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Antibodies against the PER protein of *Drosophila* label neurons in the optic lobe, central brain, and thoracic ganglia of the crickets *Teleogryllus commodus* and *Teleogryllus oceanicus*

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Abstract We describe labeling of neurons in the central nervous system of two cricket species, *Teleogryllus commodus* and *T. oceanicus*, with both mono- and polyclonal antibodies against the PER protein. Western blots reveal that the monoclonal antibodies recognize a single protein with a molecular weight of approximately 94 kDa, i.e., similar to that of the PER protein of the moth, *Anterea pernix*. Neurons and their processes are labeled both in the optic lobes and in the central brain. Processes occur in the accessory medulla, the medulla, and proximal lamina, in the central complex, in the non-glomerular neuropil, and in the retrocerebral complex, suggesting that PER-containing neurons form a widely distributed network. Neurons and processes were also labeled in the meso- and metathoracic ganglia. Four to six PER-immunoreactive (ir) neurons with processes in the accessory medulla were double labeled by an antibody against pigment-dispersion factor (PDF), a peptide that is implicated in circadian rhythmicity in *Drosophila*. In the central brain, projections of fibers labeled by the anti-PER

and anti-PDF antibodies were mainly distinct, with overlap only in a few restricted regions. In most neurons, including those projecting into the accessory medulla, PER labeling was restricted to the cytoplasm and there was no indication of circadian variation in the intensity of staining.

Keywords Circadian rhythm · Period gene · Cricket · Immunocytochemistry · *Teleogryllus commodus*, *T. oceanicus* (Insecta)

Introduction

The *period* gene plays a central role in the regulation of circadian rhythms (Konopka and Benzer 1971). Molecular and genetic studies have shown that the *per* gene product, PER, is one of several proteins that form the core of the clock mechanism (Dunlap 1999; Panda et al. 2002). Homologues of *per* have been implicated in circadian rhythms in other insects (Colot et al. 1988; Reppert et al. 1994; Sauman and Reppert 1996; Thoma et al. 2000) as well as in mammals (Siwicki et al. 1992; Bae et al. 2001; Zheng et al. 2001).

PER is associated with distinct neurons and glial cells within the brain in different insects as shown by immunocytochemistry. In the brain of *Drosophila* antibodies against PER stain three lateral clusters of neuronal somata (Ewer et al. 1992; Helfrich-Förster 1995; Frisch et al. 1996). One of these, the ventral neuron cluster (LN_v), is located between the medulla and the lateral protocerebrum (Helfrich-Förster 1995). Four small neurons in the LN_v (LN_vs) are also immunostained by an antibody against the crustacean neuropeptide pigment-dispersing hormone (PDH; Helfrich-Förster and Homberg 1993; Helfrich-Förster 1995). Incubation with the anti-PDH antiserum revealed projections of the cells into the dorsal protocerebrum. Studies on *disco* mutants, in which the LN_vs neurons are variably present, show that at least one of the neurons, and its projection to the dorsal protocerebrum, is required to maintain a robust circadian rhythm of

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locomotor activity (Helfrich-Förster 1998). In a more recent study using a GAL4-expression system Kaneko and Hall (2000) visualized additional neuronal processes of *per*-expressing neurons and described other neurons that had not been shown by immunohistochemical studies or β -galactosidase histochemistry (Helfrich-Förster 1995). Their results suggest that the network of neurons involved in circadian rhythmicity may be broader than previously suspected.

Cockroaches were the first animals in which a circadian pacemaker was localized by lesion experiments. These indicated that a critical element of the circadian oscillator resides in the medulla of the optic lobe (Nishiitsutsuji-Uwo and Pittendrigh 1968; Page 1982, 1983; Colwell and Page 1990). A similar conclusion was drawn for the location of the circadian oscillator that drives the calling song rhythm of male crickets (Loher 1972; Sokolove 1975; Sokolove and Loher 1975; Chiba and Tomioka 1987; Tomioka and Chiba 1992; Abe et al. 1997). Studies on neurons that express pigment-dispersing factor (PDF; the peptide homologue to PDH of the grasshopper *Romalea microptera*; Rao et al. 1987; Homberg et al. 1991) in cockroaches and crickets have defined more precisely the accessory medulla as the likely region of the circadian pacemaker in both insect groups (Homberg et al. 1991; Stengl and Homberg 1994; Stengl 1995). As in flies, the PDF-immunoreactive (PDF-ir) neurons project into the central brain into distinct regions (Helfrich-Förster and Homberg 1993; Helfrich-Förster et al. 1998). However, expression of PER has not yet been shown in these presumptive pacemaker neurons in either cockroaches or crickets.

A recent study on *Manduca sexta*, using two new monoclonal antibodies against PER, showed a widespread distribution of PER immunoreactivity in the optic lobes, brains, and neurosecretory cells projecting into the corpora cardiaca (Wise et al. 2002).

In *Drosophila*, *per* is also involved in an ultradian rhythm in the courtship song, which has a period of about 1 min. Mutations of *per* affect this rhythm in the same direction as their effect on circadian rhythms; in *per^l* flies, the period of the courtship-song rhythm is lengthened, in *per^s* flies it is shortened, and in *per^o* flies the rhythm dissociates into a number of short-period components (Kyriacou and Hall 1980; Alt et al. 1998). Experiments with genetically mosaic flies have shown that the effect of *per* on circadian rhythms is determined by the genotype of the head, whereas the thoracic genotype determines the influence of *per* on song rhythms (Konopka et al. 1996). In the thoracic ganglia, however, the likely site for generation of song rhythms (Konopka et al. 1996), PER immunoreactivity is found only in glial cells (Ewer et al. 1992). Crickets, also express song-related ultradian rhythms (periods of tens of milliseconds; Balakrishnan and Pollack 1996), and preliminary evidence indicates that these different rhythms may share the same control mechanisms as in *Drosophila*. Among wild-type *Teleogryllus oceanicus*, intermale variations in circadian period are positively correlated with variations in the

period of one of the rhythmic components of the calling song (Lupien and Pollack 1998).

In the present paper we describe the pattern of PER immunoreactivity in two closely related cricket species, *Teleogryllus oceanicus* and *T. commodus*, as revealed by the monoclonal antibodies described by Wise et al. (2002). We find that PER-ir somata are clustered in the medulla of the optic lobe, the dorsolateral brain, and the pars intercerebralis. PER-labeled processes are widely distributed throughout the brain but occupy some focal areas, for example, the central body. A few neurons in the thoracic ganglia, the sites of generation of song and other ultradian rhythms, are also labeled by anti-PER. Double labeling using the anti-PDF antiserum (Rao et al. 1987) can be observed in a few neurons in the accessory medulla.

We thus present for the first time the pattern of PER-ir neurons in crickets. The neurons revealed are likely to participate in the generation and distribution of circadian, and perhaps also ultradian, rhythmicity.

Materials and methods

Animals

Teleogryllus oceanicus and *T. commodus* were raised in the laboratory at 25–30°C under a 12 h:12 h light:dark cycle. Crickets had ad libitum access to Purina Cat Chow (adult formula) and water. *Teleogryllus commodus* were fed additionally once a week with organic lettuce. Adult males and some *T. commodus* females were used for immunocytochemistry; results were identical for both sexes.

Dissection and embedding

The brain, meso-, and metathoracic ganglia were dissected under cold-anesthesia at 6-h intervals, beginning at lights on. Six *T. oceanicus* and *T. commodus* were examined at each time point. For *T. commodus* three additional animals were dissected at 1, 2, and 3 h after dark. Dissected tissues were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C. The tissues were then infused overnight at 4°C in 25% sucrose in 0.1 M phosphate buffer. *Teleogryllus oceanicus* tissue was mounted for cryosectioning in a droplet of HistoPrep (Fisher). *Teleogryllus commodus* brains were aligned in a plastic mold in a warm 1.5% agar (Sigma), 5% sucrose solution. After cooling to room temperature, the agar–sucrose gel was cut into individual blocks, each containing one brain, and submerged in a 30% sucrose solution overnight at 4°C. The following day, after the blocks had sunk to the bottom, they were frozen in 2-methylbutane cooled in liquid nitrogen and sectioned immediately.

Anti-PER antibodies

Two monoclonal antibodies (5F7F6, 10C3C9), both generous gifts from Dr. K. Siwicki (Swarthmore College, Swarthmore, Pa., USA) were used. The antibodies were raised against a mixture of three recombinantly expressed PER fragments. Two were from *Drosophila*; an N-terminal fragment of ca 60 kDa, extending nearly to the end of the PAS domain, and a C-terminal fragment of ca 95 kDa, extending from the PER^S region to the C-terminus. The third PER fragment was from *M. sexta* (50 kDa) extending from the PAS domain to 24 residues downstream of the site of the PER^S mutation (Wise et al. 2002). Both antibodies recognize the C-terminal

fragment of *D. melanogaster* PER, and the fragment of *Manduca* PER but not the N-terminal *Drosophila* fragment, in ELISA as well as on Western blots. Both antibodies also label PER-expressing somata in the brains of *Drosophila* showing that they detect *Drosophila* PER (Wise et al. 2002).

