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The crayfish *Procambarus clarkii* CRY shows daily and circadian variation

María Luisa Fanjul-Moles*, Elsa G. Escamilla-Chimal, Andrea Gloria-Soria and Gabriela Hernández-Herrera

Laboratorio de Neurofisiología Comparada, Departamento de Biología, Facultad de Ciencias, Universidad Nacional Autónoma de México, México DF 11000

*Author for correspondence (e-mail: mlfm@hp.fciencias.unam.mx)

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Summary

The circadian rhythms of crayfish are entrained by blue light, through putative extra retinal photoreceptors. We investigated the presence and daily variation of CRY, a protein photosensitive to blue light spectra and ubiquitous in animals and plants, in the putative pacemakers of *Procambarus clarkii*, namely the eyestalk and brain, at different times of the 24 h light:dark cycles. Using different experimental light protocols and by means of qualitative/quantitative immunofluorescence anatomical and biochemical methods, we identified CRY immunoreactivity in cells located in the medulla-terminalis-hemiellipsoidal complex (MT-HB) and the anterior margin of the median protocerebrum (PR). The immunoreaction varied with the time of day and the two neural structures showed a semi-mirror image. The results of the biochemical analysis matched these variations. Western blotting demonstrated statistically significant circadian rhythms in brain CRY abundance, but no daily circadian CRY abundance oscillations in the eyestalk. These immunocytochemical and biochemical results link a specific photoreceptor molecule to circadian rhythmicity. We propose that CRY may be linked to the photoreception of the clock and to the generation of circadian rhythmicity.

Key words: rhythm, *Procambarus clarkii*, pacemaker, cryptochrome, circadian photoreceptor.

Introduction

Circadian rhythms are entrained by light to adapt to the daily solar cycles. The 24 h light:dark cycle (LD) is considered the most important zeitgeber for synchronization; however, the intensity and quality of light change through the daily cycle, especially around dawn and dusk, and the photo entrainment of the clock, have been shown to depend on these two factors. In a general sense, the sensory mechanisms of photo-entrainment are complex but are considered ubiquitous to all organisms (Foster and Helfrich-Forster, 2001). Over and above the classical vision (Roenneberg and Foster, 1997), however, the circadian system has evolved sensory specializations that enable it to extract time information.

The crayfish is a nocturnal crustacean that displays a variety of circadian rhythms controlled by periodic function of the nervous system (Fanjul-Moles and Prieto-Sagredo, 2003). Although some of these rhythms are well described, there is scant information about the circadian photoreceptors and the entrainment pathways that couple the clock to the daily light changes. Some authors have proposed that the photoreceptors participating in the entrainment are extra-retinal and located in the supra-esophageal ganglion (Page and Larimer, 1972, 1976; Sandeman et al., 1990), while others have proposed the sixth abdominal ganglion as a locus of circadian photoreception (Bernal-Moreno et al., 1996; Prieto-Sagredo and Fanjul-Moles, 2001). Interestingly, experiments have demonstrated that the crayfish circadian photo-entrainment depends on the quality of light (Fanjul-Moles et al., 1992; Bernal-Moreno et al., 1996; Miranda-Anaya and Fanjul-Moles, 1997), suggesting that this phenomenon rests on different photo proteins and inputs that converge on the circadian pacemakers, the eyestalk (retina and optic lobe) and the brain (Aréchiga et al., 1993).

The effect of blue monochromatic light (λ=440 nm) on the electroretinogram (ERG) and activity rhythms of crayfish (Fanjul-Moles et al., 1992; Miranda-Anaya and Fanjul-Moles, 1997), as well as the features of the phase–response curves constructed for the ERG rhythm (Inclán-Rubio, 1991; Bernal-Moreno et al., 1996), confirm the photo entrainment action of blue light, indicating the presence of a photo pigment that absorbs light in the blue spectrum (400–500 nm). This pigment could be a cryptochrome (CRY), blue/UV-A absorbing photo protein, originally discovered in plants, which has homologues in the animal kingdom (insects, mice and human; Sancar, 2003) and is associated with the circadian clock. Mounting evidence from genetic and molecular studies indicates that in insects, light acts directly on the clock through CRY (Emery et al., 1998; Stanewsky et al., 1998); although to date in mammals a photoreceptive function for these pigments has not been proved, they do participate in the feedback loop of the

circadian genes constituting the machinery of the clock (Stanewsky, 2003).

In crayfish, the molecular mechanisms involved in the generation and synchronization of circadian rhythms are practically unknown, although there is abundant but controversial information about the physiological and behavioral mechanisms underlying the generation and entrainment of the clock. The object of the present study is to contribute to this knowledge, investigating whether CRY is expressed in the putative circadian pacemakers of crayfish, the eyestalk and the brain, and whether this protein may be considered as an element of the circadian clock.

Materials and methods

Animals and experimental design

Field-collected *Procambarus clarkii* Girard, 1852 of homogeneous size and weight and in intermoult stage (N=66) were acclimatized to the laboratory for 1 month in aquaria placed under 12:12 LD cycles (lights on at 07:00 h) at constant temperature (20°C). After acclimation the organisms were divided into three batches under different LD cycle light schedules: (1) 30 animals were maintained under 12:12 LD cycles, (2) 18 animals were exposed to 24 h of constant darkness, and (3) 18 animals were exposed to 72 h of constant darkness. The first group was subdivided into three subgroups, two of which (6 animals each) were processed for histological and anatomical techniques and a third group (18 organisms) was used for biological determination. Three specimens from each group were selected at random for anatomical determinations, twice per day (N=12), and for biochemical determinations, six times per day (N=54). At each experimental time point, each organism was adapted to darkness for 30 min, anaesthetized on ice and killed. The eyestalk and the supraesophagic ganglion were dissected and processed for histological or biochemical analyses. We explored the following time points: 11:00 h and 23:00 h in organisms processed for histology; 03:00 h and 19:00 h in organisms processed for whole-mount and immunohistochemistry and 07:00 h, 11:00 h, 15:00 h, 19:00 h, 23:00 h, 03:00 h in organisms analyzed by biochemical techniques.

Whole-mount immunocytochemistry

The whole-mount technique was a modification of that previously described (Galizia et al., 1999). Dissection of the whole eyestalk and brain was performed in cold (4°C) physiological saline (Van Harreveld). The tissue was fixed with 10% formaldehyde in phosphate-buffered saline (PBS) for 12 h at 4°C and rinsed in PBS, followed by a series of alcohol solutions (50%, 70%, 90, 96% and 100% ethanol for 15 min each, xylol 5 min and 100%, 96%, 70%, 50% ethanol 15 min each). Subsequently the tissue was washed with PBS-Tween 20, pH 7.6 for 20 min, and then protein-blocked and incubated in the primary antibody diluted 1:1500 (v/v) with PBS for 48 h at 4°C. We used a commercially available antiserum generated in rabbit immunized with *Drosophila* CRY (Alpha Diagnostic International Inc., San Antonio, TX, USA). Tissues were washed in PBS for 2 h and incubated in 1:50 (v/v) secondary antiserum (goat anti-rabbit IgG labeled with Texas Red; Rockland, Gilbertsville, PA, USA) for 24 h at 4°C, followed by several washes in PBS for 2 h. Finally, the tissue was dehydrated in increasing ethanol solutions (50%, 70%, 90%, 100%, 15 min each), mounted in methyl salicylate (ICN Biomedicals, Inc., Irvine, California, USA) and viewed with a confocal Bio-Rad MRC-1024 (Bio-Rad, Hercules, California, USA) attached to a Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan).

Histological procedures

Both the eyestalk and the brain of six organisms were separately fixed in 10% formaldehyde in PBS for 12 h. The fixed material was progressively dehydrated in 50%, 70%, 98% and 100% ethanol, incubated in Paraplast for 12 h and embedded in a block for sectioning. Both organs were cut into longitudinal sections (4 μm thick) using a calibrated microtome. Serial sections were collected, deparaffinized in xylene, mounted on gelatine-coated glass slides and progressively rehydrated (100%, 96%, 70%, 50% ethanol, water). To localize CRY, the brain and eyestalk sections were processed by immunofluorescence. The sections were incubated for 12 h at room temperature in the same polyclonal rabbit anti-*Drosophila* CRY serum (dilution 1:1500 v/v). To visualize the primary immunoreaction the sections were incubated for 1 h in goat anti-rabbit IgG-Texas Red (Rockland, 1:50 v/v) at room temperature. The slices were preserved with fluorescent mounting medium (Biogenex, San Ramon, CA, USA). Control sections were (i) treated in the same way but with the antiserum omitted and (ii) treated by preadsorption of the CRY antiserum with *Drosophila* CRY peptide (Alpha Diagnostic International, Inc.). To localize CRY, the sections were examined using a Nikon Labophot 2 epifluorescence microscope.

Image analysis and confocal microscopy

The immunofluorescent sections were studied by stereological analysis as described elsewhere (Escamilla-Chimal et al., 2001). The sections were studied using a Nikon Labophot 2 epifluorescence microscope. For each histological section (at least three sections were examined), three video images of the structures were captured using the 40× objective, and digitalized by means of an image processor system (Argos 20, Hamamatsu, Hamamatsu City, Japan), captured with MGI Video Wave software (Roxio, Santa Clara, California, USA.) and analyzed stereologically using Sigma Scan Pro (*vs.* 4.01, SPSS Inc., Chicago, IL, USA).

Confocal images of whole-mount preparations were obtained using an MCR 1024 Bio-Rad laser-scanning system equipped with an Ar Kr/Ar air-cooled laser attached to an inverted Nikon TMD 300 microscope. Images were collected with a Nikon 40× objective (numerical aperture 1.0). Neurons stained with Texas Red were excited with the 568 nm line of

the laser, and emitted light was band-passed with a 605 nm filter. Serial optical sections were taken at intervals of 1–5 μm. The stacks of images were processed into stereo pairs of movies, saved as three-dimensional projections and converted to TIF format with Todd Clark's program Confocal Assistant 4.2. Further analysis to adjust brightness and contrast was performed using Adobe Photoshop 5.0 (Adobe Systems Inc., San José, CA, USA).

Biochemical determination

Protein sample preparation

Brain and eyestalk, including the retina, were carefully homogenized in 100 μl of ice-cold PBS, pH 7.4. Then, the homogenates were centrifuged at 11 000 ***g*** for 25 s at room temperature. Supernatants were stored at –71°C until analyzed. Samples were thawed at room temperature and the protein concentration was determined using the method of Bradford (1976) and standards of 3.75, 11.25, 18.75, 26.25, 37.5 μg μl^{-1} bovine serum albumin (Sigma-Aldrich Co.; St Louis, MO, USA).

Western blotting

Proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) with a 10% polyacrylamide separating gel. Each lane was loaded with 40 μg of protein except for the positive control (control peptide; Alpha Diagnostic International, Inc.) and the molecular mass standards.

Proteins resolved by SDS-PAGE were electrophoretically transferred from the gels to nitrocellulose membrane Hybond ECL (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England) by routine methods, using a Bio-Rad Mini Trans-Blot system at 100 V for 45 min. Protein loading and localization for molecular mass were revealed by staining with Coomassie Blue. Prior to immunodetection the membranes were incubated with a blocking solution containing 3% gelatin diluted in TBS (Immuno-Blot Assay Kit, Bio-Rad) for 1 h followed by two rinses with TTBS (350 μl Tween-20 diluted in 700 ml TBS). Later the blots were incubated for 12 h at room temperature with the previously described rabbit anti-CRY antiserum diluted 1:800 (v/v) in a 1% gelatin solution. Immunoblots were revealed using peroxidase-labelled anti-rabbit antibodies (Immuno-Blot Assay Kit, Bio Rad) diluted in 1% gelatin (1:3000 v/v) for 2 h. To test the specificity of the antibody it was incubated with the peptide control at 4°C for 24 h and afterwards the antibody was used for western blotting.

Gel and blots were scanned and digitalized using a HP 3400 C Scanjet scanner (Hewlett Packard, Palo Alto, CA, USA). Quantifications of the bands were performed in a computerized analyzer system using the software Sigma Scan Pro (SPSS Inc., vs. 4.01) and GeneTools (vs. 3.00.22; Syngene Division, Synoptics Group, Cambridge, UK). Briefly, the scanned images of the bands of the blots were framed to fill the stained areas on the image, the dark areas measured and the average intensity of each band determined. The criterion for selecting the immunoreactivity targets was a minimum ratio of background 0 pixels (white) and 255 pixels (black). For each experiment the data (average intensity of the immunoreactive area of the band) obtained for each time point were averaged, expressed as mean ± S.E.M. of CRY relative abundance, normalized to the maximal value obtained for the experiment, and plotted as chronograms. The raw data were statistically analyzed using a single cosinor analysis (Nelson et al., 1979) by mean of the software program COSANA (Menna Barreto et al., 1993).

Cosinor analysis

The software COSANA utilizes the cosinor statistical method described elsewhere (Castañon-Cervantes et al., 1999). On the basis of a test period (τ), cosinor analysis adjusts data to a sinusal curve and provides an objective test of whether the amplitude of the rhythm differs from zero, i.e. whether the rhythm is validated for an assumed τ. This method provides descriptive estimators for a number of different parameters of a rhythm, i.e. acrophase, mesor, amplitude and percentage of rhythmicity (PR). The acrophase is the crest time of the best-fitting mathematical function approximating data, the mesor (*M*) is the value about which oscillation occurs, and when the interval of time between data is constant, it equals the arithmetic mean of the rhythmic oscillation. Hence, in the present work *M* corresponds to the arithmetic mean of the rhythmic oscillation of CRY abundance throughout 24 h. The period is the duration of one complete cycle of the oscillation and it is expressed in units of time. In the cosinor method, the amplitude (A) is equal to half the difference between the highest and lowest values of the oscillation, i.e. it

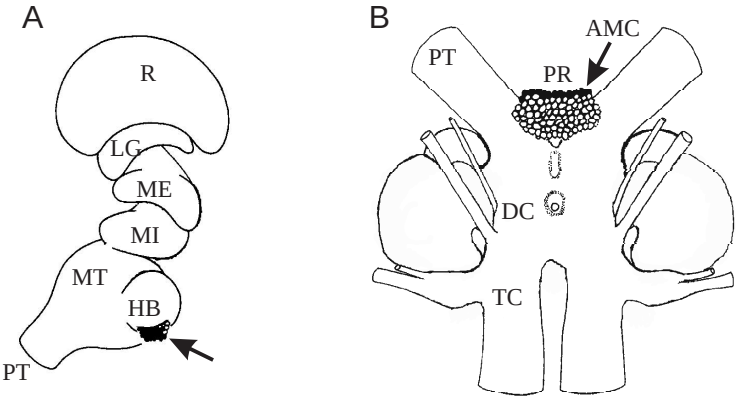

Fig. 1. (A) Schematic representation of the eyestalk of *P. clarkii*. R, retina; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; HB, hemiellipsoid body; LG, lamina ganglionaris. The dark region represents cells expressing CRY immunoreactivity in MT-HB. Note the cluster of cells located in the inferior region of the hemiellipsoid body. (B) Schematic representation of the brain of *P. clarkii*. PR, protocerebrum; PT, protocerebral tract; AMC, protocerebral anterior median cluster; DC, deuterocerebrum; TC, tritocerebrum. Note the dark region representing the protocerebral median cluster immunoreactivity.

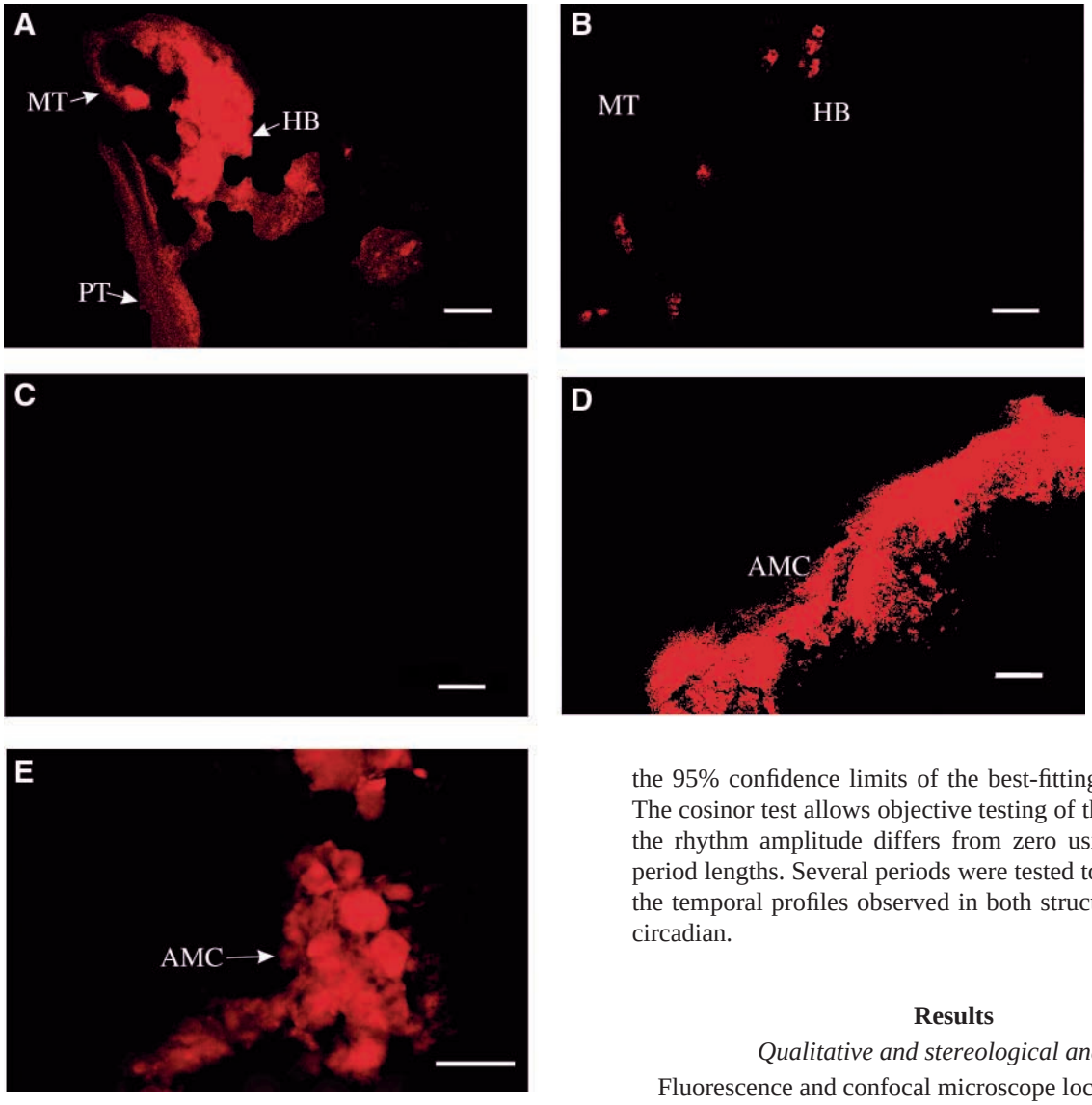

Fig. 2. (A–D) Confocal image of the three-dimensional topography of the eyestalk and brain CRY immunoreactivity of *P. clarkii* at two times of day. All the images are optical sections of whole-mount preparations. (A) MT-HB complex cells expressing CRY at 03:00 h. The hemiellipsoid body shows a cluster of cells expressing a strong CRY immunoreaction; an immureactive cell apparently located in MT seems to branch towards the HB (small arrows). Note the protocebral tract (PT) expressing a dim immunoreaction. (B) MT-HB complex at 19:00 h, showing low immunoreaction. (C) Protocerebrum at 03:00 h; note the lack of immunoreactive signal at this hour. (D) Neurons of the protocebral anterior medial cluster (AMC) expressing strong CRY immunoreactivity at 19:00 h. Scale bars, 100 μm. (E) Fluorescence micrograph of a histological section showing the cytoplasm of some cells of the protocerebral anterior medial cluster (AMC) expressing CRY immunoreactivity at 11:00 h. Scale bar, 20 μm. See text for explanation.

is the crest-to-trough difference, and the percentage of rhythmicity (PR) is the percentage of the data included within the 95% confidence limits of the best-fitting cosine function. The cosinor test allows objective testing of the hypothesis that the rhythm amplitude differs from zero using different trial period lengths. Several periods were tested to analyze whether the temporal profiles observed in both structures were indeed circadian.

Results

Qualitative and stereological analysis

Fluorescence and confocal microscope localization revealed CRY immunoreactive material in the optic lobe, at the medulla terminalis-hemiellipsoid body complex (MT-HB) and in the brain protocerebral anterior median cells (AMC) (see Fig. 1A,B). Body cells located in the base of the eyestalk in HB and the dorsal region of the median protocerebrum (PR) showed strong immunoreactivity. The immunoreaction was specific because none of the cell populations of the eyestalk or the brain gave a signal in control incubations. Stereological analysis did not reveal any day–night differences in the amount of CRY-immunoreactive material in MT-HB and PR at 11:00 h ($X_{MT\text{-}HB}$=0.19±0.02%; X_{PR}=0.12±0.02%) and 23:00 h ($X_{MT\text{-}HB}$=0.23±0.05% and X_{PR}=0.19±0.04%) or differences between both structures at both hours. However, whole-mounts performed at 03:00 h and 19:00 h (not quantified) revealed important qualitative temporal differences in the amount of immunoreactive material in both structures (Fig. 2A–D). The brain protocerebrum showed a positive CRY immunoreaction at 19:00 h accompanied by a negative immunoreaction at 03:00 h. The MT-HB showed a strong signal at 03:00 h but a slight signal at 19:00 h. Fig. 2A–D shows the confocal mirror

image of fluorescent material in both structures and at the two time points studied. Fig. 2E shows a fluorescent image of the protocebral cells at 11:00 h.

Biochemical analysis

Analysis of the extracts of crayfish brain and eyestalks revealed the presence of a protein immunoreactive to anti-CRY antibody. This protein matches the molecular mass of the cryptochrome reported for *Drosophila melanogaster*, approximately 60 kDa (Emery et al., 1998) (Fig. 3).

Chronograms showing the temporal changes in CRY relative abundance in the eyestalk are depicted in Fig. 4A–C. Western blot showed that levels of CRY oscillate daily attaining maximal values at late subjective night (03:00 h) with a deep trough coincident with the onset of light (07:00 h). Throughout the subjective day and night, the CRY protein increased steadily with only a slight decrement after the offset of light (Fig. 4A). Interestingly, when the lights were turned off, and the crayfish were submitted to 24 h darkness, levels of CRY relative abundance increased almost twofold, oscillating with a bimodal rhythmic oscillation due to the two troughs corresponding to the previous offset and onset of light (Fig. 4B). After 72 h of darkness, a very damped unimodal rhythm appears showing a 4 h phase advance (maximal peak at 2300). Cosinor analysis shown in Table 1 revealed that the level of activity (mesor) and amplitude rhythmic parameters are modified by the different experimental conditions. The mesor of the rhythm obtained under LD (50±6) increases after 24 and 72 h darkness (89±2 and 71±4.3), when the period value (see Materials and methods) changes from 24 h to 12 h and 24 h, respectively.

Temporal changes of CRY abundance in the brain under the same experimental conditions are shown in the chronograms of Fig. 4D–F. When crayfish are subjected to LD, the CRY protein in brain tends to increase during the subjective day and decrease in the subjective night, showing maximal abundance at 19:00 h. This rhythm oscillates with a 24 h statistically significant rhythm that shows a higher activity level than the eyestalk rhythm (*M*=66±5) (Table 1). After 24 and 72 h of darkness the zenith of the CRY oscillation delays for 8 h,

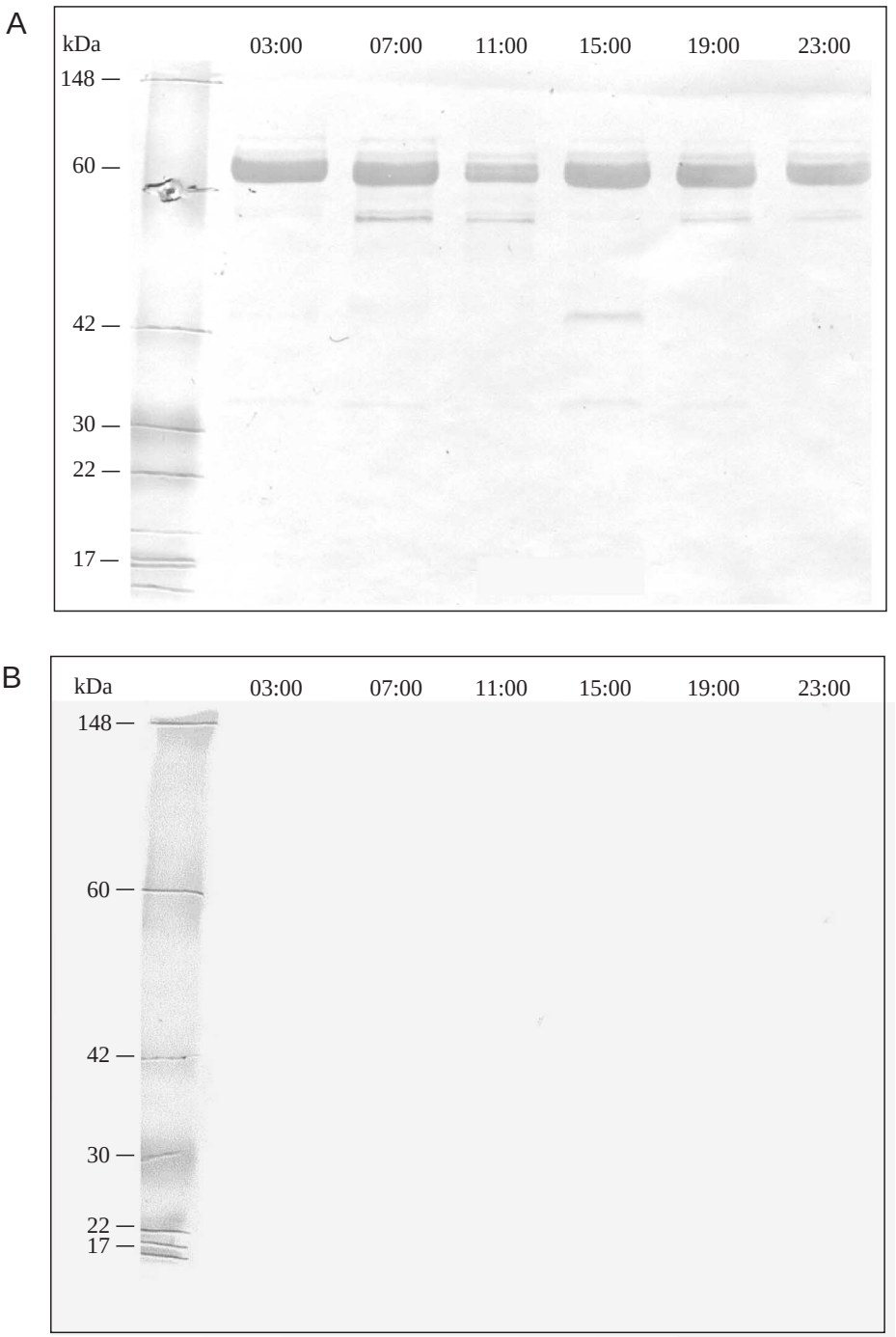

Fig. 3. Western blots showing the specificity of the the *Drosophila* CRY antibody for crayfish. (A) The anti-CRY antibody recognizes CRY in the brain of crayfish. The molecular mass of the crayfish protein matches that of the cryptochrome reported for *Drosophila* (approximately 60 kDa). At 07:00 h and 11:00 h, small bands of lower intensity appear below. (B) After incubation of the antibody with the control peptide the immunoreactive bands are not present, indicating the antibody specificity. Each lane represents a time point of sample collection from animals maintained in the dark for 72 h (*N*=3). The left side of the figure shows the position of molecular mass markers.

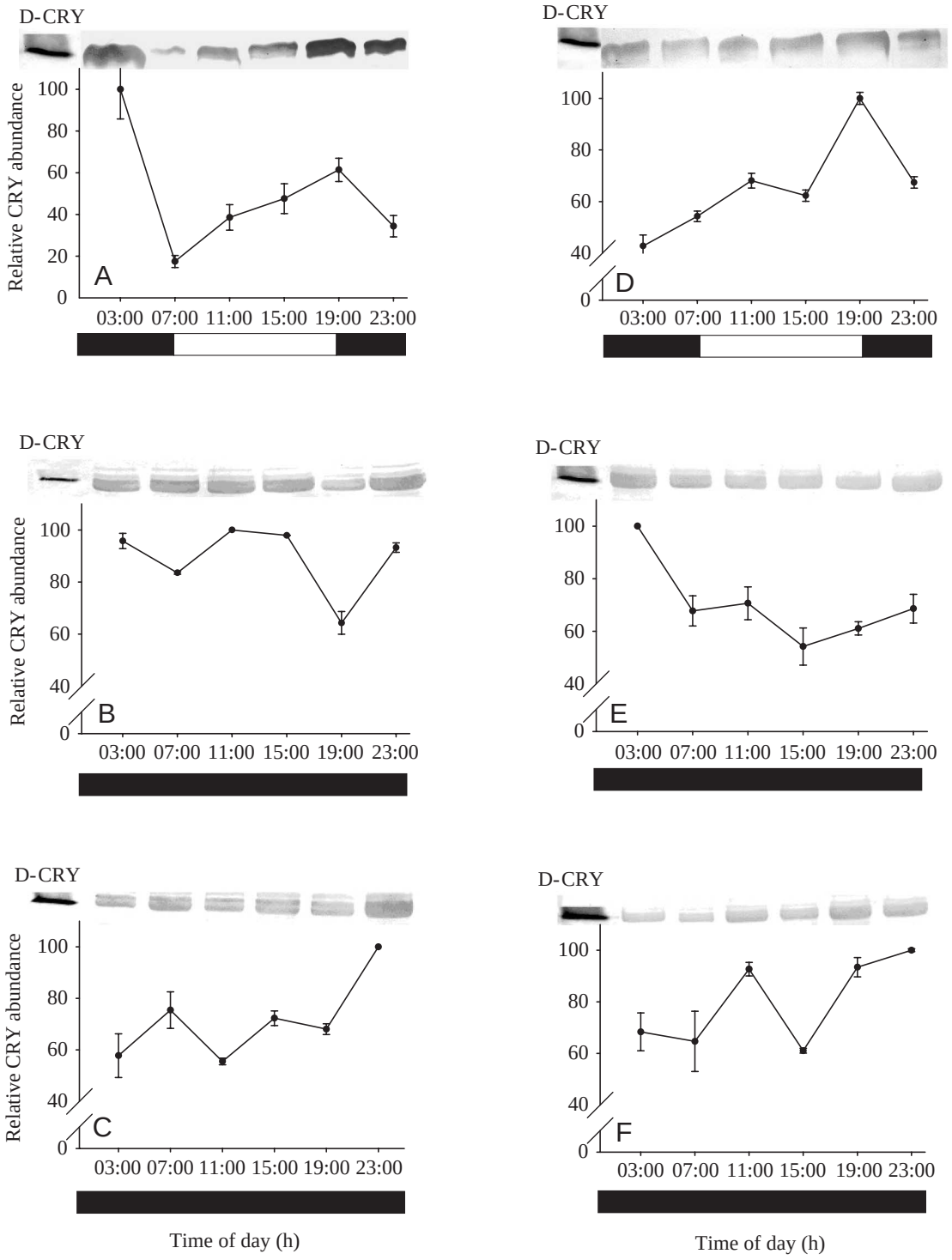

Fig. 4. Chronograms illustrating the daily rhythmic pattern of CRY abundance in *P. clarkii*. