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# The cycling and distribution of PER-like antigen in relation to neurons recognized by the antisera to PTTH and EH in *Thermobia domestica*

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**Abstract**

The cephalic nervous system of the firebrat contains antigens recognized by antisera to the clock protein period (PER), the prothoracicotropic hormone (PTTH) and the eclosion hormone (EH). The content of the 115 kDa PER-like antigen visualized on the western blots fluctuates in diurnal rhythm with a maximum in the night. The oscillations entrained in a 12:12 h light/dark (LD) cycle persist in the darkness and disappear in continuous light. They are detected by immunostaining in 14 pairs of the protocerebral neurons and are extreme in four suboesophageal neurons and two cells in each corpus cardiacum that contain PER only during the night phase. No circadian fluctuations occur in three lightly stained perikarya of the optic lobe. Five cell bodies located in each brain hemisphere between the deuto-and the tritocerebrum retain weak immunoreactivity under constant illumination. In all cells, the staining is confined to the cytoplasm and never occurs in the cell nuclei. The cells containing PER-like material do not react with the anti-PTTH and anti-EH antisera, which recognize antigens of about 50 and 20 kDa, respectively. The anti-PTTH antiserum stains in each brain hemisphere seven neurons in the protocerebrum, eight in the optic lobe, and 3–5 in the posterior region of the deutocerebrum. The antiserum to EH reacts in each hemisphere with just two cells located medially to the mushroom bodies. No cycling of the PTTH-like and EH-like antigens was detected.


*Keywords:* Circadian clock; Period; PTTH; Eclosion hormone; Neurosecretion; *Thermobia*

## 1. Introduction

Most processes in eukaryotic organisms exhibit circadian rhythms that are characterized by cyclicity with a period close to 24 h and persistence in constant darkness, temperature compensation within the physiological temperature range, and entrainment to environmental stimuli such as daily changes of light intensity (Eskin, 1979). The isolation of *period* (*per*) mutants in *Drosophila melanogaster* (Konopka and Benzer, 1971) revealed that a single gene controls the circadian behavior in adult eclosion and locomotory activity. The fruit fly *per* gene was cloned in 1984 (Bargiello and Young, 1984; Reddy et al., 1984) and later found to be expressed in dozens of neurons and hundreds of glial cells in the brain of *D. melanogaster* (Liu et al., 1988; Siwicki et al., 1988). The PER protein contents in the brain neurons, photoreceptors, and many peripheral tissues showed prominent daily fluctuations (Zerr et al., 1990). PER was detected in the cytoplasm during early night and in the nuclei late at night and early in the day. The levels of *per* mRNA also cycled, reaching a peak several hours earlier than the PER protein rose to its maximum (Hardin et al., 1990). Further studies in the fruit fly led to cloning of several additional clock genes and to the formulation of a paradigm explaining the cycling mechanism (reviewed by Giebultowicz, 2000; Williams and Sehgal, 2001). The diurnal changes in *per* expression were found to depend on the translocation of PER from cytoplasm into the nucleus.

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The first ortholog of *per* gene in an insect other than flies was cloned from the giant silkmoth *Antheraea pernyi* (Reppert et al., 1994). Similar to the fruit fly, PER protein was found to cycle in the silkmoth eyes with pronounced nuclear translocation in the photoreceptor cells. Unlike the fruit fly brain, however, PER was expressed only in eight large neurosecretory cells in the central brain of the silkmoth and was confined to their cytoplasm, never being detected in the nuclei (Sauman and Reppert, 1996a). Since the cells also contained an anti-*per* RNA, it was suggested that its interaction with *per* mRNA regulates the oscillations of PER at translation level. However, a later study made this explanation unlikely by demonstrating that the anti-*per* transcript occurs only in silkmoth females (Gotter et al., 1999). The molecular clock mechanism operating in the brain of *A. pernyi* is certainly different from that in *D. melanogaster* but its gear remains to be elucidated.

Examinations performed on the representatives of insect orders Blattaria (Reppert et al., 1994), Heteroptera (Syrová and Sauman, unpublished), Diptera (cf. Piccin et al., 2000), Lepidoptera (Reppert et al., 1994), and Hymenoptera (Toma et al., 2000) proved that the *per* gene is highly conserved. However, the distribution of cells that express the gene, the rhythm of expression, and the regulation of this rhythm are species specific. For example, the contents of *per* mRNA and the PER protein cycle in a diurnal rhythm in the photoreceptor cells of *D. melanogaster* and *A. pernyi* (Siwicki et al., 1988; Sauman and Reppert, 1996a), but are maintained at high levels throughout the light/dark cycle in the photoreceptor cells of the hawkmoth *Manduca sexta* (Wise et al., 2002). *D. melanogaster* and *M. sexta* exhibit PER cycling and nuclear translocation in various glial cells (Ewer et al., 1992; Wise et al., 2002). PER localization in the glial cells was also reported for the cricket *Teleogryllus commodus* (Honegger et al., 1991) and the beetle *Pachymorpha sexguttata* (Frisch et al., 1996), but was not found in *A. pernyi* (Sauman and Reppert, 1996a). The cytoplasm/nuclear PER cycling in certain neurons at the base of optic lobes, which is typical for *D. melanogaster* (Siwicki et al., 1988), has not been detected in other insects. On the other hand, *Drosophila* lacks PER expression in the neurosecretory protocerebral cells that were identified in *T. commodus* (Honegger et al., 1991), *P. sexguttata* (Frisch et al., 1996), *A. pernyi* (Sauman and Reppert, 1996a), and *M. sexta* (Wise et al., 2002).