Immunocytochemistry

Frozen sections (12 μm) of the *T. oceanicus* CNS were air dried on slides for at least 1 h at room temperature, rinsed with phosphate-buffered saline (PBS), blocked with 10% normal goat serum (NGS; Vector Laboratories) in PBS (room temperature, 1 h), and incubated with primary antibody (5F7F6, dilution 1:500; 10C3C9, dilution 1:100) for 36–48 h at 4°C. After washes in PBS the sections were incubated in biotinylated anti-mouse IgG (Vector; room temperature, 2 h), washed again and incubated in avidin-biotin peroxidase (Vectastain, ABC kit) for 1 h at room temperature. They were then incubated in 3,3'-diaminobenzidine (DAB) reaction solution (DAB kit; Vector) for 10 min at room temperature. Sections were then rinsed, air dried, and coverslipped with Permount (Fisher). Specimens collected at each of the four time points were processed in parallel, along with a control for non-specific binding of the secondary antibody (collected just before lights on), for which the primary antibody was omitted on half the sections. Staining was consistently observed on the anti-PER-treated sections, and was never observed on those sections that were not incubated with anti-PER. A similar protocol, including the control without primary antibody incubations, was followed for *T. commodus* with the following changes. Frozen sections were 20 μm thick, primary antibody dilutions for both monoclonals were 1:20, and preincubation after sectioning was done in 3% NGS, 0.1% BSA in PBS-0.3% TX-100. No labeling was observed in brains for which the primary antibodies had been eliminated. For DAB staining, the protocol of Bartos et al. (1994) was followed. The stained sections were dehydrated in an isopropanol series from 50% to 100%, cleared in xylene, and mounted in Permount (Fisher). Photographs were taken with a Zeiss Dialux 20 photomicroscope.

Double labeling

Double-labeling experiments were done using an antiserum against pigment-dispersion factor (PDF; Rao et al. 1987; Homberg et al. 1991) in combination with anti-PER. As fixative a 7.5% picric acid solution, 4% PFA in phosphate buffer (Petri et al. 1995) was used. The PDF antibody, used at a dilution of 1:5,000, was a generous gift from Dr. R. Rao, University of West Florida. PER labeling was detected using Cy-2 fluorescence, and PDF labeling was detected by Cy-3 fluorescence. Slides were first incubated simultaneously with the primary antibodies, washed in PBS, 0.03% TX-100, 3% milk powder, and then incubated with Cy-3-coupled goat anti-rabbit IgG (dilution 1:500 for 2 h). They were then washed three times for 10 min in PBS, embedded in glycerol, and viewed under the microscope to check for successful labeling. Coverslips were then removed, slides washed twice for 10 min in PBS followed by three 15-min washes in PBS, 0.03% TX-100, 3% milk powder, and incubation with Cy-2-coupled rabbit anti-mouse IgG (dilution 1:500 for 2 h). The slides were then washed three times in PBS, dehydrated in alcohol, cleared in xylene, and embedded in Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, Mich., USA). Photographs were taken with a Zeiss Dialux 20 photomicroscope or a confocal microscope (Zeiss LSM410; Vanderbilt University Medical Center, Cell Imaging Resource).

Western blots

Whole brains with the optic lobes attached, muscle, gut, and salivary glands were collected on dry ice and then homogenized for 2 min on ice in 0.1 mM phosphate buffer as described in Kostron et al. (1996). The homogenates were mixed with 4 \times sample buffer

with or without 2-mercaptoethanol, heated to 80°C, and loaded onto 10% polyacrylamide minigels. Approximately 35 μg total protein (BSA was used as standard) was loaded per lane. For further details, see Kostron et al. (1996). Following SDS gel electrophoresis of the tissue homogenates, gels were incubated for 1.5 h in regeneration buffer (50 mM TRIS-HCl, pH 7.5, containing 20% glycerol) and electroblotted (Towbin et al. 1979) onto Immobilon-P membranes (PVDF membranes; Millipore, Bedford, Mass., USA) using carbonate blotting buffer (for details, see Kostron et al. 1996). The membrane was then incubated overnight in primary antibody (10C3C9 or 5F7F6) at a dilution 1:20 at 4°C. Antiserum detection was visualized using enhanced chemiluminescence (ECL; Amersham) following a working protocol of Amersham.

Results

The experiments on *T. oceanicus* and *T. commodus* were performed separately and independently in two different laboratories. The results were combined after the senior authors became aware of one another's parallel efforts. Consequently, the procedures (and some of the results) differed slightly between the two species. In *T. oceanicus* both antibodies 5F7F6 and 10C3C9 were used for incubations. In *T. commodus* most of the incubations were done with the 5F7F6; 10C3C9 was used additionally at some time points. In *T. oceanicus*, antibodies were used at dilutions of 1:100 (10C3C9) and 1:500 (5F7F6), whereas in *T. commodus* dilution was 1:20. In general, larger numbers of neurons were revealed in *T. commodus*, probably because of the higher antibody concentration. Nevertheless, results were, in the main, similar between the two species. Differences between the two sets of results are noted below.

Central brain

Locations of PER-ir somata

A conspicuous pair of PER-ir somata, 30–40 μm in diameter, was located close to the origins of the lateral ocellar nerves (Fig. 1A). We refer to these as the dorsolateral neurons (DLNs). Their primary neurites project to the dorsoposterior part of the brain (Fig. 1B). In *T. commodus*, these neurons show clear cytoplasmic labeling and absence of nuclear labeling (Fig. 1C) at all circadian times except 2 h after onset of darkness, when both cytoplasmic and nuclear staining were observed with both antibodies in three of five brains dissected at this time point (Fig. 1D). Brains dissected 1 and 3 h after onset of dark did not show nuclear staining (three brains tested for each time point). In *T. oceanicus*, both cytoplasmic and nuclear labeling were observed in these cells with the 5F7F6 antibody in 10 of 12 preparations, distributed nearly equally among the four time points sampled. Only 1 of 12 preparations showed nuclear labeling with the 10C3C9 antibody (cytoplasmic labeling was observed in all 12); this cricket was killed at 2 h before the onset of darkness.

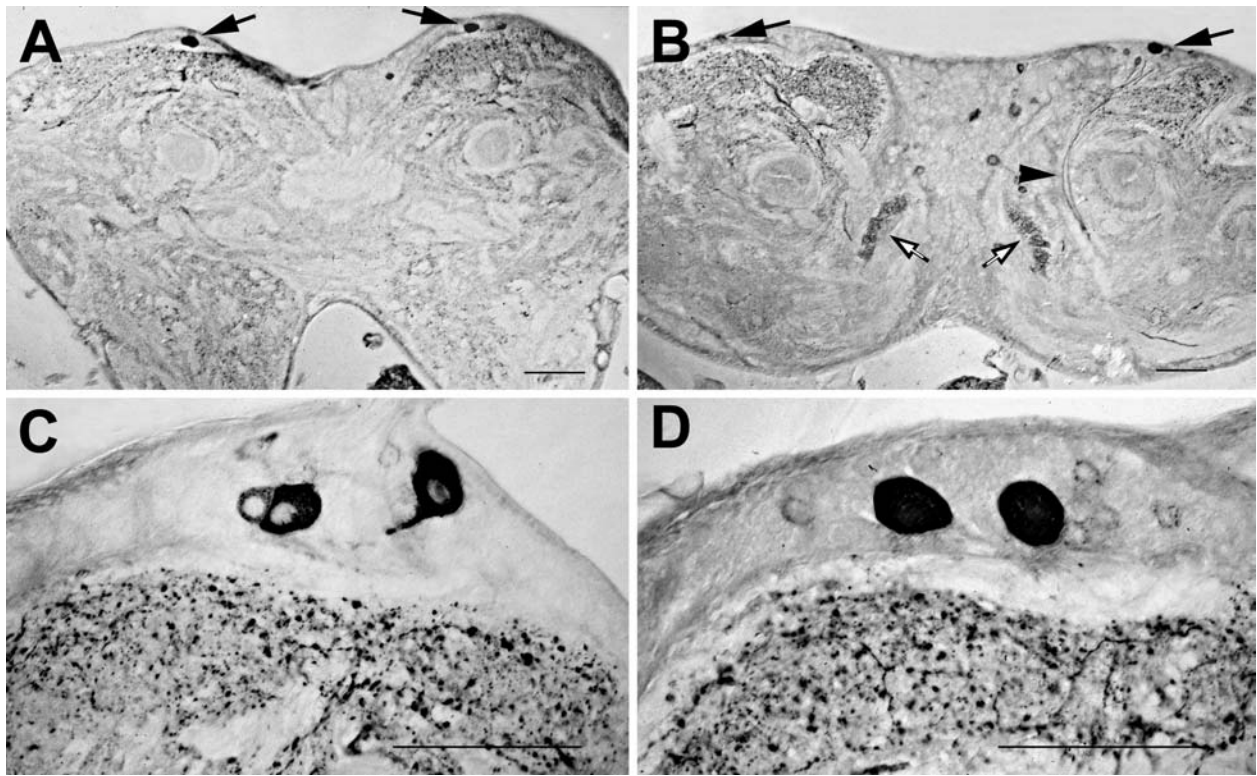


Fig. 1A–D *Telogryllus commodus*, dorsolateral neurons (DLN) with large somata close to the origins of the ocellar nerves. **A** Horizontal section, *arrows* point to two of the four cells. **B** Oblique section between horizontal and transverse plane. On the *right*, two primary neurites (*arrowhead*) project posteroventrally from the

