of the deutocerebrum. Additional 6 weakly stained cells were found in the suboesophageal ganglion of *P. regina*. The EH-positive cells were located in both species in the lateral regions of the protocerebrum and in the suboesophageal ganglion (Fig. 5 N,R,S). *N. bullata* contained in each protocerebral hemisphere just one large, distinct cell and *P. regina* 3 smaller cells that were stained less intensively. In the suboesophageal ganglion, there were 4 lateral cells in *N. bullata* and 6 more ventro-medially placed cells in *P. regina*.

DISCUSSION

Distribution of Neurons Expressing PER-Like Proteins

Among the insects, full PER sequence was established in *Drosophila melanogaster* (Citri et al., 1987) and a few related flies, in the silkmoth *Antheraea pernyi* (Reppert et al., 1994), and in the cockroach *Periplaneta americana* (Reppert et al., 1994). The PER orthologs contain 3 conserved regions with amino acid identities ranging from 46% to 57%. These sequence similarities imply that antisera raised against a particular PER are likely to recognize PER orthologs in other insects. Indeed, the anti-αPER antibody raised against the *D. melanogaster* PER reacts with the *per*-expressing neurons in *A. pernyi* (Sauman and Reppert, 1996a), and the antibodies PER 57 and PER 58, directed against 2 different regions of the *A. pernyi* PER, recognize the *per*-expressing neurons in *D. melanogaster*. Specific reaction of these antibodies with neurons that express the endogenous *per* gene was also found in the American cockroach, *Periplaneta americana* (Sehadová and Sauman, unpublished), the linden bug, *Pyrrhocoris apterus* (Syrová et al., unpublished), and the housefly, *Musca domestica* (Sauman and Kyriacou, unpublished). This makes us confident that the staining obtained with the PER-antibodies in the present study revealed the presence of PER orthologs in all insect cohorts. The variation in antisera activities among the examined species (Table 2) is interpreted as indicating random diversification of antigenic epitops in different PER orthologs.

Except for the damselfly and the goldsmith beetle, the antibodies to PER stained specific perikarya in the dorsal, and in a few cases also in other regions of the protocerebrum (Table 3). Most species also contained PER-positive cells in the optic lobes, usually in proximity of the Pfv cluster of the PDH-positive cells but never identical with these cells. In a few species, additional PER-positive cells occurred in the deutocerebral and tritocerebral regions and/or in the suboesophageal ganglion.

The total number of PER neurons in the brain-suboesophageal ganglion complex is typically higher in the apterygote and exopterygote insects (Fig. 2-4) than in the Holometabola (Fig. 5). Similarly, the simultaneous occurrence of PER cells in protocerebrum and optic lobes is common in apterygotes and exopterygotes but seldom in Holometabola. Interestingly, the goldsmith beetle contained PER-positive neurons exclusively in optic lobes (Fig. 5A), while the previously examined ground beetle *Pachymorpha sexguttata* contained neurons in both optic lobes and the central brain (Frisch et al., 1996).

Cellular Localization and Daily Fluctuations of PER

Ticking of the endogenous biological clock in *D. melanogaster* is set by the degradation rate of the TIM protein and depends on translocation of the PER/TIM dimer from cytoplasm into the nucleus (Kloss et al., 1998; Price et al., 1998). The translocation occurs in *D. melanogaster* in certain brain neurons and glia, the photoreceptive cells of compound eyes, and some other cells (Siwicki et al., 1998). It was also found in the brain glial cells in a cricket (Honegger et al., 1991), a beetle (Frisch et al., 1996), and the moth *M. sexta* (Wise et al., 2002). In the silkmoth *A. pernyi*, nuclear translocation of PER was observed in the photoreceptor cells (Reppert et al., 1994) and the midgut cells (Sauman et al., 1996) but not in the glia and the 4 pairs of neurons that express *per* gene in the brain. The homologous Ia_1 neurons of *M. sextra* contain PER both in the cytoplasm and the nucleus, without fluctuations between these 2 compartments (Wise et al., 2002). Lack of nuclear translocation of PER was reported for the brain neurons of the housefly, *Musca domestica* (Sauman and Hashimi, 1999). No clear nuclear localization of PER was detected in any species examined in the present study.