Complex organisms possess a central circadian clock that includes an input transduction pathway transmitting the environmental and other signals to the core oscillator, and one or more output pathways that relay the circadian pace of the oscillator to the effectors within and outside the cell (Carré and Kay, 1996). In insects, the output pathways must entail hormonal control over the molting process and especially its terminal phase, the ecdysis, which is often associated with a particular time of the day (Truman, 1972). The molting process is initiated by increased titer of ecdysteroids secreted from the prothoracic glands or their homologues (Gilbert and King, 1973). The glands are regulated by brain neurohormones, of which the prothoracicotropic hormone (PTTH) of Lepidoptera received most attention. PTTH is derived from just four brain cells and triggers ecdysteroid secretion from the prothoracic glands (Agui et al., 1979). The structure of PTTH was first elucidated in *Bombyx mori* (Kawakami et al., 1990). The antisera to *B. mori* PTTH were found to react with homologous neurons in other lepidopterans such as *M. sexta* (Dai et al., 1994) and this encouraged search for PTTH orthologs. They were identified in the silkmoths *Samia cynthia ricini* (Kataoka and Suzuki, 1995), *A. pernyi* (Sauman and Reppert, 1996b), and *Hyalophora cecropia* (Sehnal et al., 2002), and in the hawkmoth *M. sexta* (Shionoya et al., 2003). In *M. sexta*, it has been shown that the mode of action of recombinant PTTH corresponds to the prothoracicotropic effects of the brain extracts, proving that the isolated PTTH is the genuine stimulator of the prothoracic glands (Gilbert et al., 2000).

The terminal step of the molting process, the ecdysis, is associated with specific behavior that is controlled by a hormonal cascade terminated with the eclosion hormone, EH (Gammie and Truman, 1999). Nearly identical EH structures were found in the lepidopterans *M. sexta* (Kataoka et al., 1987; Marti et al., 1987) and *B. mori* (Kono et al., 1987), and 58% identical EH ortholog in *D. melanogaster* (Horodyski et al., 1993). Two pairs of ventrolateral protocerebral neurons were identified as the source of EH in several lepidopterans (Kono et al., 1990; Hewes and Truman, 1991; Naya et al., 1994) but in *A. pernyi*, EH was immunocytochemically localized in the medial part of the protocerebrum (Sauman and Reppert, 1996a).

Consistently with the diurnal rhythm of old cuticle shedding, the secretions of PTTH in *B. mori* (Mizoguchi et al., 2002), of an unidentified prothoracicotropin in the kissing bug *Rhodnius prolixus* (Vafopoulou and Steel, 2001), and of the EH in *M. sexta* (Reynolds and Truman, 1983), exhibit distinct circadian fluctuations. It is likely that such fluctuations are linked to the central brain clock residing in the PER-expressing neurons. The proximity of PER-containing axons to the PTTH-secreting cells in *A. pernyi* (Sauman and Reppert, 1996b) and *M. sexta* (Wise et al., 2002) suggests that PER may regulate the production of neurohormones via nervous circuits.

All studies on the central circadian clock and its possible output pathway in the insects were done on Pterygota that diverged from their apterygote ancestors 400 million years ago (Labandeira, 1998). Firebrats (Zygentoma or Thysanura *sensu stricto*) are the apterygote sister group of pterygotes that retained the capacity of molting throughout the life. In the adult firebrats,

ecdysteroids and juvenile hormone orchestrate alternations of moltings with the mating and egg deposition (Rousset and Bitsch, 1993; Bitsch and Bitsch, 1988). The moltings occur in intervals of 10–12 days and are elicited by ecdysteroid peaks that also stimulate previtellogenesis in the ovaries. Vitellogenesis is accomplished in the post-ecdysial period at a low titer of ecdysteroids and a peak of juvenile hormone. The completion of egg formation is associated with a juvenile hormone decline that continues until the next ecdysis. Oviposition takes place in about middle of the intermolt period and is followed by ecdysteroid increase evoking the next molting process.

The locomotory activity of firebrats exhibits circadian oscillations with maxima at the beginning of the dark phase in a 12:12 h photoperiod cycle (Dolezal and Sauman, unpublished). Elucidation of the clock mechanism driving this diurnal rhythm would facilitate comprehension of the differences found in clock organization among the pterygote insects and would help us to distinguish basic clock properties from the apomorphies limited to certain pterygote clades. In this study, we present evidence for a cycling PER homologue in the firebrat and examine its nuclear/cytoplasm translocation. The precise synchronization of the molting and reproductive cycles implies existence of a central coordination of the hormone secretion in the firebrat. In pterygotes, PTTH is crucial for induction of the molting process and EH elicits the ecdysis. A detection of proteins with similar antigenic properties in the firebrat would provide a lead for finding possible ancestral molecules of these neurohormones. The pattern of locomotory activity indicates that the molting and egg-laying in the firebrat is linked to certain time of the day and this encouraged us to examine association of the PTTH- and EH-like antigens with the PER-expressing cells.

## 2. Materials and methods

### 2.1. Animals

Culture of the firebrat *Thermobia domestica* (Packard), which represents apterygote insects of the order Zygentoma, was maintained in constant darkness (Rohdendorf, 1966) but the adults selected for our analyses were kept under specific illumination conditions:

1. Twelve days at 12:12 LD cycle (12 h light alternating with 12 h darkness); brains were dissected in 4 h intervals on days 11 and 12.
2. Ten days in LD 12:12, after the last lights-off in constant darkness (DD) for 2 days, samples collected on the second DD day in 4 or 6 h intervals.
3. Ten days in LD 12:12, during lights-on transferred to constant light (LL), brains dissected at ZT4 and ZT16 on days 1, 3, 5, and 7, and in 4 h intervals on day 15 after the LL exposure.

The circadian or "zeitgeber" time (ZT) was counted from the lights-on moment (=ZT0). Dim red light of 660–670 nm was used when the insects were handled during the dark phase. All insects were kept at optimal temperature of 36 °C.

### 2.2. Antibodies

Polyclonal rabbit antibodies were used. The antiserum to *M. sexta* EH was obtained from J. Truman, University of Seattle. The remaining antisera came from our stock. The dilutions of antibodies for western blot analysis and immunocytochemistry are summarized in Table 1. As an additional control for binding specificity, the anti-PTTH antibody was preincubated with 100 M excess of the original antigen prior to immunological staining. No significant signal was observed above background. For more information about the anti-PTTH antibody production and affinity purification, see Sauman and Reppert (1996b).

