Histochem Cell Biol (2005) 123: 407–418
DOI 10.1007/s00418-004-0728-3

ORIGINAL PAPER

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Light-dependent PER-like proteins in the cephalic ganglia of an apterygote and a pterygote insect species

Accepted: 27 October 2004 / Published online: 15 April 2005

Abstract Antibodies targeted to a highly conserved tetradecapeptide region of the pivotal biological clock protein PER detect in the firebrat *Thermobia domestica* a 115-kDa protein and in the cockroach *Periplaneta americana* a 110-kDa protein that are present in the cytoplasm of a small set of brain cells. A similar cytoplasmic reaction occurs with antisera to the whole PER protein of *Drosophila melanogaster*, but these antisera also react with numerous cell nuclei. On western blots, they detect an 80-kDa antigen in *T. domestica* and 70- and 80-kDa antigens in *P. americana*. No indication of antigen translocation between cell nuclei and cytoplasm was found. Nuclear staining is maintained at a high constant level in *T. domestica* held at a 12:12 h light:-dark photoperiod (LD) or in continuous light, but disappears rapidly in response to extended darkness. In *P. americana* under LD conditions, the number of immunoreactive nuclei and their staining intensity fluctuate in parallel, with maximal staining late in the day. The circadian changes are maintained in continuous light but all staining vanishes in continuous darkness. A 6-h light pulse in early night of an LD cycle induces maximal staining after about 10 h, suggesting that the effect of light on nuclear PER-like expression is indirect. The behaviour of nuclear antigens is opposite to that of the cytoplasmic PER-like proteins that persist in constant darkness and disappear in constant light. Under LD conditions, the cytoplasmic PER-like antigen cycles in *T. domestica* but remains at a steady level in *P. americana*. The sensitivity to photoregime suggests that both the nuclear and the cytoplasmic PER-like antigens are components of the biological clock.

Keywords Biological clock · Circadian rhythms · PER · *Periplaneta* · *Thermobia*

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Introduction

Various manifestations of life exhibit circadian rhythms that are synchronised with the diurnal environmental changes (Young and Kay 2001). The rhythms are controlled by endogenous clocks that run with a periodicity close to 24 h. The clock pace is entrained to daily alternation of light and darkness, is insensitive to temperature variations within physiological limits, and persists in constant darkness. Molecular clocks seem to be present in most cells and are synchronised by a master oscillator localised in the central nervous system (Giebultowicz 2000; Lowrey and Takahashi 2000).

Elucidation of the molecular mechanisms driving the clock was initiated by the discovery of *period* (*per*) mutants in the fruit fly *Drosophila melanogaster* (Konopka and Benzer 1971). PER protein was subsequently identified as a key cycling component of the circadian clocks in various animals (Dunlap 1999). The mechanism of PER cycling rests on a transcriptional feedback loop that includes at least seven proteins and is stabilised by an additional oscillating loop linked to light perception (Stanewsky 2003). Circadian changes in the PER content and its translocation from cytoplasm to the nucleus are regarded as a crucial clock feature. The translocation was discovered in the brain of *D. melanogaster* (Liu et al. 1988; Siwicki et al. 1988), but could not be detected in the PER expressing neurons of other insects (Sauman and Hashimi 1999).

In the brain of the silkmoth *Antheraea pernyi*, *per* is expressed in each hemisphere in four neurons in which

PER amount cycles in the cytoplasm and is absent in the nuclei (Sauman and Reppert 1996). The neurons in question apparently represent the Ia_1 neurosecretory cells that were shown to contain a steady level of PER in both the cytoplasm and nuclei in another moth, *Manduca sexta* (Wise et al. 2002). In this species, PER was also detected in many other brain neurons and glial cells but exclusively in the nuclei. PER-like immunoreactivity in distinct clusters of neurons was found in a number of other insect species but always either in the cytoplasm or, rarely, in the cell nuclei, without translocation between these compartments (Závodská et al. 2003a and references therein).

In our investigations on the firebrat *Thermobia domestica* (Závodská et al. 2003b) we also detected clusters of neurons that contained PER-like immunoreactivity only in cytoplasm. The intensity of immunoreactivity fluctuated in circadian fashion, reaching a maximum in the night. The oscillations persisted in constant darkness, whereas continuous illumination caused a disappearance of the antigen. In the present paper we report that *T. domestica* contains in the brain and the suboesophageal ganglion additional PER-like antigen(s) that are located in the cell nuclei. We further show that the cockroach *Periplaneta americana* also expresses a cytoplasmic and a nuclear PER-like antigen(s) that are detectable with the same set of antibodies as the antigens in *T. domestica*. Our findings suggest that the cytoplasm/nuclear translocation of the PER-like proteins may be widespread in insects but it often escapes immunocytochemical detection because it is associated with a profound change of antigenic properties.

Materials and methods

Animals

Investigations were performed on adult firebrats *T. domestica*, representing apterygote insect order Zygentoma, and adult cockroaches *P. americana* from the pterygote insect order Blattaria. The insects came from our standard cultures reared at a 12:12 h photoperiod (12 h light and 12 h darkness) and species specific optimal temperatures 36 and 28°C, respectively. Experimental insects were kept under four different illumination regimes for 10 days at least. The time was measured from the lights-on point that is referred to as 0 Zeitgeber time (ZT) in animals exposed to a photoperiod, and circadian time (CT) in insects that had been entrained to lights-on at this time point and then kept in constant darkness or constant light. The dim red light of 660–670 nm wavelength was used when the insects were handled during the dark phase. All investigations were carried out minimally in nine replicas and included both sexes. The illumination cycles were as follows:

1. LD: 12 h light alternating with 12 h darkness, i.e. standard rearing conditions. Brains for semiquantitative immunocytochemical analysis were dissected at ZT 0, 4, 8, 12, 16 and 20. In *T. domestica*, dissections were done on days 11 and 12, and in *P. americana* on days 11 through 14. Western blot analysis was performed on day 11 at ZT 4, 10, 16 and 22 in *T. domestica*, and at ZT 4 in *P. americana*.
2. DD: Constant darkness ensuing after a night phase of the preceding LD cycles. Samples were collected during the first full DD cycle in *T. domestica*, for immunocytochemistry at 4-h intervals and for the western blotting at 6-h intervals. In the cockroach, immunocytochemical investigations were done at 4-h intervals for 3 days in DD.
3. LL: Constant light ensuing after a light phase of preceding LD cycles. The brains for immunocytochemistry were dissected from the firebrats every 4 h on day 15 and from the cockroaches every 4 h on day 9. The samples for western analysis were taken from the firebrat at CT 4 and CT 16 on days 1, 3, 5 and 7.
4. PS (phase shift, cockroaches only): 6 h illumination applied during the dark phase (from ZT 14 to ZT 20) of an entrained LD cycle, followed by a DD cycle during which brains were taken every 4 h for cytochemistry.