PER present in the 4 pairs of the dorsal brain neurons in *A. pernyi* appears to be axonally transported to other brain regions and to the corpora cardiaca (Sauman and Reppert, 1996a). This is consistent with the notion that the neurons in question are neurosecretory cells of the Ia_1 group with axonal projections to the ipsilateral corpora cardiaca (Wise et al., 2002). We noted presence of PER-positive fibers in the brain and often also in the suboesophageal ganglion of all examined insects. Passing of such fibers into the corpora cardiaca was most clearly seen in the mayfly and the stonefly. It is justified to propose that PER, in addition to being a component of the intracellular clock mechanism, acts as a humoral clock output released in the retrocerebral neurohaemal organs.

Association of the PDH-Positive Neurons with Optic Lobes

The brain master clock is synchronized with environmental circadian changes via a pair of pacemakers, which were identified with the accessory medulla and adjacent perikarya expressing PDH orthologs (Homberg et al., 1991). As far as we know, only *M. sexta* was reported to lack PDH-positive cells in the optic lobe (Wise et al., 2002). We found a cluster of PDH-positive cells close to the accessory medulla (i.e., typically on the Pfv side of the optic lobe) in all examined species. The number of cells in the cluster varied in different species from 6 to 20 (Table 3). The mayfly and both locust species contained 2 additional PDH cell clusters at the distal edge of medulla (Fig. 2E; Fig. 3 A,D). Fibers of the PDH cells formed a semifunnel over the frontal medulla surface and in some species continued over the lateral medulla face to terminate in the lamina. One or 2 bundles of fibers ran from the Pfv cluster to the lateral protocerebrum where they branched and gave rise to arborizations. One or 2 branches continued medially to form bilateral connections of the optic lobes. Morphological evidence for a linkage of the bilateral optic lobes is available for the cohorts Thysanura (Závodská, unpublished), Polyneoptera (Homberg et al., 1991; Fig. 3G), Paraneoptera (Fig. 4D), and Holometabola (Helfrich-Förster et al., 1998).

Sauman and Reppert (1996a) found in *A. pernyi*—and we did in the mayfly, one of the bugs, and one of the blowflies—PDH cells in the central brain (Table 3). These cells were probably not associated with the optic lobe pacemaker. Their function may be similar as proposed for the PDH neurons located in the abdominal ganglia of a locust, in which PDH modulates neuronal circuits in the terminal abdominal ganglion and is also released into the haemolymph (Persson et al., 2001). However, we did not detect any sign of PDH transport to the retrocerebral neurohemal organs.

Pacemaker Linkage to the Master Clock

The loss of rhythmicity in the locomotor activity of cockroaches following removal of the PDH neurons

from the optic lobes demonstrated their importance for the endogenous clock setting (Stengl and Homberg, 1994). In *D. melanogaster*, PDH neurons co-express the clock genes *per* (Helfrich-Förster, 1995), *tim* (Hunter-Ensor et al., 1996; Kaneko et al., 1997), and *doubletime* (Kloss et al., 1998). However, no colocalization of PDH with the products of clock genes was observed in the beetle *P. sexguttata* (Frisch et al., 1996) and the moth *A. pernyi* (Sauman and Reppert, 1996a), and it is unlikely to occur in any of the species examined in the present study. The PER-positive and the PDH-positive neurons were close to one another in the bristletail, damselfly, waterstrider, beetle, and the blowfly *P. regina*, but the appearance and exact positions of the perikarya revealed that the expressions of PER and PDH did not overlap. It seems that the co-expression of PDH with PER in *D. melanogaster* is an exceptional phenomenon.

Some fibers of the PDH neurons ran from the optic lobe centripetally (Homberg et al., 1991) and ramify in the region of calyces (Fig. 3G; Fig. 4 D,M). The importance of PDH supply to the central brain is evident from the disturbance of the rhythmic behavior in *D. melanogaster* subjected to ectopic expression of introduced *pdh* gene in the neurons projecting to the dorsal protocerebrum (Helfrich-Förster et al., 2000). This suggests strongly that the meshwork of PDH axons in the central brain is essential for a signal transduction between the pacemakers in the optic lobes and the master clock located in specific protocerebral neurons.