### 2.3. Western blot analysis

Samples of 30 trimmed heads (the antennae, the mouth parts, and the scales were removed) of adult firebrats were homogenized in a single-detergent lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1.0% Nonidet P-40, 100 μg/ml PMSF, 2 μg/ml Aprotinin). The homogenate was centrifuged (12,000 *g*, 5 min at 4 °C) to eliminate cellular debris, and a 12 μl aliquot of the supernatant was taken for protein quantification with the Bradford assay (Bio-Rad). The remainder was mixed with equal volume of SDS-PAGE gel-loading buffer, boiled for 5 min, and loaded on 10% SDS—polyacrylamide gel. Equal amounts of total protein per lane were applied. High molecular weight standard mixture 30–200 kDa and prestained SDS-PAGE Standards 18–106 kDa (Sigma-Aldrich) were used to estimate the molecular weights of proteins. After electrophoresis, the proteins were transferred by semidry electroblotting onto a polyvinylidine difluoride membrane (Sigma-Aldrich). The strip with markers was stained with 0.1% Coomassie Blue R-250 in 50% methanol. The remaining membrane was wetted with distilled water, blocked for 1 h at room temperature (RT) with 5% nonfat dry milk in phosphate-buffered saline (PBS), and then subjected to immunostaining. Incubation with primary antibody (for dilutions in PBST, see Table 1) was carried out overnight at 4 °C under gentle agitation. After washing in PBST (3 × 10 min at RT), the membranes were incubated with goat anti-rabbit IgG-HRP-conjugated antibody (Jackson ImmunoResearch), which was diluted 1:10,000 in PBST, for 1 h at RT, and washed

Table 1
The antisera (all raised in rabbits) and their dilutions for cytochemistry and western blot analysis. See text for the species and antigen abbreviations

| Antigen | Antibody and author(s) | Antiserum dilution | |
|---|---|---|---|
| | | Cytochemistry | Western blots |
| *A. pernyi* PER | 57/10w; Sauman and Reppert, 1996a | 1:200 | 1:10,000 |
| *A. pernyi* PER | 58/10w; Sauman and Reppert, 1996a | 1:200 | 1:10,000 |
| *A. pernyi* PTTH | 274/IV-A; Sauman and Reppert, 1996b | 1:500 | 1:10,000 |
| *M. sexta* EH | J. Truman, University of Seattle | 1:2000 | 1:20,000 |

five times for 10 min with PBST. The enzymatic activity of horseradish peroxidase (HRP) was visualized by chemiluminiscent reaction using ECL western blotting detection reagent (Bio-Rad) and X-ray film. Relative amount of the antigen was assessed from the intensity of immunostaining.

### 2.4. Immunocytochemistry

The brain-suboesophageal ganglion complex was dissected in sterile insect saline and immediately fixed in Bouin–Hollande solution without acetic acid but supplemented with 0.7% mercuric chloride (Watson et al., 1993). Overnight fixation at 4 °C was followed by dehydration in ethanol series (three times 30 min in 70%, twice 30 min in 95%, and twice 20 min in absolute ethanol), saturation with chloroform (twice 20 min), and embedding in paraplast. Sections 4–10 μm thick attached to microscopic slides were deparaffinized in xylene (twice 10 min) and brought to distilled water through an ethanol series. The slides were treated with Lugol's iodine followed by 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). To prevent unspecific immunoreaction, the sections were bathed in 10% normal goat serum in PBST (30 min at RT) prior to overnight incubation with primary antibody (diluted appropriately in PBST) in a humidified chamber at 4 °C. After rinsing with PBST (three times 10 min at RT), the samples were incubated for 1 h at RT with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, diluted 1:1000 in PBST). Following washings in PBST (three times 10 min at RT) and once in 0.05 M Tris–HCL (pH 7.4, 10 min at RT), the HRP enzymatic activity was visualized with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M Tris–HCl, pH 7.4). Stained sections were dehydrated and mounted in DPX—mounting medium (Fluka). The mounted sections were viewed and photographed under Zeiss Axioplane 2 microscope equipped with Nomarski (DIC) optics and CCD camera.

The levels of staining were subjectively scored in randomized slides with an intensity scale ranging from + (traces) to ++++ (maximum staining), absence of immunoreactivity was marked as –. Ten animals were examined and evaluated at each time point. Preimmune sera, and in the case of PTTH also antisera saturated with 100 M excess of the respective antigen, were used in the control reactions that were always negative.

## 3. Results

### 3.1. Antisera to PER recognize a specific protein that undergoes circadian fluctuations

Both antisera to PER stained specifically a protein of about 115 kDa on the western blots of protein extracts prepared from the firebrat heads (Fig. 1). The expression level of this protein at different times was examined with the antiserum 57/10w. In insects kept in a 12:12 LD cycle, the content of the PER-like protein fluctuated in diurnal rhythm, being maximal during scotophase and minimal during the photophase (Fig. 1, LD). The fluctuations were maintained with the same periodicity in firebrats transferred to constant darkness (Fig. 1, DD). It should be mentioned that such a persistence of circadian oscillations upon transfer to constant darkness is one of the features characterizing biological clocks.

Western analysis of the brain-suboesophageal ganglion extracts was also performed at ZT4 and ZT16 in insects kept for 8, 11, 13, and 15 days in constant light. The results demonstrated dramatic suppression of the 115 kDa antigen (Fig. 1, LL). The circadian oscillations were abolished and the protein became undetectable.

### 3.2. PER-like immunoreactivity in specific neurons

Both antisera to PER reacted with about 50 neurons in the brain-suboesophageal ganglion complex of the firebrat (Fig. 2A,H). No differences were found between males and females. One superior and one more internal pair of large neurons were stained in the *pars intercerebralis* (Fig. 2B). Short but distinct axonal projections could be traced from these cells. Each hemisphere further contained three cells in the dorsolateral protocer-

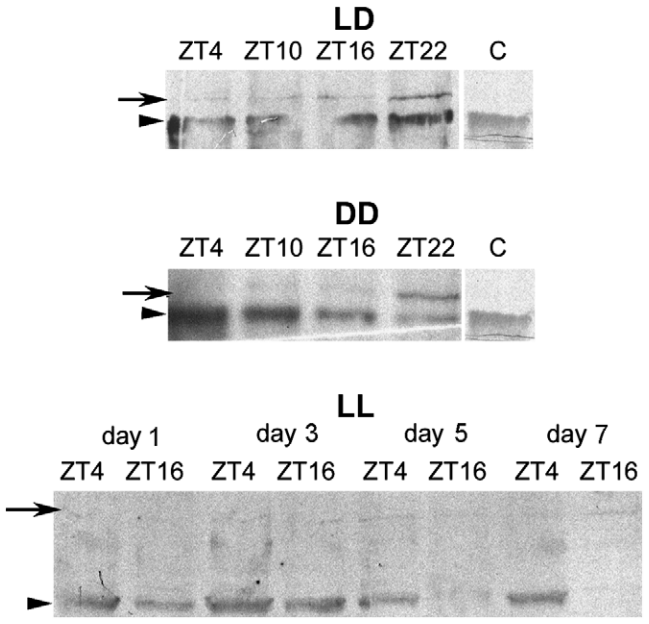


Fig. 1. Western blot analysis of the PER-like antigen in the head extracts of firebrats kept under 12:12 h light/dark cycle (LD) for 12 days or transferred on day 10 either to constant darkness (DD, analyses 1–2 days later) or constant light (LL, analyses on indicated days). Antiserum 57/10w was used and the times of analyses (ZT) are given in hours after the actual or the entrained lights-on point. Band C shows an aliquot of the ZT22 sample stained with the secondary antiserum alone. Arrows indicate the specific PER-immunoreactive band corresponding to about 115 kDa, and arrowheads mark a band reacting non-specifically with the secondary antiserum.

ebrum (Fig. 2C) and three weakly stained cells in deep optic lobe layer between the lobula and the medulla. About 14 small cells occurred at the base of the protocerebrum (Fig. 2D) and five larger cells were located at the border between the deuto- and the tritocerebrum (Fig. 2E). At specific zeitgeber or circadian times (see below), four PER-immunoreactive cells could be detected in the posterior-ventral region of the suboesophageal ganglion (SOG; Fig. 2F) and two cells in each corpus cardiacum (Fig. 2G). In all these cells, PER staining was confined to the cytoplasm and was never detected in the nuclei.

Dispersed staining indicative of fine fibers occurred in the dorsolateral protocerebrum and between the medulla and the lamina of the optic lobes. A narrow band of fibers seemed to traverse protocerebrum at the level of the central body, and another band seemed to run under the lateral brain surface into a distinctly stained fiber arborization in the suboesophageal ganglion.

### *3.3. Immunocytochemical data confirm cycling of the cytoplasmic PER-like antigen*

PER immunoreactivity was examined in insects kept in the 12:12 h light/dark (LD) cycle. Ten animals were analyzed every 4 h around the clock. The intensity of PER-staining was found to undergo profound circadian fluctuations, being consistently higher during the night than during the light period (Table 2; Fig. 2A,H). The most notable changes occurred in specific neurons located in the pars intercerebralis (Fig. 2Ba,Bc), the dorsolateral protocerebrum (Fig. 2Ca,Cc), and the base of protocerebrum (Fig. 2Da,Dc). The intensity of staining in these perikarya was weak (+) to moderate (++) at ZT0–ZT8, considerable (+++) at ZT12 and ZT20, and strong (++++) at ZT16. The oscillations of staining intensity in axonal projections correlated with those in the corresponding perikarya. No circadian oscillation was detected in the staining of PER-positive cells between the deuto- and the tritocerebrum. Three cells in each optic lobe were also weakly stained at all time points (Table 2). The PER-positive cells in SOG and the corpora cardiaca were consistently detectable at ZT16, occasionally also at ZT12 and ZT20 (Table 2; Fig. 2Fc,Gc) but no PER labeling was detected in these cells during the light period of the day (Table 2 Fig. 2Fa,Ga). Fine arborization of stained fibers was observed in the SOG throughout the LD cycle (Fig. 2Fa,Fc).

### *3.4. The cytoplasmic oscillations of PER-like antigen are free-running*

To verify the endogenous character of the detected daily rhythm in PER expression, we examined PER immunoreactivity in 4 h intervals in a DD (constant darkness) 24 h cycle. We found that the neuron-specific patterns of circadian changes in the staining intensity were maintained (Table 3). Prominent oscillations continued in the neurons of protocerebrum and in the SOG (Fig. 2A,H), with the same phase and amplitude during subjective day and subjective night as under the preceding LD conditions (cf. Table 2). Staining intensities of the PER-positive perikarya in the pars intercerebralis (Fig. 2Bb,Bd), the dorsolateral protocerebrum (Fig. 2Cb,Cd), and at the base of protocerebrum (Fig. 2Db,Dd) were weak to moderate at ZT0–ZT8 and strong from ZT12 to ZT20. The staining was maximal at ZT16. Also similar to the LD conditions, the intensity of axonal staining within the central brain correlated with oscillations in the cytoplasmic PER staining in the corresponding perikarya. The PER-positive cells in the deuto- and the tritocerebrum and in the optic lobes did not exhibit any significant oscillations in their immunostaining (Fig. 2Eb,Ed). The group of PER-positive cells in the SOG and the pair of cells in each corpus cardiacum were detected only during the subjective night, and showed maximal staining at ZT20 (Table 3; Fig. 2Fd,2Gd). No PER-immunoreactive cells were found in the SOG and in the corpora cardiaca during the subjective day (Table 3; Fig. 2Fb,Gb).