DLN (*arrows*). *Open arrows* depict the labeled protocerebral bridge. **C, D** DLN at higher magnification. The section in **C** is from a brain dissected at the middle of the light period. Nuclear staining in **D** in a section from a brain dissected 2 h after start of dark. Note the dense fiber tangles. Scale bars 100 μm

In the pars intercerebralis (PI) a large number of smaller somata, 10–20 μm in diameter, were labeled (Fig. 2A). In one *T. commodus* brain, 87 somata were counted in the PI. In addition, three bilateral pairs of larger somata, 25–30 μm in diameter, were consistently found in the PI in *T. commodus*, and one bilateral pair in *T. oceanicus*. The single pair in *T. oceanicus*, and the most posterior of the three pairs in *T. commodus*, contribute a tuft of fibers to the most dorsal part of the protocerebral bridge (PB; Figs. 2B, 3E). Two bilaterally paired somata were stained near the base of the optic stalk and one group of 3–4 somata in the lateral wedge between the proto- and deutocerebrum. Their neurites enter the dorsal (mechanosensory) part of the deutocerebrum (Fig. 2C). A group of 4–5 somata were labeled in the lateral cortex between the deuto- and tritocerebrum. Figure 2D shows one of these neurons to give rise to extensive branches in the tritocerebrum.

PER-ir fiber projections

PER-ir processes occupy many regions of the central brain. The abundance of fibers in the non-glomerular neuropil of the brain made it impossible to trace the arborizations of single neurons; however focal areas of

projections could be discriminated. These are the central body (CB; Fig. 3A, B) the medial tips of the β -lobes of the mushroom bodies (bL_i labeled only in *T. commodus*; Fig. 3C, D) and the PB (Fig. 3E). In the CB dense arborizations were observed in the fan-shaped section (Fig. 3A, B), as well as a few fibers in the lower division of the CB and the lateral accessory lobes (not shown). These projections probably originate from neurons with somata in the PI that also send projections into the PB (Figs. 2B, 3E). The projections in the β -lobes have fine punctate processes. PER-ir fibers ramify extensively in the entire ventromedial and ventrolateral region of the brain surrounding the β -lobes, medial parts of the pedunculi, and extending to the edges of the PI (Figs. 2A, 3F). Especially in this region, many of these fibers bear large conspicuous varicosities (Fig. 3D, F, see also Fig. 5A). PER-ir fibers cross the midline ventral of the PB (Fig. 3E), anterior and ventral of the CB (Fig. 3C), and connect the dense projections surrounding the α -lobes. PER-ir fibers are absent from the major optic commissures that contain axons of neurons connecting the two optic lobes (Honegger and Schürmann 1975; Homberg et al. 1991). The superior lateral protocerebrum contains only a few PER-labeled fibers that project into the optic tract toward the medulla. The inferior lateral protocerebrum is almost devoid of such projections. PER-

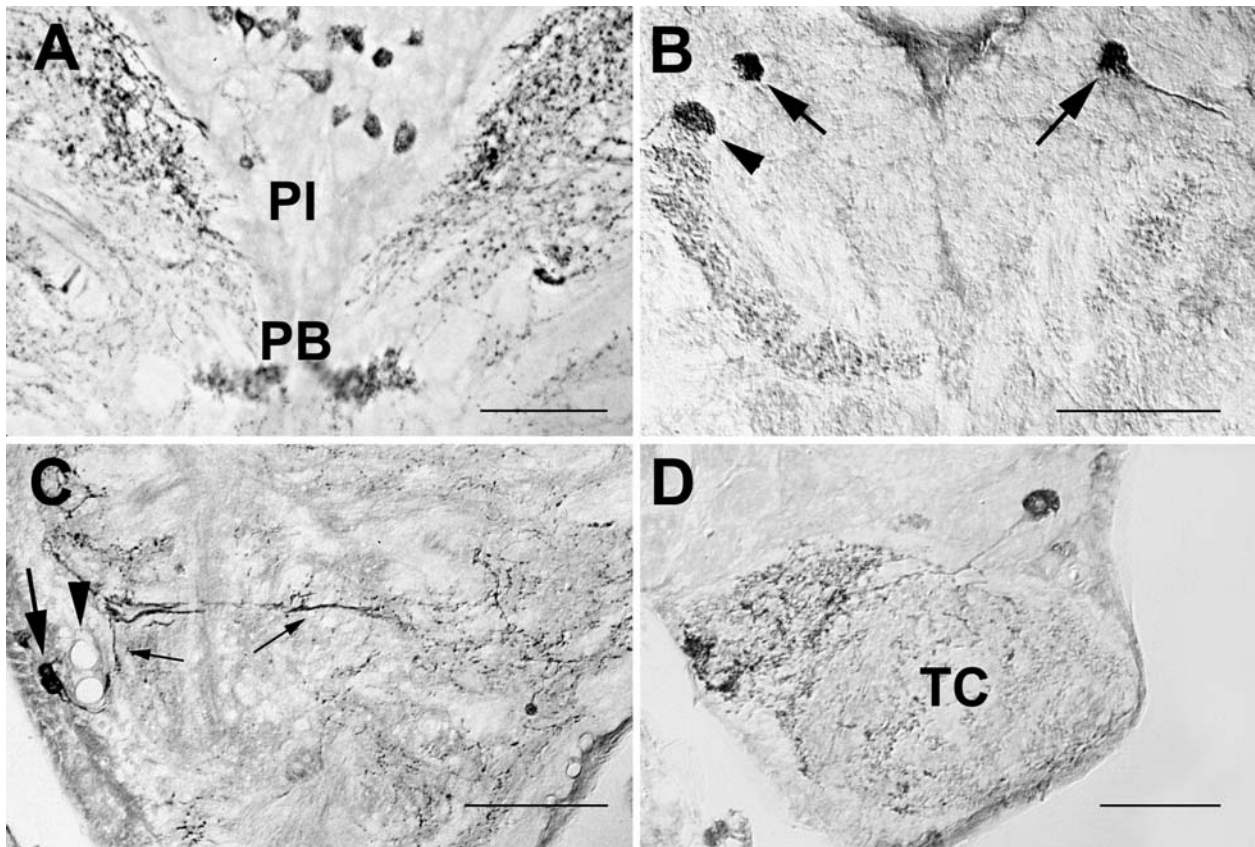


Fig. 2A–D Somata labeled reproducibly in the central brain of both *Teleogryllus* species. **A** Small labeled neurons in the median pars intercerebralis (PI), horizontal section. **B** One recurring pair of somata (arrow) in the dorsoanterior PI. These neurons have a dense tuft of dendrites (arrowhead) in the most dorsal portion of the protocerebral bridge (PB); up is dorsal. **C** Three of four somata

(arrows) in the cortex between proto- and deutocerebrum projecting into the dorsal deutocerebrum. Small arrows depict primary neurites circumventing a trachea (arrowhead). **D** One of four somata which project into the tritocerebrum (TC). In the upper left some glomeruli of the antennal lobes, devoid of PER-immunoreactive (ir) projections, are seen. Scale bars 100 μ m

ir projections run from anterior to posterior in the dorsal brain and project toward the tritocerebrum at the medial sides of the two brain hemispheres. Among these are collaterals of the neurites of the DLNs. The tritocerebrum contains a densely labeled fiber mesh that originates in part from the neurons with somata in the lateral tritocerebrum (Fig. 2D). The deutocerebrum contains only a few labeled fibers in the antennal lobe between the glomeruli, but the antennal mechanosensory region in the dorsal part of the deutocerebrum contains many labeled fibers and arborizations. Fibers of variable diameter were labeled in the circumesophageal connectives and their tissue sheath in *T. commodus* (Fig. 5B).

PER immunoreactivity in the optic lobes

In *T. commodus*, the anti-PER antibodies labeled 14–19 somata with 20–30 μ m diameter, located along the proximal rim of the medulla (number of labeled somata counted in five medullae of *T. commodus*). Six to seven of these somata, clustered together at the anteriomedial cortex (Fig. 4A), send dense tufts of small arborizations

into the accessory medulla (AME; Fig. 4B). In *T. oceanicus* six neurons were labeled in most of the 24 optic lobes investigated. In addition to the projections to the AME, processes from these cells run along the anterior face of the medulla and project through the first optic chiasm to the proximal layer of the lamina (Fig. 4A–C) in both species. Other fine fibers occupy the medullar neuropil in strata-like fashion (Fig. 4C). Labeled fibers project through the optic stalk, bypassing the lobula (Fig. 4D).

PER immunoreactivity in the retrocerebral complex

PER-ir processes were found in the main organs of the retrocerebral complex, i.e., in the corpora cardiaca (CC; Fig. 5C, D), parts of the corpora allata (CA; Fig. 5F), the frontal ganglion (Fig. 5E), and the recurrent nerve connecting to the hypocerebral ganglion (not shown). Four to five PER-ir profiles travel from the brain to the CC through NCCII (Fig. 5D). Coarse processes and varicosities occur throughout the CC, but are particularly prominent at the cortex of the gland (Fig. 5C, D).

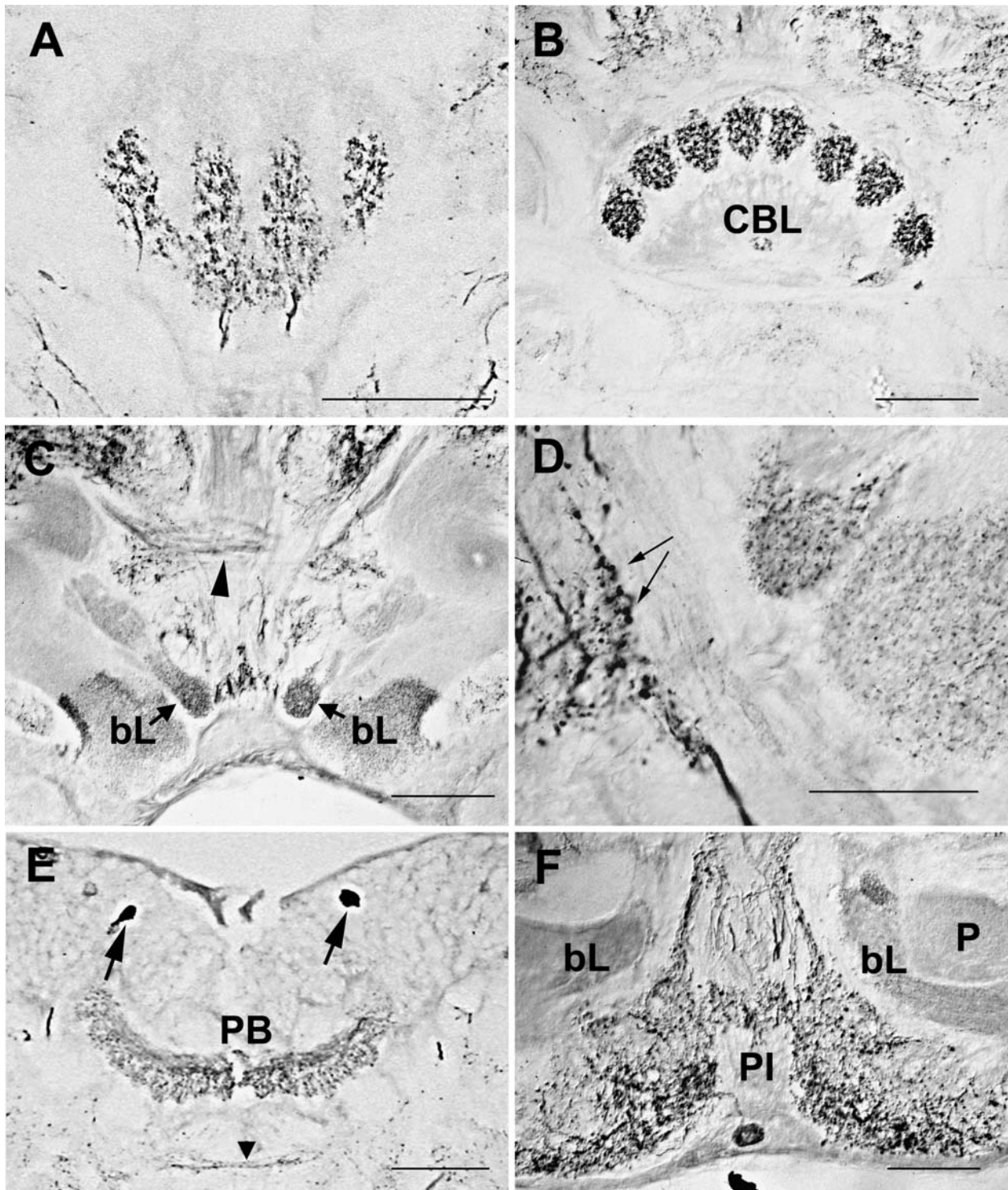


Fig. 3A–F PER-ir projections in the central brain of *T. commodus* (**B–D, F**) and *T. oceanicus* (**A, E**). **A, B** Central body, transverse section in **A** and horizontal section in **B**. Note the dense fiber staining in the fan-shaped part of the upper division. The lower division (**CBL**) contains no fibers in the section plane of **B**. **C** Extrinsic projections in the tip of the β -lobe (**bL**). Arrows point to the subcompartment of the β -lobe receiving projections from the KIII Kenyon cells (Schürmann et al. 2000). Arrowhead points to crossing fibers in the anteroventral protocerebrum. **D** Comparison of the fine, punctuated projections in the β -lobe at right and the

coarse fibers in the non-glomerular neuropil carrying large varicosities (arrows). **E** Protocerebral bridge (**PB**); arrows point to the two somata that project with a fiber tuft into its most dorsal extension (compare with Fig. 2B, showing the homologous somata in *T. commodus*); arrowhead points to fiber crossing the midline as in **C**. **F** Dense fiber tangle in the ventral part of the non-glomerular neuropil. Note medial soma in the pars intercerebralis (**PI**) with primary neurite extending from it. **P** Pedunculus of the mushroom body. Scale bars 100 μ m

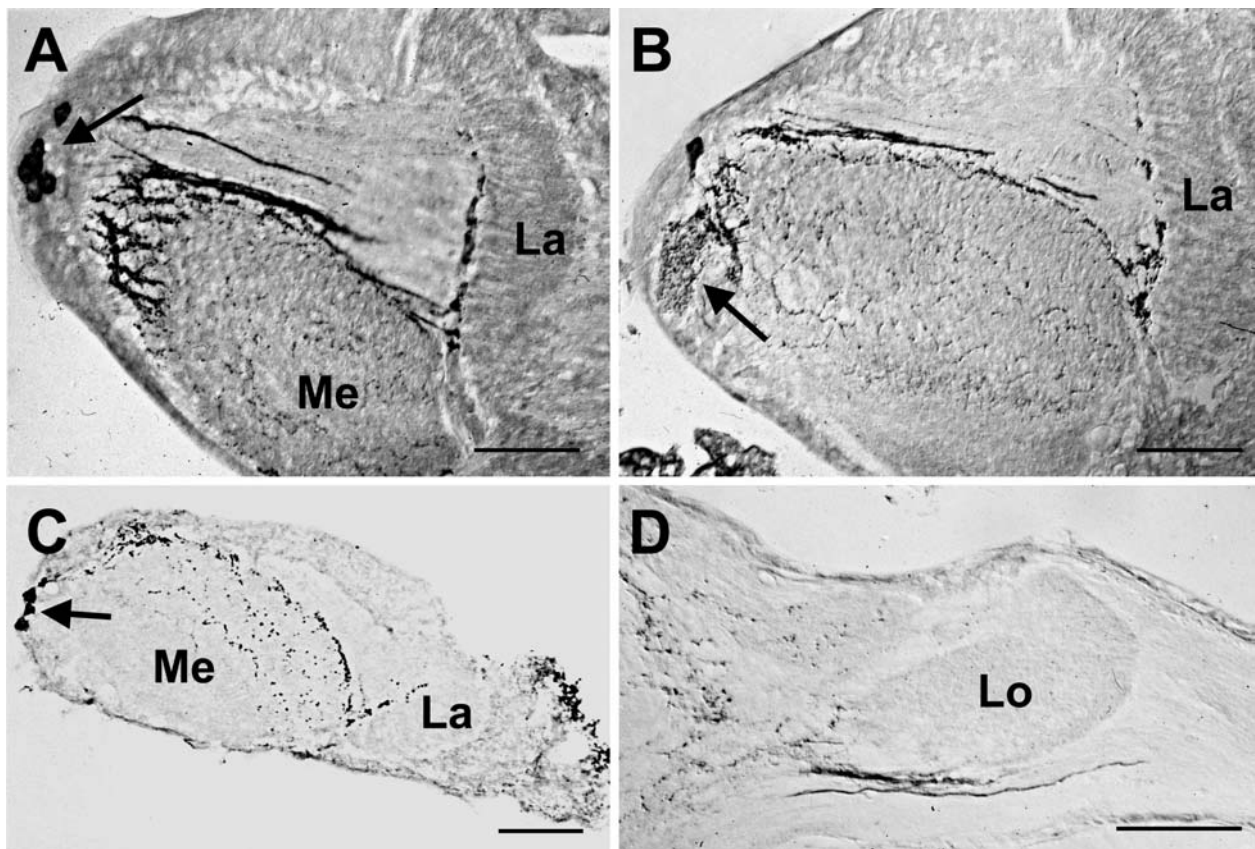


Fig. 4A–D Optic lobes of *T. commodus* (**A**, **B**, **D**) and *T. oceanicus* (**C**). **A** Cluster of five accessory medulla (AMe) somata (*arrow*), and PER-ir projections in the medulla (*Me*) and its anterior face, extending to the proximal rim of the lamina (*La*). Note the non-

labeled nuclei. **B** Projections of the soma cluster shown in **A** in the accessory medulla (*arrow*). **C** The fine projections in the medulla are stratified, also seen in **B**. **D** PER-ir fibers bypass the lobula in the optic stalk. The central brain is to the left. Scale bars 100 μ m

Processes travel from the CC to the CA through NCA1. Within the CA, processes are restricted to regions close to the site of entry of NCA1 (Fig. 5F). Labeled somata were not found in either gland. In addition, PER-ir fiber bundles enter the frontal ganglion through the frontal connectives and spread in the frontal ganglion neuropil (Fig. 5E) to project through the recurrent nerve (examined only in *T. commodus*; data not shown). Their probable projections in the hypocerebral ganglion were not investigated. The large somata in the frontal ganglion were not labeled. The intense innervation of the retrocerebral complex indicates a neurosecretory function of some of the PER-ir neurons.