Clock Control over Neurohormonal Signaling

The productions of PTTH (Vafopoulou and Steel, 2001) and EH (Reynolds and Truman, 1983) exhibit distinct circadian fluctuations, suggesting that they are under central clock control. The structure of PTTH, which is a regulator of ecdysteroid hormone secretion, was elucidated in 5 lepidopteran species, including *Bombyx mori* (Kataoka et al., 1987) and *M. sexta* (Shionoya et al., 2000) that belong to different suprafamilies. The PTTH molecules of these 2 species are 64% identical. Structural similarities are manifested by the cross-reactivities of antibodies; for example, antibodies to *B. mori* PTTH react in *M. sexta* exclusively with the 2 pairs of neurons that produce the endogenous PTTH (Dai et al., 1994).

In our present study, the PTTH antibody recognized specific sets of neurons in all examined species except locusts. Immunoreactive neurons were mostly localized in the protocerebrum and included cells resembling by their position the 2 pairs of neurosecretory cells producing PTTH in Lepidoptera. The PTTH-positive cells usually lay in proximity of the PER neurons, similarly as reported for *A. pernyi* (Sauman and Reppert, 1996a). The numbers of PTTH-positive protocerebral neurons were limited to 2 to 3 pairs in the bristletail, bugs, and the honeybee. Simoes et al. (1997) demonstrated that the honeybee contains several PTTH-positive neurons at the start of metamorphosis, but the number is later reduced to just 2 pairs, which are apparently identical with those we found in adult honeybee workers. Five and more pairs of PTTH-positive neurons were found in the stonefly, the blowflies, the beetle, and the caddisfly (Table 3). The finding in blowflies is consistent with an earlier report on a high number of PTTH-positive neurons in *D. melanogaster* (Žitnan et al., 1993). Axonal transport of the PTTH-like material to the corpora cardiaca, indicative of release into the hemolymph, was detected only in the stonefly. Unusual was the location of PTTH-positive neurons in the suboesophageal ganglion and not protocerebrum in the mayfly (Fig. 2 E,H) and damselfly (Fig. 2I).

Antiserum to *M. sexta* EH (Kataoka et al., 1987; Marti et al., 1987) was used to detect EH-like antigens. The sequence of *M. sexta* EH is nearly identical to that of *B. mori* EH (Kono et al., 1987) and 58% identical with the EH of *D. melanogaster* (Horodyski et al., 1993). The antisera to *B. mori* or *M. sexta* EH were shown to react with homologous neurons in different families of Lepidoptera. Our results demonstrate that EH-like proteins occur in specific neurons of most insect orders. In Lepidoptera, EH is primarily expressed by 2 pairs of neurons in the ventro-lateral protocerebrum (Copenhaver and Truman, 1986; Naya et al., 1994). In the insects of other orders, a single pair of EH-positive cells in comparable position was detected only in the backswimmer. The mayfly, the bugs, the goldsmith beetle, and the blowflies possessed 2 to 8 pairs of cells with EH-like material in dorso-lateral protocerebrum. The backswimmer and the beetle contained additional EH-positive cells in optic lobes and the beetle and the blowflies in the suboesophageal ganglion. In the bristletail, EH-neurons were confined to optic lobes and in the caddisfly to the suboesophageal ganglion. No EH-positive cells occurred in the damselfly, the stonefly, locusts, and the honeybee.

Conservation of the Master Clock Regulatory Mechanisms in Insect Phylogeny

The results of our study indicate that basic components of the brain master clock are similar throughout the class of Insecta but that their locations and possibly also the function mechanisms differ. PER-like antigens were detected in some neurons of all species so far examined, but cyclic PER translocation from cytoplasm to nuclei and PER co-expression with PDH is known only in the ventro-lateral neurons of *D. melanogaster.*

The clock output, which secures synchronization of various oscillating processes, embraces control over the production of neurohormones such as PTTH and EH. Antigens related to these neurohormones were detected in most insects, including apterygotes, indicating that PTTH and EH represent molecules with ancient antigenic epitopes. They seem to have been lost or substantially modified in the evolution of only a few insect groups. Both anti-PTTH and anti-EH sera failed to react in Orthoptera and the anti-EH serum also in Odonata, Plecoptera, and Hymenoptera. The PTTH- and EH-like antigens are never produced in identical cells and never co-localize with either PER- or PDH-like antigens. The neurons expressing PTTH-like or EH-like antigens are commonly located in dorsal protocerebrum, usually not far from some of the PER-expressing neurons. This seems to be a default distribution pattern that was modified in some insect clades. Notable modifications include dislocation of the PTTH-producing cells to the suboesophageal ganglion in both orders of Palaeoptera and dislocation of the EH neurons from the dorsal to the ventral brain region in Lepidoptera.

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