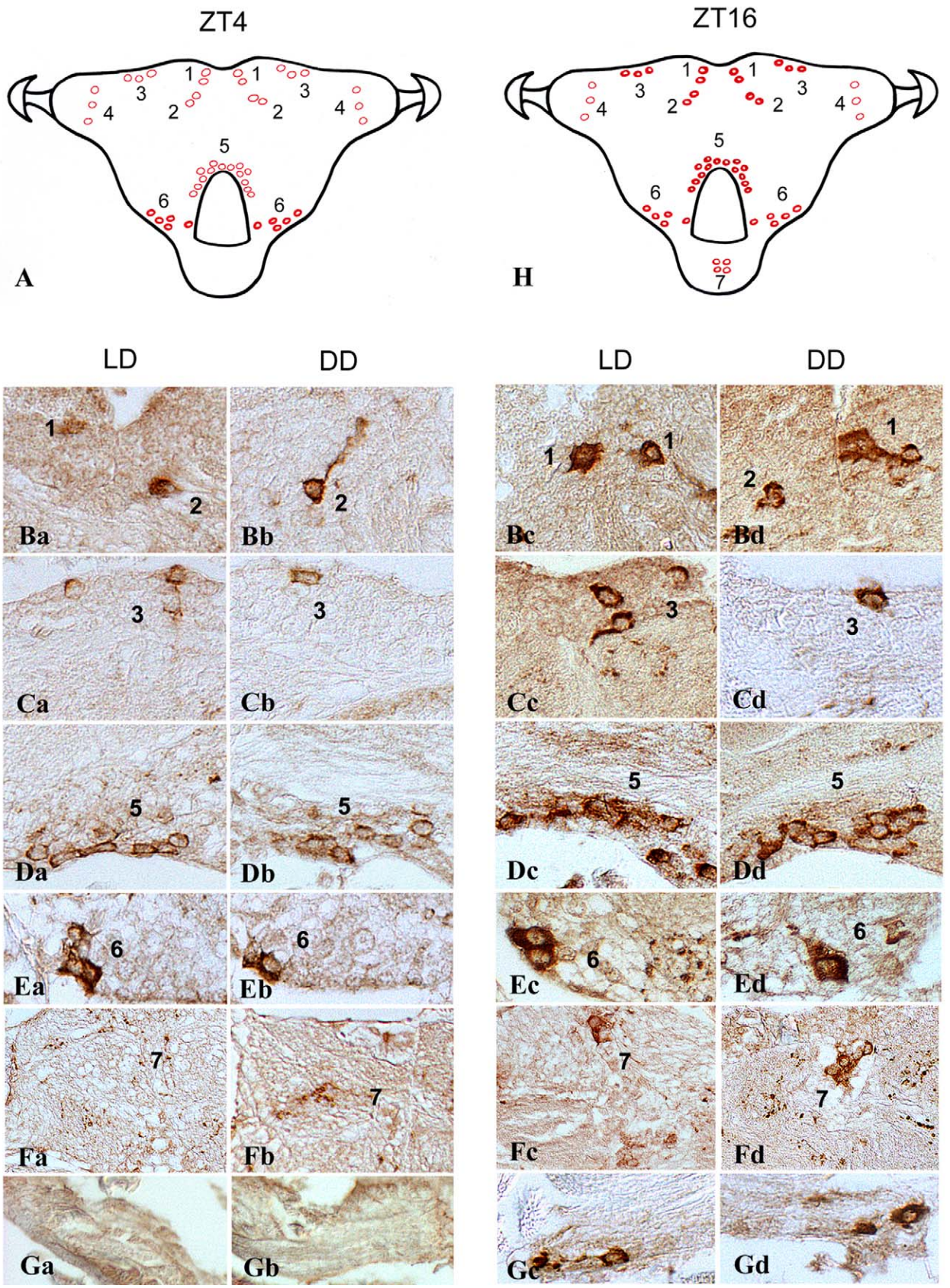
ZT4
ZT16
A
H
LD
DD
LD
DD
Ba
Bb
Bc
Bd
Ca
Cb
Cc
Cd
Da
Db
Dc
Dd
Ea
Eb
Ec
Ed
Fa
Fb
Fc
Fd
Ga
Gb
Gc
Gd

Table 2
Numbers of perikarya and the intensities of their staining with antibody to PER in the firebrats kept under 12:12 h light/dark cycle for 12 days

| Perikarya location | *C* | ZT0 | ZT4 | ZT8 | ZT12 | ZT16 | ZT20 |
|---|---|---|---|---|---|---|---|
| Pars intercerebralis | 4 | + | ++ | ++ | +++ | ++++ | +++ |
| Lateral protocerebrum | 3 | ++ | ++ | ++ | +++ | ++++ | +++ |
| Base of protocerebrum | 7 | ++ | ++ | ++ | +++ | ++++ | +++ |
| Deuto-/tritocerebrum | 5 | +++ | +++ | +++ | +++ | +++ | +++ |
| Optic lobe | 3 | + | + | + | + | + | + |
| Suboesophageal ganglion | 2 | − | − | − | + | +++ | ++ |
| Corpus cardiacum | 2 | − | − | − | ++ | +++ | + |

Circadian zeitgeber time (ZT) is given in hours after lights-on (analysis was done in 4 h intervals). *C* indicates number of pairs of the immunostained neurons. Their staining intensity was quantified subjectively as absent (−), weak (+), moderate (++), considerable (+++), and strong (++++).

Table 3
Numbers of perikarya and the intensities of their staining with antibody to PER in the firebrats transferred from the 12:12 h light/dark cycle to constant darkness on day 10 and analyzed 1–2 days later

| Perikarya location | *C* | ZT0 | ZT4 | ZT8 | ZT12 | ZT16 | ZT20 |
|---|---|---|---|---|---|---|---|
| Pars intercerebralis | 4 | + | ++ | ++ | +++ | ++++ | +++ |
| Lateral protocerebrum | 3 | ++ | ++ | ++ | +++ | ++++ | +++ |
| Base of protocerebrum | 7 | ++ | ++ | ++ | +++ | ++++ | +++ |
| Deuto-/tritocerebrum | 5 | +++ | +++ | +++ | +++ | +++ | +++ |
| Optic lobe | 3 | + | + | + | + | + | + |
| Suboesophageal ganglion | 2 | − | − | − | + | ++ | +++ |
| Corpus cardiacum | 2 | − | − | − | + | ++ | +++ |

For explanation, see Table 2.