Intensity of cytoplasmic PER immunoreactivity does not show circadian cycling

In *T. oceanicus*, staining intensity with anti-PER antibodies was compared across specimens that were killed at four time points (0, 6, 12, and 18 h after lights on). We assessed this semiquantitatively in the brain by rating staining intensity using a subjective scale (0=no staining, 4=intense staining; the specimens were coded so as to obscure, during the rating process, the

times at which they were killed). Staining intensity was scored separately for the following structures: DLN processes in the CB, processes in the PB, optic lobe somata, optic lobe neuropil processes, and CC/CA. There was no statistically significant circadian cycling, either when the scored structures were considered separately (Kruskal-Wallis test, $P>0.07$ for DLNs, $P>0.18$ for all other structures; probabilities not corrected for multiple comparisons) or when staining intensity was pooled across structures ($P=0.57$). In the optic lobe, the brain structure most closely tied to circadian rhythms in crickets (Abe et al. 1997), neither the somata ($P=0.20$) nor the neuropil processes ($P=0.53$) showed any indication of circadian variation in staining intensity.

PER immunoreactivity in thoracic ganglia of *T. oceanicus*

The anti-PER antibodies labeled both somata and processes in the meso- and metathoracic ganglia; the prothoracic ganglion was not examined. The numbers and positions of the somata are shown in Fig. 6A. Processes were found mainly at mid-to-posterior regions of the ganglia. Many of the processes exhibit varicosities

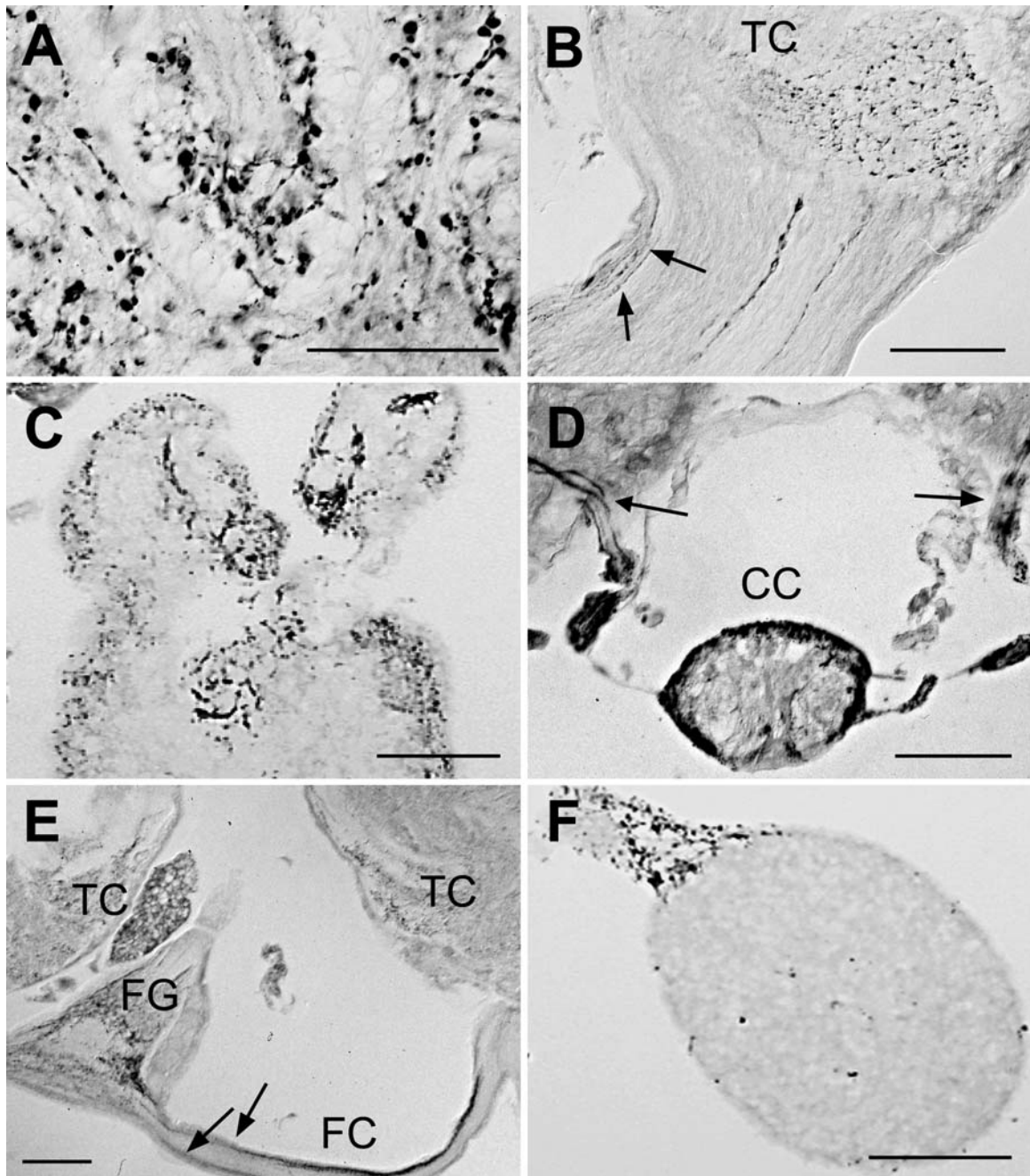


Fig. 5A–F PER-ir projections in brain, connectives, and the retrocerebral complex. **A, B, D, E** from *T. commodus*; **C** and **F** from *T. oceanicus*. **A** PER-ir projections in the non-glomerular part of the central brain bearing large varicosities. **B** Axons in the circumesophageal connective leaving the tritocerebrum (TC). Note the fine labeled profiles in the tissue sheath of the connective

(arrows). **C** PER-ir profiles in the corpora cardiaca. **D** Arrows at left and right point to labeled axons in the NCCII connecting the brain with the corpora cardiaca (CC). **E** Labeled fibers (arrows) project through the frontal connective (FC) from the tritocerebrum (TC) to the frontal ganglion (FG) and spread there. **F** Corpora allata with the NCA1 containing labeled profiles. Scale bars 100 μ m

(Fig. 6B). Processes were widely dispersed throughout the neuropil, but appeared to avoid the central regions (Fig. 6C, D). Thoracic ganglia of *T. commodus* were not examined.

Double labeling using both the anti-PER antibodies and antiserum against PDF

In *Drosophila*, the PER-expressing pacemaker neurons are also immunoreactive to PDH (Helfrich-Förster 1995, 1998). We therefore investigated, in *T. commodus*, whether the subset of PER-ir neurons found in the

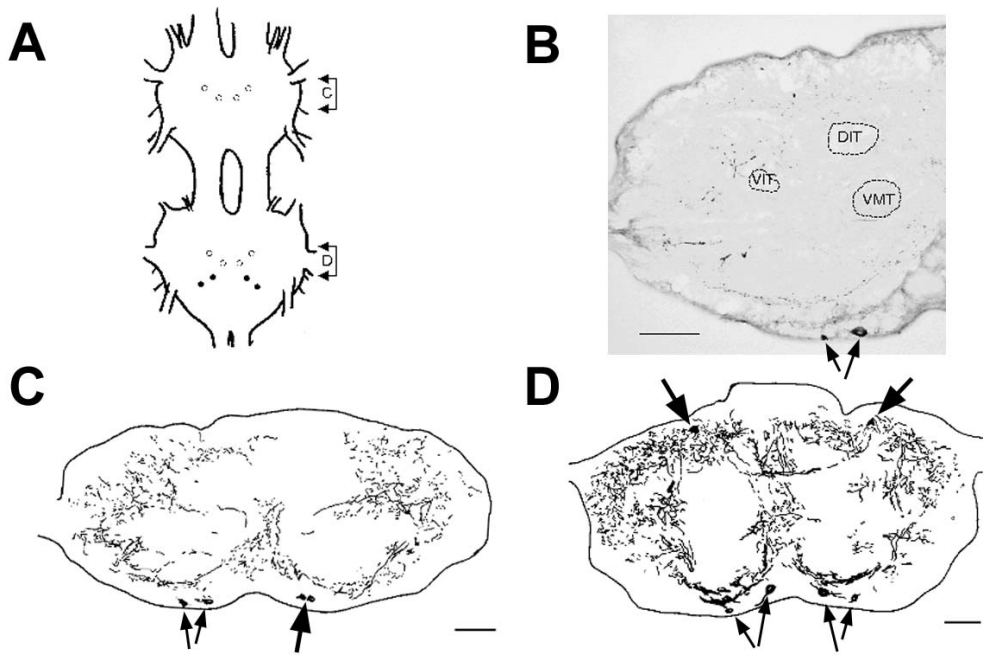


Fig. 6A–D Anti-PER staining in the thoracic ganglia of *T. oceanicus*. Positions of somata are marked by *arrows* in **B–D**. **A** Schematic drawing of positions of immunoreactive somata; *open circles* ventral, *filled circles* dorsal. Regions indicated by *brackets* correspond approximately to the transverse sections represented in **C** and **D**. **B** Stained processes and somata (*arrows*) in the neuropil