Antibodies

Available antibodies to PER were prepared with antigens derived from different insect species (Table 1). The antigens included highly conserved tetradecapeptide of the PER S-region, a stretch of about 350 amino acids

Table 1 Primary antibodies

Code	Antigen	Antibody type	Reference
PER-S	*Drosophila melanogaster* S-region KSSTETPPSYNQLN	Polyclonal, rabbit	Siwicki et al. (1988)
57/10w and 58/10w	*Antheraea pernyi* S-region KSSTETPLSYNQLN	Polyclonal, rabbit	Sauman and Reppert (1996)
PaPER	PAS + S region (340 amino acids) of *Periplaneta americana* PER	Polyclonal, rabbit	Sehadová et al. (2004)
5F7F6 and 10C3C9	PAS + S region (358 amino acids) of *Manduca sexta* PER	Monoclonal, mouse	Wise et al. (2002)
αPER	*D. melanogaster* full-length PER	Polyclonal, rabbit	Vosshall and Young (1995)

encompassing a PAS domain and the S-region, and the whole recombinant PER, respectively (for the structure of PER protein see Baylies et al. 1993). In the present work we used polyclonal rabbit antibodies 57/10w and 58/10w, which had been raised against the PER S-region of *A. pernyi* (Sauman and Reppert 1996), and two α-PER antibodies, which had been prepared against a full-length recombinant PER of *D. melanogaster* (Vosshall and Young 1995). Since the antibodies always reacted identically, they are referred to under a single denomination αPER. The 57/10w and 58/10w antisera were used at a 1:200 dilution for immunocytochemistry and a 1:20,000 dilution for the western blotting; the corresponding dilutions of the α-PER antibodies used were 1:1,000 and 1:10,000, respectively.

Immunocytochemistry

Brain–suboesophageal ganglion complexes were dissected from the water-anaesthetised animals in sterile saline and fixed overnight at 4°C in Bouin-Hollande solution without acetic acid but supplemented with 0.7% mercuric chloride (Levine et al. 1995). Standard techniques were used for tissue dehydration, embedding in Paraplast, sectioning to 6–10 μm, deparaffinisation and rehydration. The sections were treated with Lugol's iodine followed by 7.5% solution of sodium thiosulphate to remove residual heavy metal ions, and then washed in distilled water and phosphate-buffered saline supplemented with 0.3% Tween 20 (PBS-Tw). The non-specific binding sites were blocked with 10% normal goat serum in PBS-Tw for 30 min at room temperature (RT). Incubation with the primary antibody diluted with PBS-Tw was done in a humidified chamber overnight at 4°C. Normal goat serum was used instead of the primary antibody in the control staining. After a thorough rinsing with PBS-Tw (three times for 10 min at RT), the sections were incubated for 1 h at RT in a 1:1,000 solution of the goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Jackson Immunoresearch) in PBS-Tw. Following rinsing with PBS-Tw (three times for 10 min at RT) and with 0.05 M TRIS-HCl, pH 7.5 (10 min at RT), the HRP enzymatic activity was visualised with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M TRIS-HCl, pH 7.5). Stained sections were dehydrated, mounted in DPX mounting medium (Fluka), and viewed and photographed under a Zeiss Axioplan microscope (Zeiss) equipped with Nomarski (DIC) optics and a CCD camera.

Immunostaining quantification

Circadian variations in the number of labelled cells and the intensity of their staining were examined in serial sections of the brain and the suboesophageal ganglion. The proportion of PER-positive cells was assessed from the size of ganglia regions containing immunoreactive nuclei. No staining was classified as 0 and the staining encompassing 30%, 60% and 90% of the cells as stages A, B and C, respectively (10% of the cells never reacted with tested antibodies). Assessments were facilitated by the regularity of the spatial immunostaining pattern (cf. Fig. 4). The staining of cell nuclei in *T. domestica* was an all-or-none response, whereas in *P. americana* the staining intensity varied from zero to weak, moderate and strong. Since these variations followed the same temporal and spatial pattern as the changes in the proportion of the immunoreactive cells, it was possible to include them in the 0, A, B and C classification.

The quantification of the number of immunoreactive cells and, in the case of *P. americana*, in the intensity of immunostaining, were done in three sets of preparations, each including three series of ganglia dissected at ZT 0, 4, 8, 12, 16 and 20. All preparations of every series were processed simultaneously, but separately from the parallel time series. The entire immunocytochemical procedure, from tissue dissection to staining evaluation, was standardised in respect to time, temperature and other conditions. The length of exposure to DAB was set to 15 min based on preliminary runs with preparations from animals killed at ZT 4. Resulting staining was examined independently by two researchers, first in randomised samples and then by comparing preparations from different circadian times of individual time series. The results were eventually verified by confronting preparations from diverse series but of the same circadian time.

Western analysis

Western analysis was performed on samples comprising 30 trimmed heads (the antennae, mouth parts and scales were removed) of adult firebrats or ten dissected complexes brain–suboesophageal ganglion of adult cockroaches. The samples were homogenised in 200 μl 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 the supernatant mixed with 50 μl 5×SDS-PAGE loading buffer, boiled for 5 min and loaded on 10% SDS-polyacrylamide gel (12 μl per lane). 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 separated proteins that were transferred onto a nitrocellulose membrane (Sigma-Aldrich) by semidry electroblotting. The strip with markers was stained with 0.1% Coomassie Blue R-250 in 50% methanol and the remaining membrane was processed for immunostaining. It was treated for 1 h at RT with 5% non-fat dry milk in phosphate-buffered saline supplemented with 0.1% Tween 20 (PBS-Tw) to block unspecific IgG

binding sites and then incubated overnight at 4°C and under gentle agitation with the primary antibody in 5% non-fat dry milk in PBS-Tw. After washing in PBS-Tw (three times 10 min at RT), the membranes were incubated for 1 h at RT with the goat anti-rabbit IgG labelled with HRP (Jackson Immunoresearch) diluted 1:10,000 in PBS-Tw, and then washed three times for 10 min in PBS-Tw. The immunoreaction was revealed by detecting the enzymatic activity of bound HRP either with a chemiluminescent reaction (ECL Western Blotting Detection System; Amersham) or with the standard DAB staining employing hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M TRIS-HCl, pH 7.5).