### 3.5. The cytoplasmic oscillations of PER-like antigen are suppressed by constant light

Immunocytochemical search for PER-like material was also done in the firebrats exposed to constant light for 2 weeks (Fig. 3A). Very few and only slightly stained cells were found (Table 4). No PER-like material was detected in the protocerebrum (Fig. 3B–D), the SOG (Fig. 3F), and the corpora cardiaca in any of the time points. Weak cytoplasmic staining persisted in a group of cells at the border between the deuto- and the tritocerebrum (Fig. 3E). Dispersed staining indicative of fine fibers also occurred in this region around the clock.

Fig. 2. The localization of cells reacting with the 57/10w antiserum. Insects were kept in a 12:12 h light/dark cycle (LD) for 12 days or transferred to constant darkness (DD) on day 10 and analyzed 1–2 days later at 4 h (ZT4) and 16 h (ZT16) after the real or subjective lights-on point. Schematic diagrams (A, H) show the positions and the maximal staining intensities (indicated by the thickness of drawing lines) of the PER-positive perikarya located in pars intercerebralis (1), deeper protocerebral region (2), lateral protocerebrum (3), optic lobe region between the lobula and the medulla (4), basal protocerebrum (5), between the deuto- and the tritocerebrum (6) and in the suboesophageal ganglion (7). Photos of the immunopositive cells (numbered as in diagrams A and H) are shown in sections through pars intercerebralis (B), dorsolateral protocerebrum (C), the base of protocerebrum (D), region between the deuto- and the tritocerebrum (E), suboesophageal ganglion (F), and corpus cardiacum (G). Magnification B–G, 160×.

### 3.6. The PTTH- and EH-like immunoreactivities

The anti-PTTH-antibody stained about 35 perikarya in the firebrat brain (Fig. 3G). In each hemisphere, two cells were detected in a deep layer of the dorsolateral region (Fig. 3H), about five cells at the base of protocerebrum (Fig. 3K), and 3–5 cells posteriorly at the border between the deuto- and the tritocerebrum (Fig. 3L). Four PTTH-positive cells occurred ventroanteriorly at the base of each optic lobe (Fig. 3I) and four cells dorsoanteriorly of the lobula (Fig. 3J). No PTTH-positive axons were detected and only dispersed staining indicated connections between the left and the right protocerebral groups of PTTH-positive neurons. The two groups of PTTH-positive cells in the optic lobe seemed to be linked with one another and also with the PTTH-positive neurons in the ventral and ventroposterior brain regions. No significant staining of any cell or fiber was detected with the antibody that had been preincubated with 100 M excess of the original PTTH antigen.

The antibody to EH stained just two cells located anteriorly to the corpora pedunculata in each brain hemisphere (Fig. 3G,M). The EH-like antigen was confined

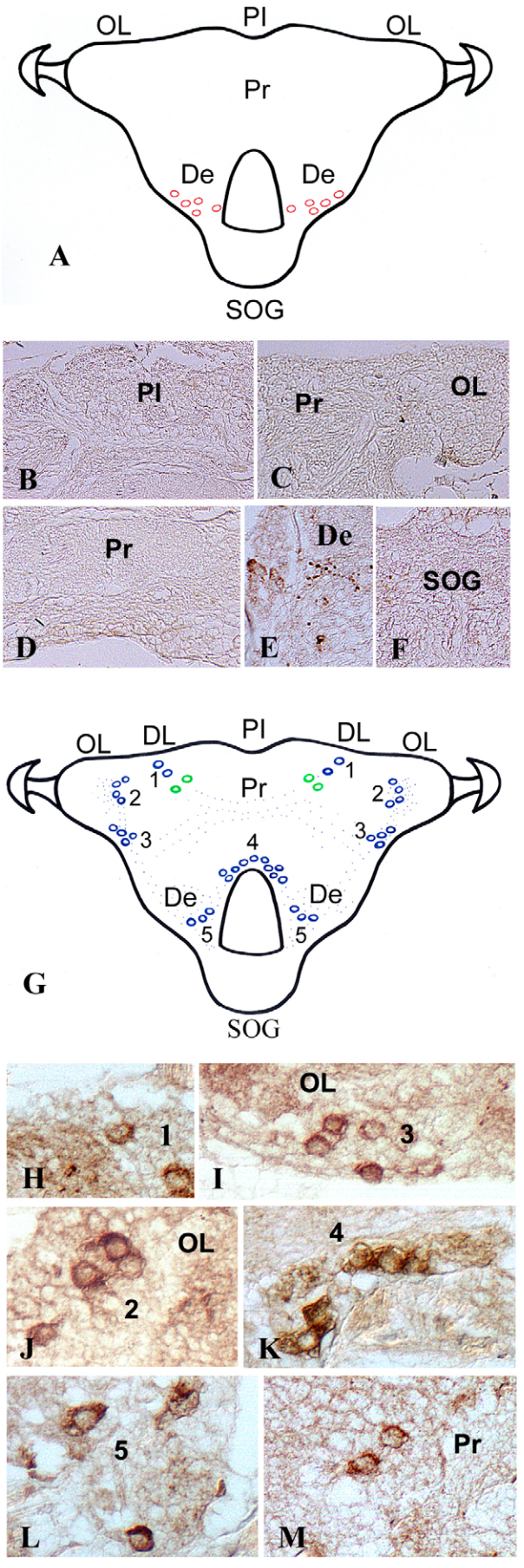


Fig. 3. PER-, PTTH-, and EH-immunoreactivities in the brain-suboesophageal complex of firebrats. Schematic diagram (A) illustrates the topography of cells stained with the PER-antiserum in firebrats kept in constant light for 2 weeks. The photographs demonstrate absence of PER-immunostaining in pars intercerebralis (B), dorsolateral protocerebrum and the base of optic lobe (C), basal protocerebrum (D), and suboesophageal ganglion (F). Slight immunoreactivity occurred only in the cells between the deuto- and the tritocerebrum (E). Schematic diagram (G) shows the topography of cells containing PTTH-like (blue) and EH-like (green) antigens. The PTTH staining occurred in specific cells in the lateral protocerebrum (group 1), anterio-dorsal (2) and anterio-ventral (3) regions of the optic lobe, basal part of the protocerebrum (4), and at border between the deuto- and the tritocerebrum (5). The EH staining was confined to two cells shown in green located anteriorly to the mushroom bodies. The photographs show PTTH-positive cells in the dorsolateral protocerebrum (H), the anterio-ventral (I) and anterio-dorsal (J) regions of the optic lobe, the base of protocerebrum (K), and at the border between the deuto- and the tritocerebrum (L). The EH-positive cells in the lateral protocerebrum are shown in photo (M). Magnification B–F, 80×; G–M, 160×. Abbreviations: De, deutocerebrum; DL, dorsolateral protocerebrum; OL, optic lobe; PI, pars intercerebralis; PR, protocerebrum; SOG, suboesophageal ganglion.