of the mesothoracic ganglion. *DIT*, *VIT*, *VMT* Dorsal and ventral intermediate tracts, and ventromedial tract. **C**, **D** Reconstruction of locations of processes and somata (*arrows*) in mesothoracic (**C**) and metathoracic (**D**) ganglia. Each drawing superimposes processes from several successive sections, corresponding to the regions indicated in **A**. *Scale bars* 100 μm

medulla of the optic lobe, which project to the AMe (the best candidates for pacemaker neurons in crickets; see Introduction), are also labeled by antibodies against PDF, the homologous peptide to PDH of *R. microptera* (Rao et al. 1987). Three optic lobes were examined. In one, double labeling was observed in six somata close to the AMe; in another, four somata were double labeled. The third optic lobe was examined by confocal microscopy. Double labeling was observed in two somata in one 20- μm section and in fibers projecting into the medulla (Fig. 7C). As the figure shows, the relative intensities of staining with the two antibodies differed among the two somata. In addition, labeling within the cytoplasm is non-uniform, suggesting that that PER and PDF might be differentially compartmentalized.

In the optic lobes of two brains incubated with only the anti-PDF antiserum, 10–12 neurons were labeled. These were located in the same region as the double-labeled neurons described above and, like them, had projections in the AMe. This suggests that only approximately 50% of the PDF-ir neurons are PER-ir.

In the central brain double labeling of fibers has not been investigated in detail. However, it was readily apparent that major differences exist between projections of fibers staining with anti-PER and anti-PDF. Figure 7A and B show that PDF-ir processes are prominent throughout the superior and medial protocerebrum, and in one optic commissure running across the anterior roof of the CB. Serial sections showed that PDF-ir fibers run in

four commissures. They also project into the frontal ganglion from a small posteriomedial fiber tangle in the tritocerebrum. The PDF-ir somata were not detected in any area of the central brain, and therefore these projections must be from the AMe somata that are not immunoreactive to PER. For further details of projections of PDF-ir neurons we refer to Homberg et al. (1991). As can be seen in Fig. 7A and B the nuclei of all somata were labeled by the anti-PDF antiserum. We consider this a fixation artifact, because the labeling intensity in the nuclei was increased if only 4% paraformaldehyde was used for fixation instead of a mixture of picric acid and PFA as used in this study (see Materials and methods).

Western blots

Both anti-PER antibodies detected a single protein band at approximately 94 kDa in blots of SDS-polyacrylamide gels loaded with homogenates of the brain, the esophagus, muscle, and salivary glands and run both under reducing and non-reducing conditions (tested only in *T. commodus*). Figure 8 shows one blot where the homogenates of brain and esophagus were investigated with both antibodies. The molecular weight of 94 kDa is nearly identical to that of the *Anterea pernix* PER protein (Reppert et al. 1994). This result indicates that all labeled neurons in the *T. commodus* brain express the identical protein, and that this is very likely to be PER. Based on

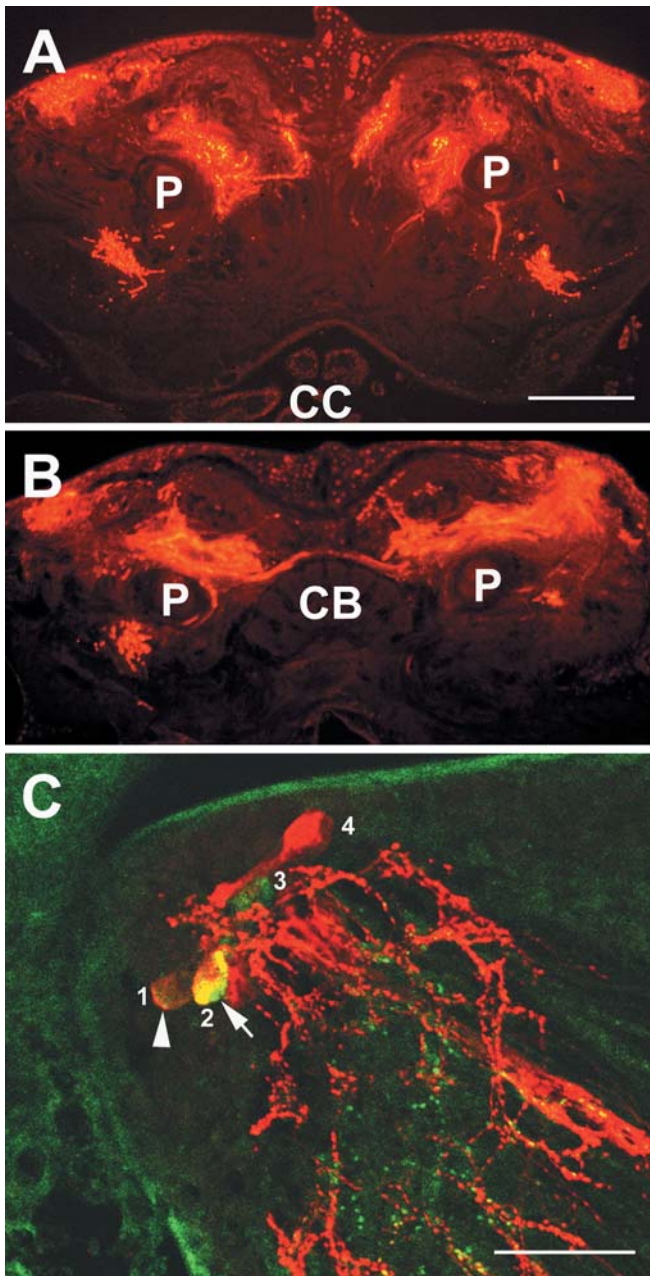


Fig. 7A–C *Teleogryllus commodus*, PDF immunofluorescence in the central brain (A, B) and PDF/PER double labeling in the medulla of the optic lobe (C). PDF is detected by red (Cy-3), PER is detected by green fluorescence (Cy-2), and overlap of both colors generates yellow (C). A, B Twenty-five- μm horizontal sections. Note that nuclei of neuronal somata in the PI are labeled. The pedunculi (P) of the mushroom bodies serve as landmarks. CC Corpora cardiaca; no PDF-labeled fibers project into this gland. B Section of the same brain as A but 40 μm posterior of section shown in A. Note the fibers in the optical commissure anterior of the central body (CB). No profiles are labeled in the CB. C Confocal microscopy of a section of the medulla of the optic lobe. Optical section is 2.7 μm . Overlap of PER and PDF is indicated by yellow. Four somata (1–4) are labeled. Soma 3 is labeled only green (PER) and soma 4 is labeled only red (PDF). Somata 1 and 2 are labeled both for PDF and PER. Soma 2 is in the optimal focal plane. Arrowhead in soma 1 depicts one of the small yellow areas; arrow in soma 2 points to an only green area. Note also that nuclei are stained red in all somata. The fiber tufts of these neurons in the AMe are in another section plane. The red fluorescent fibers project centrifugally toward the lamina. Note that some fine fibers appear in yellow. Scale bars 200 μm in A, B; 50 μm in C

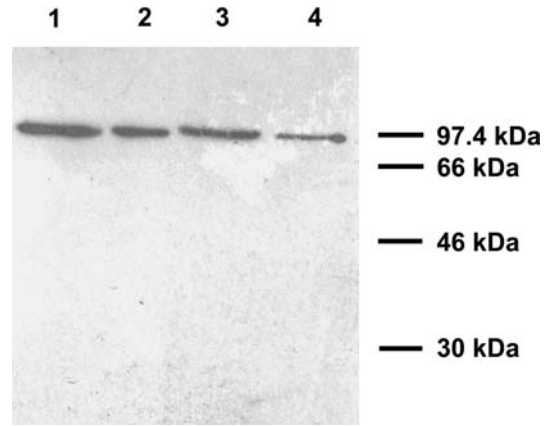


Fig. 8 Western blot of *T. commodus* homogenates of brain (lanes 1, 3) and esophagus (lanes 2, 4) after non-reducing SDS-PAGE. Incubation with 5F7F6 (lanes 1, 2) and 10C3C9 (lanes 3, 4), both diluted 1:20

the similarity of labeling patterns, it seems likely that the same, or a similar, protein is recognized in *T. oceanicus*.