Results

The firebrat

The antisera 57/10w and 58/10w reacted in the cephalic ganglia of the firebrat with a cycling cytoplasmic antigen present in a small number of neurons (described in detail by Závodská et al. 2003b). The αPER antibodies also labelled the cytoplasm of a few cells (Fig. 1A). Their distribution and the circadian pattern of immunostaining, which was often detectable only during the night phase, leave no doubt that they are identical with those revealed with the 57/10w and 58/10w antisera. In addition to this limited and cycling cytoplasmic staining, the αPER antibodies reacted strongly with the nuclei of up to 90% of all brain cells. Nuclear staining occurred in the optic lobes around lobula and medulla and including cells lying between these two regions (Fig. 1B), in the dorsolateral and central protocerebrum (Fig. 1C), at the base of protocerebrum, in the deuto- and tritocerebrum, and between the latter brain regions (Fig. 1D). The proportion of immunoreactive nuclei was about 80% in the suboesophageal ganglion, where the staining occurred especially in the posterior-ventral region (Fig. 1E). About the same proportion of nuclei was stained in the *corpora cardiaca* (data not shown).

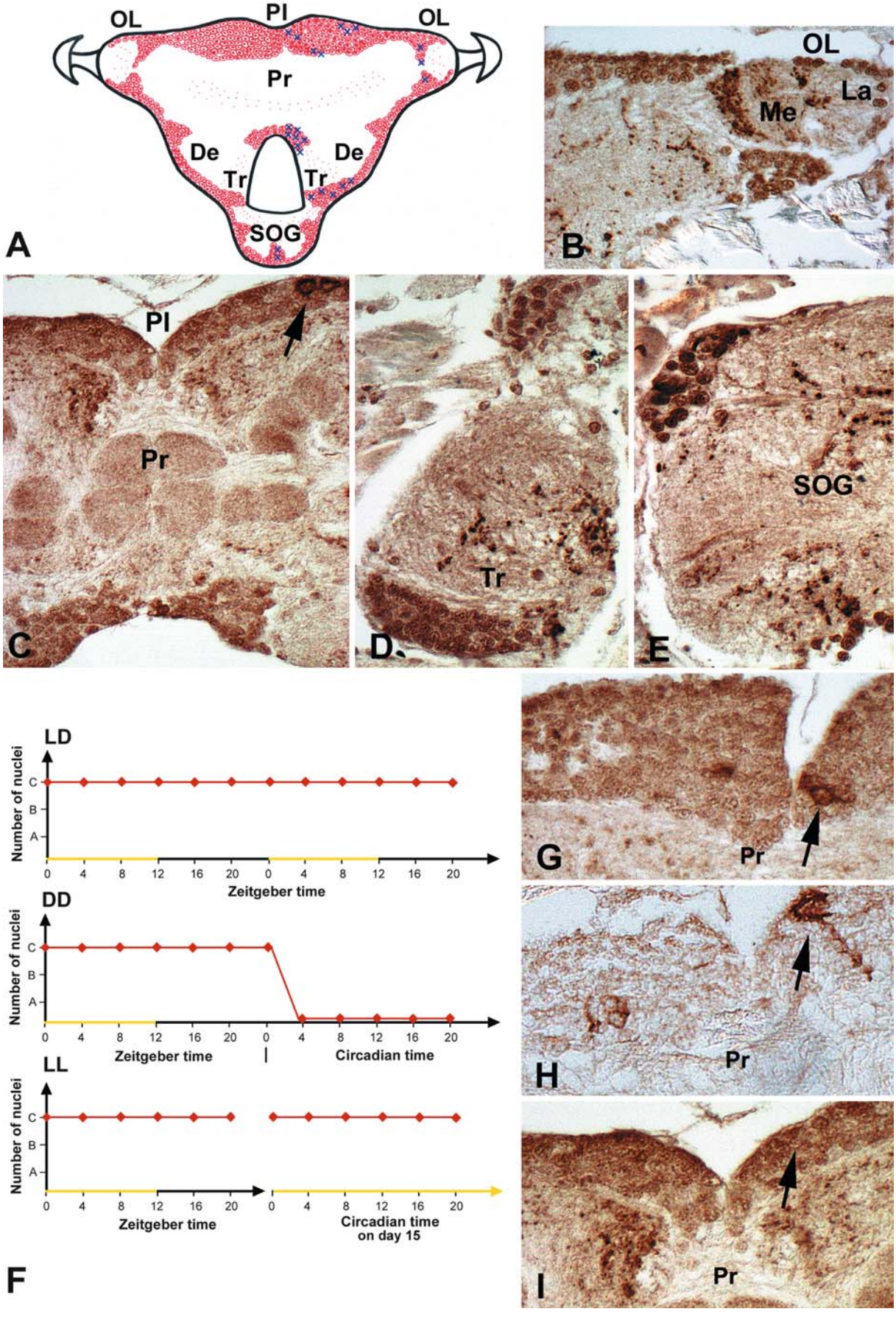
Nuclear staining did not exhibit any daily rhythm in either intensity or the number of immunoreactive cells in firebrats reared under the LD photoperiod. Strong staining persisted, without any perceivable fluctuations, at all examined ZT time points (Fig. 1F *graph LD*, G). Upon a transfer to constant darkness, however, very slight staining (classified as 0 with our scale) occurred at CT 4 and 8 in the first DD cycle and no reaction was detectable afterwards (Fig. 1F *graph DD*, H). By contrast, strong nuclei staining was found at all time points tested in the insects held in constant light (Fig. 1F *graph LL*, I). This behaviour of the nuclear antigen contrasted with the cytoplasmic PER-like staining that occurred in a small set of cells in the night phase of LD cycles (Fig. 1G *arrow*), persisted in constant darkness (Fig. 1H *arrow*) and vanished in continuous light (Fig. 1I *arrow*).

Fig. 1A–I Reactivity to the α-PER antibodies in adult *Thermobia domestica*. **A** Diagram of the distribution of cells with the nuclear (*red circles*) and cytoplasmic (*blue marks*) PER-like staining in the cephalic ganglia. **B–E** Nuclear immunostaining in **B** optic lobe, **C** central protocerebrum (*arrow* indicates cells with additional cytoplasmic staining), **D** tritocerebral region and **E** suboesophageal ganglion. **F** All or no nuclear immunostaining (*C* ca 90% of nuclei stained, *B* 60%, *A* 30%) in insects kept in two cycles of 12 h light and 12 h darkness (*LD*), permanent darkness (*DD*), permanent light (*LL*). *Yellow sections* of the *x*-axis mark the light phase and *black sections* the dark phase. **G–I** Nuclear labelling in the neurons of central protocerebrum at ZT 16 in **G** 12:12 h photoperiod, **H** continuous darkness and **I** constant light. *Arrow* indicates cells with both nuclear and cytoplasmic staining; the latter persisted in the darkness (**H**) and was absent in the light (**I**). *OL* Optic lobe, *PI pars intercerebralis*, *Pr* protocerebrum, *De* deutocerebrum, *Tr* tritocerebrum, *SOG* suboesophageal ganglion, *Me* medulla, *La* lamina. Magnification in **B–E**, **G–I** ×160

The dependence of PER-like immunoreactivity in the cell nuclei on light was confirmed with western blot analysis. Under the 12:12 LD cycle, a single immunoreactive band of the same intensity was detected in the blots of brain extracts prepared from the insects killed at ZT 4, 10, 16 and 22 (Fig. 2A, F). The molecular weight of the immunoreactive protein was assessed as about 80 kDa. Very slight reaction was detected at CT 4, and no reaction at later times in the insects transferred to constant darkness (Fig. 2B). On the other hand, a very strong 80-kDa band occurred at all CT points in insects kept for 1–7 days in constant light (Fig. 2C). By contrast, the cytoplasmic antigen of about 115 kDa, which was revealed with the 57/10w and 58/10w antisera, cycled under the 12:12 photoperiod and in constant darkness but disappeared in continuous light (Závodská et al. 2003b).

The cockroach

Similarly to the firebrat, αPER antibodies reacted strongly with the nuclei of most neurons and at certain times also with the cytoplasm of a small set of cells in the brain and the suboesophageal ganglion of cockroaches kept under LD illumination cycles (Fig. 3A). Also similar to the firebrat, the 57/10w and 58/10w antisera stained exclusively the cytoplasm of cells that were located in similar positions and occurred in similar numbers as the cells responding with cytoplasmic staining to the αPER antibodies (data not shown). The nuclear staining with αPER antibodies occurred around the lobula, medulla and lamina neuropil; arrays of stained nuclei also extended from the surface of lamina to the pigment cell layer (Fig. 3B). A very distinct band of immunostaining bordered external lamina surface. All parts of the central brain showed widespread nuclear staining that was particularly distinct in the peripheral regions (Fig. 3C). Immunopositive nuclei were also located in the circumoesophageal connectives and in the suboesophageal ganglion where they were stacked in several layers around lateral neurilemma and formed a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large mass in the mid-posterior region (Fig. 3D). Nuclear immunostaining was further observed in the cells of the *corpora cardiaca* (Fig. 3E) and *corpora allata*.

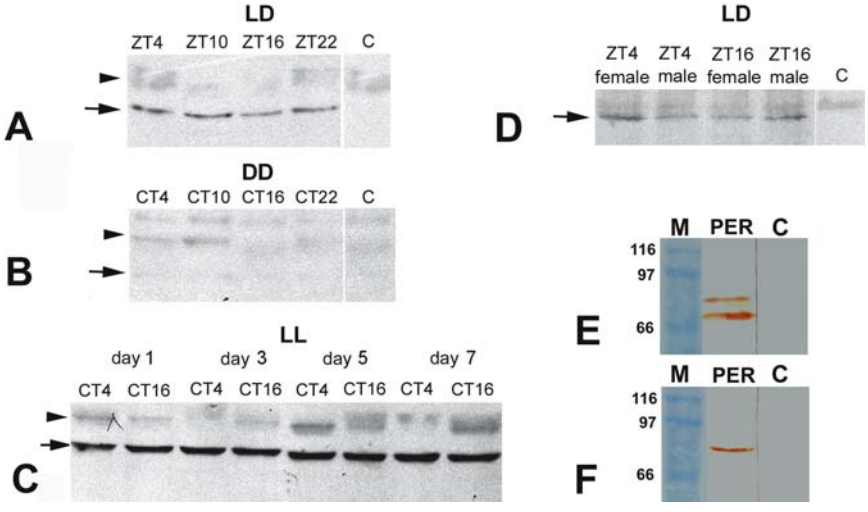
An examination at 4-h intervals through the LD 12:12 h cycles revealed robust circadian changes in the number of stained nuclei and the intensity of staining (Fig. 3F *graph LD*). The score of immunostaining increased during the light phase to a maximum between ZT 8 and ZT 12, when about 90% of all nuclei were labelled, and diminished rapidly in the dark phase, when a weak staining persisted only in a row of cells bordering external lamina surface. This was the only staining detectable at ZT 16; 4 h later a few positive nuclei appeared also laterally and posteromedially in the suboesophageal ganglion. Subsequent increase in the number of stained nuclei followed a regular pattern (Fig. 4). The numbers increased slightly with the day breaking at ZT 0 and substantially 4 h later, when the staining spread to the circumoesophageal connectives and the tritocerebrum. An increase in the intensity of immunostaining was obvious in the row of cells on the external lamina surface. The number of immunopositive nuclei rapidly increased in the second half of the light phase and at ZT 8 the label occurred in all parts of the brain and the suboesophageal ganglion, as well as in the *corpora cardiaca* and *corpora allata*. This wide distribution and high intensity of nuclear staining persisted until ZT 12 and was reduced when the lights went off.

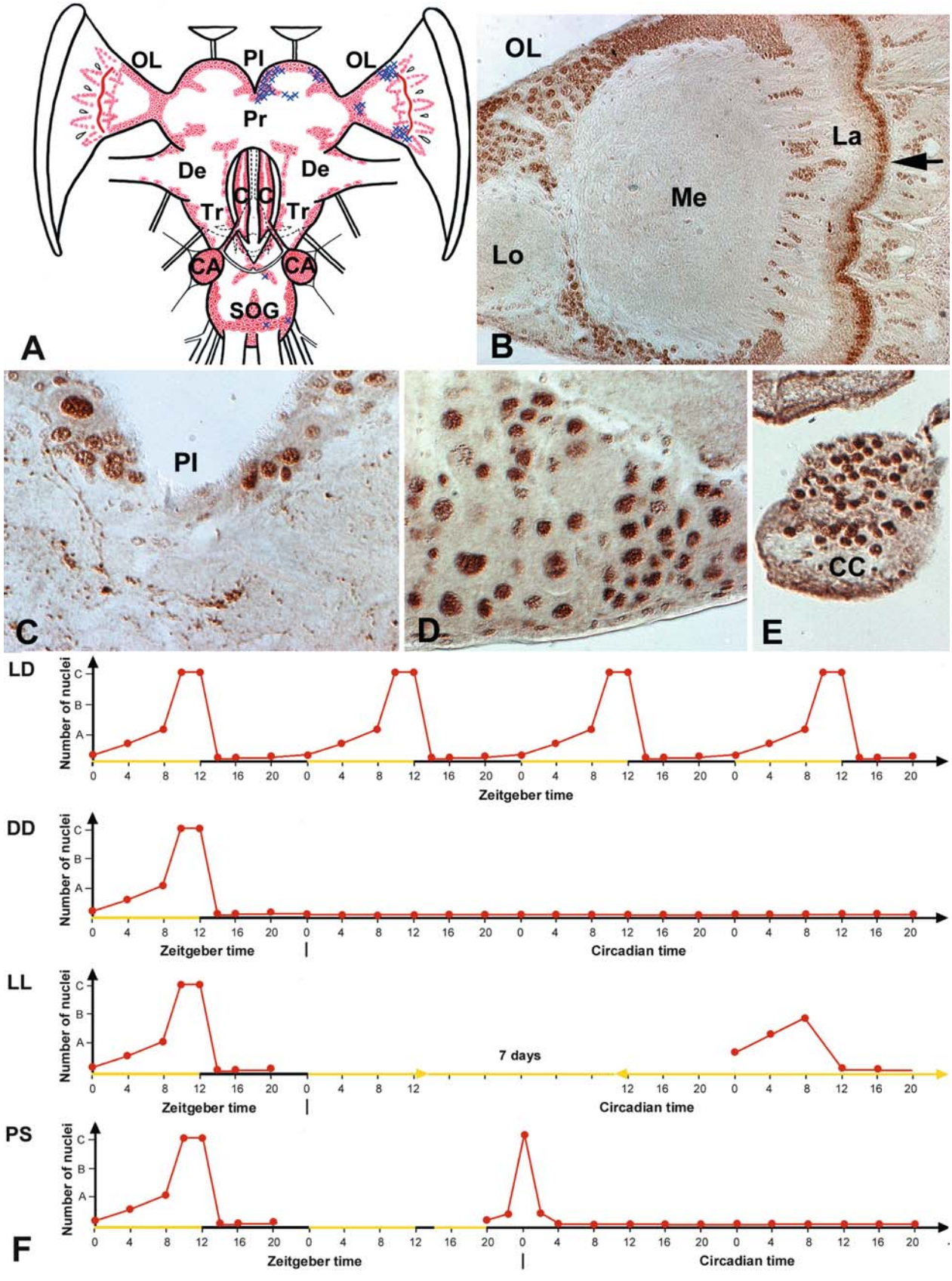
Fig. 2A–F Immunoreactivity to the αPER (**A–C**, **E**, **F**) and 57/10w (**D**) antisera on the western blots. **A–D** Chemiluminescent detection in the head extracts of *T. domestica* kept in **A** 12:12 h light/dark cycle (*LD*), **B** constant darkness (*DD*) and **C** constant light (*LL*), and **D** in the cephalic ganglia extract of *Periplaneta americana* kept in the 12:12 h light/dark cycle. *CT* Circadian time, *ZT* Zeitgeber time, *C* control staining without the primary antibody, *arrowhead* non-specific staining, *arrow* specific immunostaining. **E** DAB immunostaining of proteins extracted at ZT 4 of the LD cycle in *P. americana* and **F** in *T. domestica*. Antibody binding was detected in two protein fractions in *P. americana* and a single fraction in *T. domestica*. *M* Markers (116, 97 and 66 kDa), *PER* reactivity with the α-PER antiserum, *C* control reaction without the primary antiserum

Fig. 3A–F Reactivity to the α-PER antibodies in adult *P. americana*. **A** Diagram of the distribution of cells with the nuclear (*red circles*) and cytoplasmic (*blue marks*) PER-like staining in the cephalic ganglia. **B–E** Nuclear immunostaining in **B** optic lobe including cells on the external lamina surface (*arrow*), **C** central protocerebrum, **D** suboesophageal ganglion and **E** *corpora cardiaca*. *C Corpora cardiaca*, *CA corpora allata*, *De* deutocerebrum, *La* lamina, *Lo* lobula, *Me* medulla, *OL* optic lobe, *PI pars intercerebralis*, *Pr* protocerebrum, *SOG* suboesophageal ganglion, *Tr* tritocerebrum. Magnification **B** ×80, **C–E** ×160. **F** Daily changes in the number of immunopositive nuclei (*C* ca 90% of nuclei stained, *B* 60%, *A* 30%) in the ganglia of cockroaches kept in 12:12 h light:dark cycles (*LD*), constant darkness (*DD*), constant light (*LL*) and exposed to a 6-h light pulse during scotophase of an LD cycle followed by darkness (*PS*). *Yellow sections* of the *x*-axis mark the light phase and the *black sections* the dark phase

The decline in the number of immunopositive nuclei after the onset of darkness between ZT 12 and ZT 16 was investigated at 1-h intervals. Antigen dissipation was surprisingly fast, with all staining (except a slight labelling of the cell external to the lamina) disappearing within 2 h after lights-off, i.e. at ZT 14. A few weakly stained nuclei reappeared under LD conditions in the suboesophageal ganglion in anticipation of the dawn at ZT 20. When the darkness continued (DD conditions), the immunoreactivity of these cells and of the cells on the lamina surface persisted as long as our observations continued through three DD cycles. No other cells were stained under the DD conditions at any circadian time (Fig. 3F *graph DD*).

The insects transferred from the LD conditions to constant light (LL) were examined after 8 days. On day 9, immunostaining occurred during the subjective day and disappeared in subjective night. Some individuals possessed a few immunopositive nuclei already at CT 0 and/or at CT 12, but in most cases the staining occurred in about 30% of cells at CT 4, 60% at CT 8, and was gone at CT 12 (Fig. 3F *graph LL*). Hence, the circadian cycle of immunostaining was retained but damped and shortened after 8 days in constant light. The distribution of stained nuclei was similar but the staining intensity was reduced in comparison with the

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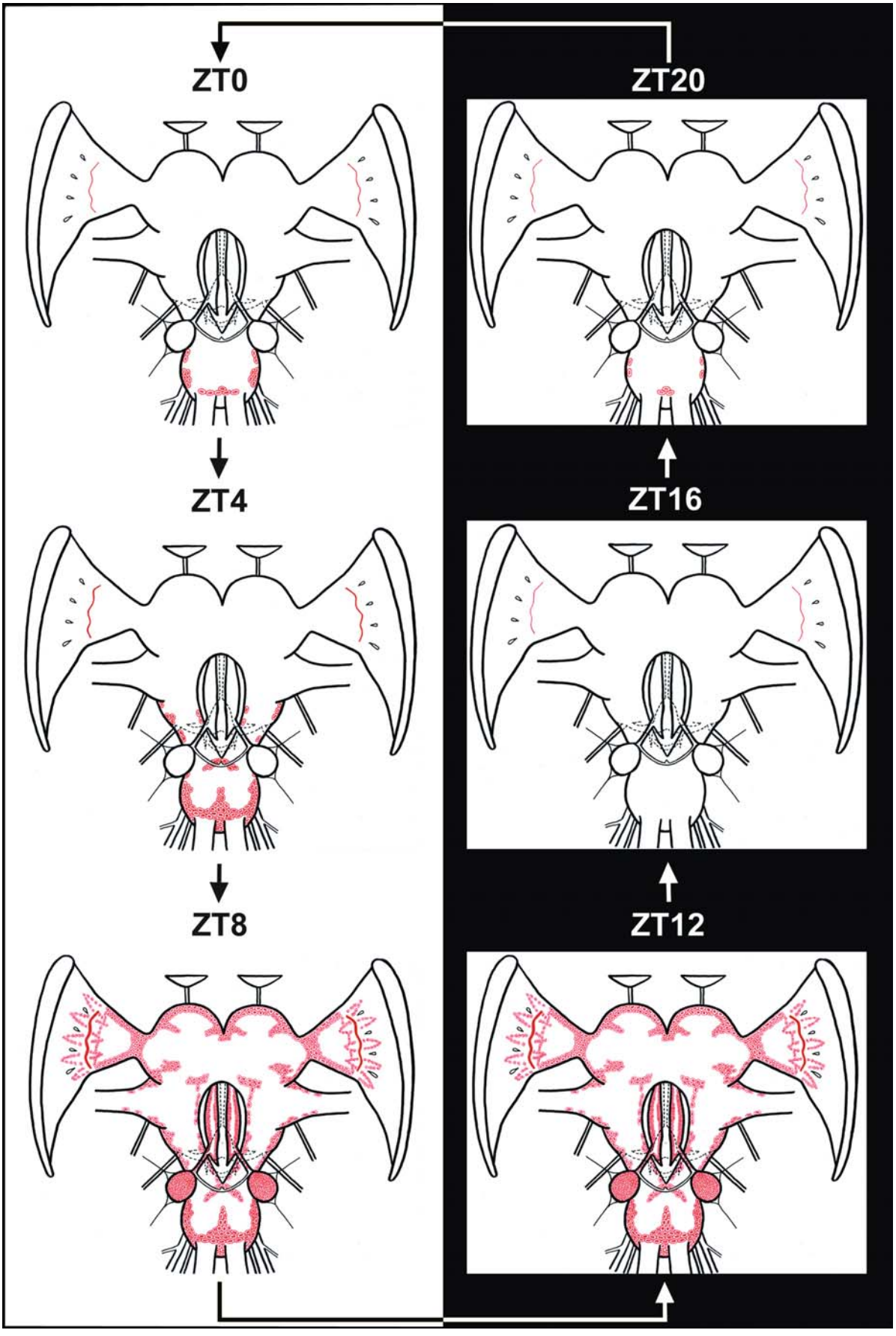
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Fig. 4 Schematic drawing of daily changes in the number and distribution of PER-positive nuclei in the cephalic nervous complex of adult *P. americana*. Each illustration represents indicated Zeitgeber time point of a standard 12:12 h LD cycle

LD conditions. Interestingly, the weak nuclear staining that occurs at CT 20 in both the LD and DD animals was not detected at this time in the LL insects, possibly due to cycle shortening.

In the phase shift experiment, the dark phase of an LD cycle was interrupted with a light pulse lasting from CT 14 to CT 20; thereafter the insects were kept in darkness. The pulse reset antigen fluctuations: the number of positive nuclei reached a peak as early as CT 0, i.e. about 10 h after the extraordinary lights-on signal and about 10 h before the normal timing of this peak. The period of 10 h between the lights-on and the maximal immunoreactivity is similar as in the standard LD cycles, when maximal staining occurs in the second half of the light period (Figs. 3F *graph LD*, 4). In the phase shift experiment, the number of stained nuclei peaked 10 h after the lights-on in spite of darkness. The duration of the peak was shortened, possibly due to darkness, but its magnitude was not affected (Fig. 3F *graph PS*).

For the western analysis, brains with attached suboesophageal ganglia were dissected at ZT 4 from cockroaches reared under LD conditions. The αPER antibodies reacted with fractions corresponding to 70 and 80 kDa proteins (Fig. 2E) and the 57/10w and 58/10w antisera with a single fraction at 110 kDa (Fig. 2D).

Discussion

Antibodies to PER often stain only one cell compartment

The cytoplasm/nuclear translocation of the PER protein, which is regarded as a key feature of the molecular clock (Stanewsky 2003), has been demonstrated in certain *Drosophila* brain cells with a variety of antibodies. The antibodies anti-PER-S (Siwicki et al. 1988) and 57/10w and 58/10w (Sauman and Reppert 1996) were raised against PER S-region of *D. melanogaster* and *A. pernyi*, respectively (Table 1). The region is close to the per^{Short} mutation in *D. melanogaster* and includes 14 amino acid residues. In a comparison of sequences available for 12 insect species in the GenBank we found maximally three residue replacements in this region; *D. melanogaster* and *A. pernyi* differ by a single residue (data not shown). Not surprisingly, the anti-PER-S antibody (Siwicki et al. 1988; Ewer et al. 1992) and the 57/10w and 58/10w antibodies detect in *D. melanogaster* identical patterns of the cytoplasm/nuclear PER translocation (Sauman, unpublished). However, studies on other insects yielded different results (Table 2).

In the cricket *Teleogryllus commodus* (Honegger et al. 1991) and the beetle *Pachymorpha sexguttata* (Frisch et al. 1996), the anti-PER-S antibody stains primarily the cytoplasm, and rather exceptionally the nuclei of certain neurons and glial cells; no sign of label cycling between the two compartments was seen. Antigen translocation between the cytoplasm and nucleus was detected with the 57/10w and 58/10w antibodies in the photoreceptors and in the midgut cells of *A. pernyi*, whereas the immunoreactive Ia_1 brain neurons were stained in the cytoplasm only (Sauman and Reppert 1996). Likewise, one or both of these antibodies stained exclusively the cytoplasm in small sets of neurons in nine other insect species (Závodská et al. 2003a), including the honeybee for which additional independent data are available (Bloch et al. 2003). In this paper we show that the antisera in question stain specifically the cytoplasm in small sets of neurons in *T. domestica* and *P. americana*.

Nuclear staining in most neurons, glial cells and many photoreceptor nuclei was obtained in *M. sexta* with two monoclonal antibodies that apparently recognise a stretch of about 360 amino acid residues (including the S-region), which is 46% homologous between *D. melanogaster* and *M. sexta* (Wise et al. 2002). In the Ia_1 neurosecretory cells the antibodies stained both the nuclei and the cytoplasm but no antigen translocation between these two compartments was detected. Identical antibodies reacted in two cricket species in the cytoplasm of distinct groups of neurons in different parts of the cephalic nervous system (Lupien et al. 2003). Rather inconsistent nuclear staining was found in a group of dorsolateral neurons; in one cricket species it occurred 2 h after the onset of darkness, while in the other it was linked to the light phase. An antibody raised to the homologous PER region of *P. americana* labelled in the moth *Bombyx mori* (45.5% sequence identity) exclusively the cytoplasm in a small set of neurons, including the Ia_1 cells, in which the staining exhibited distinct circadian oscillations (Sehadová et al. 2004).

The αPER antibodies were raised against the whole recombinant PER of *D. melanogaster* and in this species they reveal the typical PER cycling between cytoplasm and nucleus (Vosshall and Young 1995). In a comparative study with diverse insect species (Závodská et al. 2003a), the αPER antibodies either did not react (six species) or reacted exclusively in the cytoplasm of a small set of neurons (another six species). Identical cells were stained with the 57/10w and 58/10w antibodies only in three species of the latter group. In the honeybee, antibodies very similar to the αPER stained only the nuclei in some lamina cells and in *corpora cardiaca*, and either nuclei with cytoplasm or only the cytoplasm in many brain cells (Bloch et al. 2003). A widespread nuclear reactivity of the αPER antibodies associated with light sensitivity and circadian oscillations of the antigens has so far been observed only in *T. domestica* and *P. americana*.

Table 2 Immunocytoreactivity of various anti-PER antibodies in the previously examined species. Antibodies are specified in Table 1. (*CB* central brain, *OL* optic lobe, *P* protocerebrum, *CC corpora cardiaca*, *SOG* suboesophageal ganglion)

Antibody	Species (order)	Stained cells	Compartment	Oscillation	Reference
PER-S, 58/10w, 57/10w, αPER	*Drosophila melanogaster* (Diptera)	Brain neurons and glia, photoreceptors	Cytoplasm and nucleus	Cycling and translocation	Siwicki et al. (1988); Sauman (unpublished)
58/10w	*Lepismachilis y-signata* (Archaeognatha)	Neurons in P and OL	Cytoplasm	???	Závodská et al. (2003b)
58/10w	*Siphlonurus armatus* (Ephemerida)	Neurons in P	Cytoplasm	???	Závodská et al. (2003b)
58/10w	*Perla burmeisteriana* (Plecoptera)	Neurons in CB	Cytoplasm	???	Závodská et al. (2003b)
PER-S, 5F7F6	*Teleogryllus commodus* (Orthoptera)	Neurons in P, whole brain glia	Cytoplasm, in few cells nucleus	None	Lupien et al. (2003)
58/10w	*Phormia regina* (Diptera)	Neurons in P and SOG	Cytoplasm	???	Závodská et al. (2003b)
58/10w, 57/10w, αPER	*Neobellaria bullata* (Diptera)	Neurons in P and SOG	Cytoplasm	???	Závodská et al. 2003b
PER-S	*Pachymorpha sexguttata* (Coleoptera)	Neurons in CB and OL, glia in OL	Cytoplasm, in few cell nuclei	None	Frisch et al. (1996)
58/10w	*Pachnoda marignata* (Coleoptera)	Neurons in OL	Cytoplasm	???	Závodská et al. (2003b)
58/10w, 57/10w, αPER	*Apis mellifera* (Hymenoptera)	Neurons in CB, OL and CC Neurons in CB	Cytoplasm and/or nucleus Only cytoplasm	Slight in intensity	Bloch et al. (2003) Závodská et al. (2003b)
58/10w, 57/10w	*Hydropsyche contubernalis* (Trichoptera)	Neurons in P, OL and SOG	Cytoplasm	???	Závodská et al. (2003b)
58/10w, 57/10w	*Antheraea pernyi* (Lepidoptera)	Ia_1 cells in P; photoreceptors	Cytoplasm in neurons; both in photoreceptors	Cycling; translocation in photoreceptors	Sauman and Reppert (1996)
5F7F6, 10C3C9	*Teleogryllus oceanicus* (Orthoptera)	Neurons in CB and OL	Cytoplasm, in few cell nuclei	None	Lupien et al. (2003)
5F7F6, 10C3C9	*Manduca sexta* (Lepidoptera)	Ia_1 cells in P; most neurons and glia, photoreceptors	Cytoplasm and nucleus; only nucleus	None	Wise et al. (2002)
PaPER	*Bombyx mori* (Lepidoptera)	Ia_1 cells in P	Cytoplasm	Cycling	Sehadová et al. (2004)
αPER	*Ischnura elegans* (Odonata)	Neurons in CB and OL	Cytoplasm	???	Závodská et al. (2003b)
αPER	*Schistocerca gregaria* (Orthoptera)	Neurons in P	Cytoplasm	???	Závodská et al. (2003b)
αPER	*Locusta migratoria* (Orthoptera)	Neurons in P and OL	Cytoplasm	???	Závodská et al. (2003b)
αPER, 58/10w	*Notonecta glauca* (Hemiptera)	Brain neurons and OL	Cytoplasm	???	Závodská et al. (2003b)
αPER	*Gerris palludum* (Hemiptera)	Brain neurons and OL	Cytoplasm	???	Závodská et al. (2003b)

Complementary circadian behaviour of the nuclear and cytoplasmic PER-like antigens

Our study shows that the αPER antibodies recognise an 80-kDa antigen in the head extract of *T. domestica* and 70- and 80-kDa antigens in the cephalic ganglia extract of *P. americana*. In the immunocytochemical preparations of the cephalic ganglia, distinct reactivity occurs in the nuclei of most neurons and weak reactivity in the cytoplasm of a small subset of neurons. Cytoplasmic staining is also detectable with the 57/10w and 58/10w antibodies that recognise a 110-kDa antigen in the ganglia extract of *P. americana* and a 115-kDa PER-like antigen in the head extracts of *T. domestica* (Závodská et al. 2003b).

In *T. domestica*, the cytoplasmic PER-like antigen exhibits prominent daily oscillations, being low or undetectable during the light phase and reaching a peak during the night (Závodská et al. 2003b). Similarly to *D. melanogaster* and other insects, the PER cycling persists in constant darkness and is abolished in constant light when the antigen disappears. In *D. melanogaster*, however, PER protein in the cytoplasm reaches a maximum late in the day and in the night it is confined to the nucleus (Liu et al. 1988; Siwicki et al. 1988). The nuclear PER-like protein of *T. domestica* does not cycle and its presence requires periodic or constant illumination. Nearly all neurons of the cephalic ganglia contain the nuclear PER-like protein continuously in the 12:12 h photoperiod or under constant illumination but lose it within 4 h if the dark phase is extended.

The cytoplasmic PER-like protein is present persistently in *P. americana* kept under a 12:12 h photoperiod or in constant darkness but disappears readily in those exposed to constant light (Sehadová, unpublished). This is in sharp contrast to the nuclear PER-like antigens of this species that exhibit distinct circadian fluctuations in virtually all neurons of the cephalic nervous system, being maximal at the end of the day and absent in the night. The cycling persists in continuous light but in constant darkness the nuclear antigen vanishes.

The complementary cycling behaviour and reversed light-dependence of the nuclear and the cytoplasmic PER-like antigens in *T. domestica* and *P. americana* (Table 3) indicate that they may be functionally linked components of the circadian clock. Both cycling and light entrainment are basic properties of the clock elements (Hall 1995; Lee et al. 1996). In *D. melanogaster*, the cyclic changes in the PER amount and its cytoplasm/nucleus translocation are controlled by complex interactions with other clock molecules (Stanewsky 2003). Proteins such as CRYPTOCHROME (CRY), which are intimately linked to photoreception, affect the half-life of some of the clock proteins and thereby synchronise the endogenous clock with the environmental oscillations of day and night (Helfrich-Förster et al. 2001). For example, the TIMELESS protein TIM, which stabilises PER and facilitates its movement into the nucleus, is degraded upon a light-dependent interaction with CRY. The degradation of TIM leads to the breakdown of PER whose level is also controlled by a direct interaction with CRY (Rosato et al. 2001). We imagine that the apparent disappearance of the cytoplasmic PER-like proteins in *T. domestica* and *P. americana* upon exposure to continuous light may be regulated in a similar way. However, the light dependence of nuclear PER-like staining indicates the existence of an additional light-signalling pathway.

In the phase shift experiment, the number of immunoreactive cell nuclei rose sharply 10 h after the light switching, i.e. nearly half a day earlier than it corresponded to the established circadian rhythm. The light pulse was undoubtedly responsible for this shift but the long period of 10 h elapsing between the pulse and the immunostaining peak as well as the peak occurrence in the dark phase are consistent with the paradigm that light does not affect PER directly. The phase advance is presumably due to the light-induced production of a regulatory protein(s) that either turns on the expression or modifies the conformation and thereby alters the antigenic properties of nuclear PER-like antigen. The length of time for which the nuclear PER-like antigen remains detectable seems to correspond to the duration of the preceding illumination period. An untimely light pulse of 6 h induced nuclear PER-like antigen for about 8 h, while in the standard 12:12 h photoperiod it is present for ca 12 h. It seems that the hypothetical regulator of nuclear PER-like staining is active as long as the light is on.

The results indicate that apparent absence of the cytoplasm/nuclear movement of a PER-like antigen, which has been observed in many insects (Table 2), cannot be taken as evidence that the role of PER in the molecular clock gear is fundamentally different from the *Drosophila* model. Our detection of cytoplasmic and

Table 3 Comparison of PER-like antigens detected with the αPER and with the 57/10w and 58/10w antibodies

Species	Antigens	Abundance	Cellular localisation	Behaviour under different light regimes		
				LD 12:12 h	DD	LL
Thermobia domestica	80 kDa[a]	Many cells	Nucleus	Persisting	Disappears	Persisting
	115 kDa[b]	Few cells	Cytoplasm	Cycles, peaks in night	Persisting cycles	Disappears
Periplaneta americana	70 + 80 kDa[a]	Many cells	Nucleus	Cycles, peaks during day	Disappears	Persisting cycles
	110 kDa[b]	Few cells	Cytoplasm	Persisting	Persisting	Disappears

[a]Detected with αPER antibody
[b]Detected with 57/10w and 58/10w antibodies

nuclear PER-like antigens with different antibodies opens a possibility that some species either contain two PER homologues or, more likely, that a PER protein occurs in antigenically different conformations in the two cell compartments. In a *Drosophila* cell line it has been shown that cell nucleus harbours PER in inactive and active states and that the level of gene suppressive PER activity is not always reflected in the intensity of immunostaining (Nawathean and Rosbash 2004). PER activity depends on phosphorylation (Edery et al. 1994) that may render the molecule undetectable by certain antibodies but reactive with some others. We assume that the reported contradictions concerning nuclear and cytoplasmic staining in diverse insect species are due to differences in the PER structure and conformation. The rapid disappearance of PER-like nuclear staining in *T. domestica* is consistent with the notion that phosphorylation is involved. The identification of apparently cytoplasmic and nuclear PER-like moieties on the western blots opens the door to elucidating the mutual relationship of elusive antigens confined to just one cell compartment.

Acknowledgements We thank Dr. Michael Young of Rockefeller University for the generous gift of antibodies. Our work was supported by grant A5007205 from the Grant Agency of the Academy of Sciences and was performed in the frame of the Institute Program Z50070508.

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