to the cytoplasm of these cells and no staining was found outside the perikarya. We did not have EH antigen to saturate the antibody for verifying the specificity of immunostaining. However, its intensity and sharp localization, and the detection of a single antigen on the western blots suggest that it is not coincidental.

The PTTH-like and EH-like antigens were characterized and their expressions at two time points assessed with the aid of western blot analysis of the brain-suboesophageal ganglion extracts. The tissues were dissected at ZT4 and ZT16 from firebrats kept under 12:12 LD conditions. A distinct band of apparent molecular weight about 50 kDa was detected with the anti-PTTH antibody at both times (Fig. 4). A clear signal was also obtained with the anti-EH antibody that recognized a band in the region of the 20 kDa marker (Fig. 4). Staining intensity of the band was similar at the two time points.

## 4. Discussion

Full PER sequence was established in several insect species including the fruit fly *D. melanogaster* (Citri et al., 1987) and a few related flies, the cockroach *Periplaneta americana* (Reppert et al., 1994), the silkmoth *Antheraea pernyi* (Reppert et al., 1994), and the honeybee *Apis mellifera* (Toma et al., 2000). Partial sequencing of the *per* gene in several other species confirmed that all PER orthologs contain six conserved domains (Huang et al., 1993). The anti-PER antisera used in our study (57/10w and 58/10w) were directed against a tetradecapeptide corresponding to the “peptide S” region in the second conserved domain of *D. melanogaster* PER (Sauman and Reppert, 1996a). The amino acid sequence of this short part of PER orthologs

Table 4
Numbers of perikarya and the intensities of their staining with antibody to PER in the firebrats transferred from the 12:12 h light/dark cycle to continuous light on day 10 and analyzed 15 days later

| Perikarya location | *C* | ZT0 | ZT4 | ZT8 | ZT12 | ZT16 | ZT20 |
|---|---|---|---|---|---|---|---|
| Pars intercerebralis | 4 | – | – | – | – | – | – |
| Lateral protocerebrum | 3 | – | – | – | – | – | – |
| Base of protocerebrum | 7 | – | – | – | – | – | – |
| Deuto-/tritocerebrum | 5 | + | + | + | + | + | + |
| Optic lobe | 3 | – | – | – | – | – | – |
| Suboesophageal ganglion | 2 | – | – | – | – | – | – |
| Corpus cardiacum | 2 | – | – | – | – | – | – |

For explanation, see Table 2.

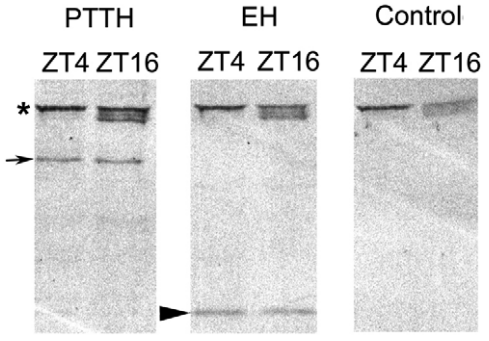


Fig. 4. Detection of PTTH-like and EH-like antigens on the western blots of proteins extracted at 4 h (ZT4) and 16 h (ZT16) after lights-on from the firebrats kept under a 12:12 h photoperiod for 12 days. The anti-PTTH antiserum recognized a single specific band of about 50 kDa (arrow) and the anti-EH antiserum a band of about 20 kDa (arrowhead) at both time points. Staining of one or two high molecular components (asterisk) was non-specific, as revealed by analyses performed without the primary antisera (Control).

of the fruit fly, cockroach, silkmoth, and the honeybee is 97% identical. The specificity of the 57/10w and 58/10w antisera was confirmed in the tests on *A. pernyi* (Sauman and Reppert, 1996a), *P. americana* (Sehadová, personal communication), and the linden bug *Pyrrhocoris apterus* (Syrová and Sauman, unpublished). In all these species, the antisera reacted exclusively in neurons in which expression of the endogenous *per* gene was proved by in situ hybridization.

The western blot analysis of the firebrat head extracts disclosed PER-immunoreactivity in a protein band of about 115 kDa (Fig. 1). This size is consistent with the 95–135 kDa range of PER orthologs as deduced from the *per* cDNAs identified in different insect species (Citri et al., 1987; Reppert et al., 1994; Toma et al., 2000). The immunocytochemical examinations revealed PER protein expression in about 50 cells that are located in the central brain, the optic lobes, the suboesophageal ganglion, and the corpora cardiaca of the firebrat (Fig. 2). The total number of PER-positive cells found in *T. domestica* is considerably lower than in the cephalic ganglia of *D. melanogaster* (Siwicki et al., 1988; Ewer et al., 1992). It is higher, however, than in most insects which typically contain from 6 to about 60 PER-immunopositive cells in each half of the brain-suboesophageal ganglion complex (Závodská et al., 2003). Some PER-positive cells are located in the dorsal protocerebrum in nearly all species, and location in the optic lobes is also common (Závodská et al., 2003). The occurrence of PER-positive neurons in other parts of the cephalic nervous system, as seen in *T. domestica*, is rather unusual.

The firebrat PER protein exhibited circadian oscillation in its abundance, which is a basic property of true clock elements (Aronson et al., 1994; Hall, 1995), suggesting that the 115 kDa protein is a genuine PER ortholog. In the 12:12 h LD cycle, the PER levels were undetectable or low during the light period and increased to a peak during the night (Fig. 1). These oscillations in the abundance of the PER protein were confirmed by immunocytochemistry (Fig. 2). They were clearly seen in the protocerebral neurons and were extreme in the neurons of the suboesophageal ganglion and the corpora cardiaca, in which PER was detectable only in the night period (Table 2). The daily pattern of PER oscillations in the protocerebral neurons of *T.domestica* is similar to that described for the eight PER-positive protocerebral neurons in the silkmoth *A. pernyi* (Sauman and Reppert, 1996a). The complete disappearance of the PER antigen in the corpora cardiaca and the suboesophageal ganglion of *T. domestica* during the light phase may have a counterpart in the honeybee *Apis mellifera*, in which certain protocerebral neurons are visualized with the anti-PER antiserum only under certain conditions (G. Bloch, personal communication).

It has been well established that the entrained circadian rhythms persist in constant darkness, whereas continuous light disrupts oscillations of the molecular components of the circadian clock and the overt circadian rhythm is abolished (Dunlap, 1999). Consistently with this general rule, the circadian oscillation of the firebrat PER protein continued in constant darkness (Table 3; columns DD in Fig. 2), but no PER protein was detectable in the firebrats kept under constant light

(Fig. 1). Slight immunostaining persisted in the non-cycling cells located between the deuto- and the tritocerebrum but was lacking in all other neurons (Fig. 3; Table 4).

A model of the molecular clock mechanism was elaborated for *D. melanogaster* (reviewed by Giebultowicz, 2000; Williams and Sehgal, 2001). It postulates PER binding to another clock protein called timeless (TIM), and translocation of the resulting dimer into the cell nucleus. For several cell types in *D. melanogaster* and for the photoreceptor cells in *A. pernyi*, it has been shown that the circadian translocation of PER from the cytoplasm to the nucleus is associated with circadian changes in the intensity of the cytoplasmic PER staining. These circadian changes are free-running in the darkness and disappear in continuous light. The 115 kDa PER-like protein of *T. domestica* exhibits diurnal oscillations in its cytoplasmic contents but it was never found in the cell nuclei. Similar cytoplasmic oscillations without a nuclear translocation of PER were detected in the protocerebral neurons of the silkmoth *A. pernyi* (Sauman and Reppert, 1996a), the hornworm *Manduca sexta* (Wise et al., 2002), the cockroach *P. americana* (Sehadová, personal communication), and the bug *P. apterus* (Syrová and Sauman, unpublished). There are two possible explanations for the lack of nuclear PER staining in these insects. The PER binding to a partner protein in the nucleus prevents PER recognition by antibodies such as 57/10w and 58/10w(1), or the PER never moves into the nucleus and the clock motion is based on a very different molecular mechanism than in *Drosophila* (2). We cannot distinguish between these two possibilities.

In addition to the perikarya, clear oscillations in the staining intensity were detected in the processes of the protocerebral PER-positive cells under both LD and DD conditions. Axonal transport of the PER protein to other brain regions and to the corpora cardiaca was demonstrated in *A. pernyi* (Sauman and Reppert, 1996a). Consistently with this observation, the PER-producing cells in *M. sexta* protocerebrum were identified as neurosecretory cells of group $Ia_1$ that send axons to the ipsilateral corpora cardiaca (Wise et al., 2002). PER-positive fibers were also detected in the corpora cardiaca of the mayfly *Siphlonurus armatus* and the stonefly *Perla burmeisteriana* (Závodská et al., 2003). Strong PER-immunoreactivity was found in the corpora cardiaca of the cockroach *P. americana* (Sehadová, personal communication). These results suggest that PER may impose circadian regulation on the release of neurohormones that are liberated from the retrocerebral complex corpora cardiaca–corpora allata. The endogenous PER-positive cells in the corpora cardiaca, which have so far been found in the firebrat only, may play a similar role.

Consistent detection of a 50 kDa protein with the anti-PTTH antiserum and of a 20 kDa protein with the anti-EH antiserum suggests that both antisera recognize in *T. domestica* very specific antigens. Their uniqueness is emphasized by their localizations in just a few neurons. The anti-PTTH and anti-EH antisera react in small, separate sets of brain neurons in the representatives of many insect orders (Závodská et al., 2003). The PTTH-like immunoreactivity typically occurs in the dorsolateral protocerebrum, where two immunopositive cells were found in the firebrat. However, the firebrat seems to be unique by having some PTTH-immunoreactive cells at the base of optic lobes. The EH-immunopositive cells were detected in about two-thirds of the examined insect orders (Závodská et al., 2003). They are always low in number and in a few species are located in the dorsolateral protocerebrum as in the firebrat.

The antigens detectable in the firebrat with the anti-PTTH and anti-EH antisera probably bear structural similarities to the respective hormones but differ from them in size. The PTTH orthologs known from five lepidopteran species consist each of slightly more than 100 amino acids, and their size is about four-fold less than can be deduced from the apparent 50 kDa size of the PER-like antigen in *T. domestica*. Similarly, all known EH orthologs contain a little more than 60 amino acids whereas the EH-like antigen found in *T. domestica* seems to be about three times larger. These size differences should not be surprising in view of considerable structural diversification of PTTH just within Lepidoptera (see Shionoya et al., 2003; Sehnal et al., 2002) and of EH between the holometabolous orders Lepidoptera and Diptera (cf. Horodyski et al., 1993). We regard the detection of a PTTH-like and EH-like antigens in the firebrat not as an indication of the existence of hormone homologues in this species but as a cue to investigations on the phylogenic origin of these hormones. Our finding of an apparent PER ortholog in the firebrat confirms widespread occurrence of this clock component in the eukaryotes and suggests that PER translocation from cytoplasm to the nucleus, which has been demonstrated in certain cells of some Holometabola, evolved late in pterygote phylogeny.

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