Labeling of neurons with an antiserum against the PER-S region of *Drosophila*

In an earlier study, the anti-PER-S antiserum directed against a peptide just downstream of the *per^s* mutation (Siwicki et al. 1988) was used on the *T. commodus* brain (Honegger et al. 1991, 1995). This antiserum labeled one bilateral pair of neurons with somata in the dorsolateral protocerebrum close to the origin of the lateral ocelli (Fig. 9A, B), and glial cells in the neural sheath of the entire brain including the optic lobes (Fig. 9D, F). No neuronal somata were labeled in the optic lobes. In the PI some large somata were lightly labeled, but not their processes. Although double labeling using this anti-PER-S antiserum and the two monoclonal antibodies was not possible (the anti-PER-S is no longer available), we hypothesize, based on similar soma positions, that the dorsolateral cells labeled by anti-PER-S are identical with the DLNs described in the present paper (compare Figs. 1A and 9A; see also Discussion). PER-S-ir projections were observed in all regions of the CNS. They occupied strata in the medulla (Fig. 9F), the entire non-glomerular neuropil of the central brain (Fig. 9D), the CB (Fig. 9C), and the CC (Fig. 9E) including the entire retrocerebral complex. No fibers were found in the β -lobes or the PB. Many small fiber projections could be found in the connectives and their tissue sheath, extending to the abdominal ganglia. These cells closely resembled in gross morphology those described by Sauman and Reppert (1996) for *A. pennii*. Western blot analysis showed that the anti-PER-S labeled a 220-kDa protein, suggesting that a protein different from PER was labeled in *T. commodus* in cells that are also immunoreactive to PER.

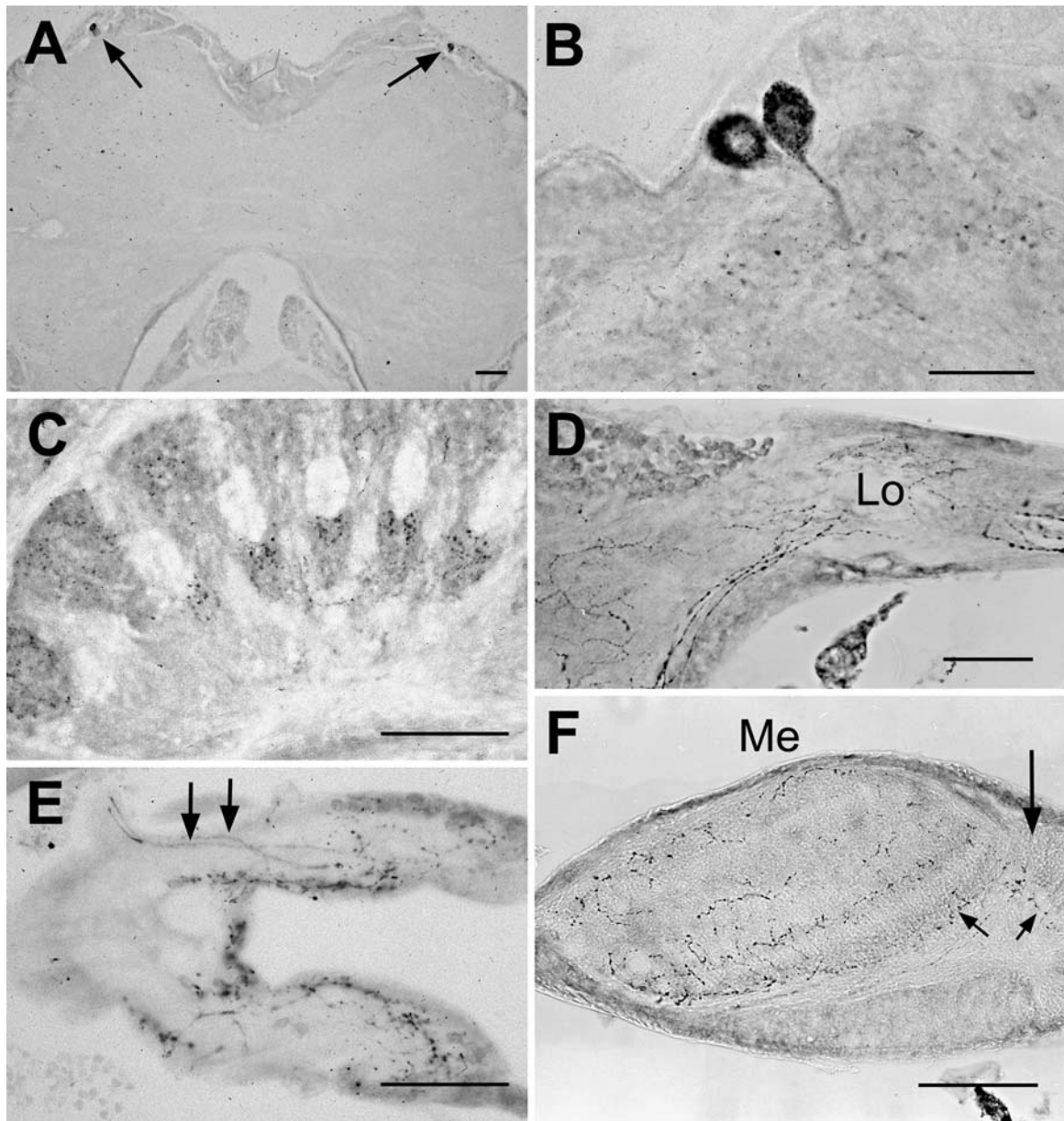


Fig. 9A–F Anti-PER-S immunolabeling in the *T. commodus* brain. **A** One pair of large somata close to the origin of the lateral ocellar nerves (compare with the DLN in Fig. 1A). **B** The somata at higher magnification. Nuclei are not stained. **C** Labeled fibers in the fan-shaped part of the upper division of the central body. **D** Varicose projections in the lateral brain and the optic stalk containing the lobula (*Lo*) and connecting to the medulla at *right*. Note the labeling of a large number of glial cells in the upper part of the

brain and in the tissue sheath of the optic stalk. **E** Labeling in the corpora cardiaca. Two labeled axons (*arrows*) project into one of its two lobes. **F** Strata-like projections in the medulla (compare with Fig. 4B, C). Some projections travel along the rim of the medulla and continue at the proximal rim of the lamina as in Fig. 4 (*arrows*). Also here the tissue sheath is labeled. *Scale bars* 100 μm in **A, D–F**; 50 μm in **B, C**

Discussion

The period gene is highly conserved among insect species (Panda et al. 2002), and antibodies directed against the PER protein have been shown to label distinct neurons in the brains of flies, beetles, and moths (Siwicki et al. 1988; Frisch et al. 1996; Sauman and Reppert 1996; Wise et al. 2002). Crickets have been the focus of a number of studies of circadian rhythmicity and its neural control

(Loher 1972; Sokolove and Loher 1975; Chiba and Tomioka 1987; Tomioka and Chiba 1992), and preliminary reports demonstrated PER-ir in four neurons in the dorsolateral brain (Honegger et al. 1991, 1995). We now show, in two closely related cricket species that monoclonal antibodies directed against two epitopes in the region between the end of the PAS domain and the C-terminus of the PER protein (Wise et al. 2002) label four to six somata in the medulla of the optic lobe with

projections into the AMe, a region that had previously been identified as the site of the circadian pacemaker in crickets (Chiba and Tomioka 1987; Tomioka and Chiba 1992), as well as neuronal somata and their projections in several regions of the central brain. No labeling of glial material was found. Our Western blot results show that the two monoclonal antibodies detect a single protein at approximately 94 kDa. A molecular mass of 94.799 kDa has been estimated for the *A. pernix* *per* homolog cDNA (Reppert et al. 1994). Together with the control experiments conducted by Wise et al. (2002) to characterize the two monoclonal antibodies that were used in our study, these data support the hypothesis that PER is detected in all labeled neurons in the cricket brain. The fact that each of the monoclonal antibodies detects only a single epitope and both antibodies detect a PER-like protein of approximately 94 kDa in Western blots, indicates strongly that the antibodies bind to a conserved region of PER (see also the discussion of Wise et al. 2002).

PER-ir neurons projecting into the AMe

The two anti-PER antibodies labeled a cluster of six to seven somata in *T. commodus*, and six somata in *T. oceanicus*, with dense tufts of projections in the AMe and processes in the optic lobes. The anti-PDF antiserum labeled about ten neurons with identical positions and projections. Since antibodies against both PDH and PER label pacemaker neurons and their projections in *D. melanogaster* (Helfrich-Förster and Homberg 1993; Helfrich-Förster 1995, 1998), we anticipated a coexpression of both PER and PDF in cricket pacemaker cells in the medulla. Four to six somata were double labeled by antibodies against the two proteins, whereas up to seven PER-ir neurons with projections in the AMe were counted. Thus, a subset of the PER-ir cells with projections to the AMe also express PDF.

Projections of PER-ir neurons in the central brain

The most conspicuous PER-ir projections were found in the fan-shaped upper part of the CB, the PB, and in the entire ventral part of the brain extending to the α -lobes of the mushroom body. Projections in a restricted area of the medial tip of the β -lobes were also labeled. These PER-ir cells might modulate the output from Kenyon cells to their targets.

We assume that PER-ir neurons with somata in the medial PI send their projections into both the PB and the CB; similar projections have been shown in other studies (Williams 1975; Homberg et al. 1991; Würden and Homberg 1995; Vitzthum and Homberg 1998; Schürmann et al. 2000). Support has accumulated that the CB serves a variety of functions, including visual integration, and control of motor activities like walking and flight (Homberg 1985; Strauss and Heisenberg 1993; Bausenwein et al. 1994; Heisenberg 1994; Ilius et al. 1994;

Homberg and Müller 1995). The PB receives input from ocellar interneurons, which make connections to the optic lobes (Honegger and Schürmann 1975). We could verify that two bilateral PER-ir neurons in the PI contribute with a fiber tuft to the labeling in the PB. The labeled fiber projections in the glomerular neuropil regions thus, may indicate that input/output pathways exist that are involved in the circadian control of sensory-motor integration.

The non-glomerular neuropil of the ventral part of the brain, extending to the α -lobes, shows a dense mesh of labeled fibers. This region also contains the dendrites of descending neurons that control calling (Hedwig 2000), suggesting a possible anatomical substrate for the circadian control of calling. The large varicose structures labeled with anti-PER throughout the brain suggest furthermore that PER-expressing neurons may release their products in a paracrine fashion, i.e., they may modulate the responsiveness of populations of other neurons within the brain.

A comparison with earlier labeling studies (Honegger et al. 1991) using the anti-PER-S antiserum (Siwicki et al. 1988) indicates that the DLNs have projections in the CB and in the general non-glomerular neuropil, and send centrifugal fibers into the medulla and proximal lamina of the optic lobe. The strata-like projections in the medulla observed in the present study may derive, therefore, from the DLNs. Labeling in the CB by anti-PER-S was sparse, indicating that the profuse labeling detected there by the monoclonal anti-PER antibodies must be due to additional neurons. The projections in the β -lobes that we describe are definitely not from the DLNs. The anti-PER-S antiserum detects a 220-kDa protein in Western blots of brain homogenates. Thus, the anti-PER-S antiserum must have detected a protein different from PER in four cells of the central brain which also express PER.

PER-ir processes in neurosecretory structures

PER-ir processes in the retrocerebral complex, especially in the CC, have been reported in several insects (Frisch et al. 1996; Sauman and Reppert 1996; Wise et al. 2002). In *T. commodus* we find a maximum of four axons projecting through each NCCII to the CC. The labeling studies with anti-PER-S showed that the axons of the two DLNs take this route. Thus, only two additional neurons seem to contribute to the intense varicose PER labeling in the CC via the NCCII. However, the projections from the tritocerebrum into the frontal ganglion and from there into the recurrent nerve suggest that some neurons may take this alternative route to the CC.

Our data and those from other groups therefore suggest that the neurosecretory system forms part of an efferent pathway to distribute circadian timing. The DLNs labeled by anti-PER-S and the monoclonal antibodies in this study may be homologous to the PER-ir neurons described by Sauman and Reppert (1996) for *A. pernix* and/or the Ia₁ cells described by Wise et al. (2002) for *M. sexta*. Sauman and Reppert (1996) discussed convincingly

that the PER product is too large to be released into the blood circulation but may affect the release of neuropeptides or transmitters. Wise et al. (2002) showed that the PER-ir Ia_1 neurosecretory cells with projections into the CC are also labeled by antisera against either leu-enkephalin, FMRFamide, or corazonin. They suggest that release of neuropeptides is probably the route by which the Ia_1 cells influence circadian rhythms. We have not carried out double-labeling studies with antisera directed against different peptides. Lesion studies showed, however, that the DLNs are not necessary to maintain circadian calling activity in *T. commodus* (Honegger et al. 1995).

PER- and PDF-ir projections in the central brain

Approximately 10–12 neurons with projections in the AMe were labeled with anti-PDF antiserum, and these appear to be the source of all of the anti-PDF labeling in the central brain. About half of these cells were also PER-ir. The remaining PER-negative cells must then be the source of fibers in the brain that were PDF-positive, but PER-negative. These include projections in the protocerebral commissures, and in the superior and medial protocerebrum. Labeling with both antibodies occurred only in the ventromedial and ventrolateral regions of the brain, and in the frontal ganglion. It is likely that the PDF-ir projections in the protocerebral commissures provide the coupling pathway between the loosely coupled bilateral pacemakers (Wiedenmann 1983, 1984) in the optic lobes (Homberg et al. 1991; Stengl and Homberg 1994). The function of the PDF-ir projections in the central brain is not clear. However, Stengl (1995) showed that PDH-ir terminals in the central brain that survived after optic lobe transection are sufficient for the maintenance of circadian calling in *T. commodus*. In addition, Petri and Stengl (1997) showed that injection of PDH in the vicinity of the AMe shifts the phase of the circadian pacemaker in cockroaches.

PER immunoreactivity in the thoracic ganglia

A novel finding in our study was PER-like immunoreactivity in neurons of the thoracic ganglia (Fig. 6). Our interest in the possibility of *per* expression in thoracic neurons stems from the fact that song rhythms are generated in the metathoracic ganglion, and that motor output to muscles used for singing arises mainly from the mesothoracic ganglion (Hennig 1990; Hennig and Otto 1995/96). If, as preliminary results suggest (Lupien and Pollack 1998), ultradian and circadian rhythms share control features in crickets, then *per* expression in thoracic neurons is not unexpected. Although immunocytochemistry does not detect PER in neurons of the thorax of adult *Drosophila*, expression of reporter genes driven by the *per* promoter is evident in thoracic neurons (Kaneko and Hall 2000). PER is found in glia of the

thoracic ganglia, and it has been suggested that this may influence *Drosophila* song rhythm indirectly, by affecting the function or development of song-generating neural circuits (Konopka et al. 1996). In crickets, no glial labeling was found.

Cytoplasmic localization of PER immunoreactivity and absence of its circadian cycling

In *Drosophila*, PER, in complex with the protein TIM, translocates from the cytoplasm into the nucleus where, through their interaction with the transcriptional activators CLK and CYC, they suppress their own transcription (Panda et al. 2002). Nuclear localization is thus a fundamental feature of PER's function as a clock molecule in *D. melanogaster*. In *T. commodus*, there are indications that nuclear entry of PER occurs in the DLNs in the early night. In *T. oceanicus* nuclear and cytoplasmic staining in these cells was observed throughout the day. There was no indication of nuclear staining, or of circadian variation in intensity of cytoplasmic staining, in any of the other PER-ir cell groups, including those in the medulla. Lack of nuclear entry of PER immunoreactivity has been observed also in other insects (Frisch et al. 1996; Sauman and Reppert 1996; Wise et al. 2002). Several interpretations for this result are possible. First, nuclear localization of PER might not be required to drive circadian rhythms, as discussed also for *A. pernix* (Sauman and Reppert 1996) and *M. sexta* (Wise et al. 2002). Second, the amount of PER entry into the nuclei of pacemaker neurons may be too small in crickets and the other insects to be detected by the antibodies. In the present case, this might be due in part to limited cross-reactivity of the antibodies to the cricket PER. Third, although nuclear PER (presumably dimerized with TIM) is detectable in *Drosophila*, dimerization might mask the site on cricket PER that binds the antibodies, a not unlikely possibility, given that the sequences of PER and TIM in crickets, though presumably conserved, may not be identical to those of *Drosophila* and *Manduca*.

We did not observe any glial labeling with the anti-PER antibodies. This result deviates from that of other insects (Zerr et al. 1990; Ewer et al. 1992; Wise et al. 2002). As for neuronal nuclei, the level of PER in cricket glia may simply be below the detection threshold. A second possibility is that post-translational processing of PER in glial cells renders it undetectable by the antibodies we used.

Relevance of multiple PER-ir neurons in the cricket brain

We suggest that PER immunoreactivity in the cricket brain is a "signature label" that identifies the neurons connected in a network that controls circadian behavioral output. PER immunoreactivity in axons and terminals of many neurons had only been shown before in beetles (Frisch et al. 1996). There is no obvious functional

explanation for this phenomenon. The core of the circadian oscillator may be the four to six neurons of the AMe that are double-labeled by anti-PDF and anti-PER. A possible explanation for the occurrence of PER in the other neurons described here is to distribute circadian timing, both to control motor output and also to synchronize peripheral oscillators (Plautz et al. 1997; Giebultowicz 2000), and to modulate sensory input (Krishnan et al. 1999). PER is unlikely to act as transmitter or secreted peptide, but it might modulate release of these substances. A second possible role of PER in central neurons is to generate circadian rhythmicity in the absence of the optic lobe pacemakers. In *Drosophila*, there is evidence that the central neurons may not only distribute circadian oscillations but also contribute to control rhythmic behavior (Kaneko and Hall 2000). Results from Rence and Loher (1975) suggest a similar role of central neurons in *T. commodus*. With both optic lobes removed *T. commodus* males can be entrained by 12 h:12 h temperature cycles to display circadian calling for a few cycles.

In summary, our results suggest that the PER network may exert its effects on three different levels: (1) via fast neuronal connections inside the brain and down the connectives, for driving motor activities, (2) via paracrine release to modulate both sensory input and motor output, and (3) via hormonal outputs to synchronize other rhythmic outputs of non-neuronal tissue.

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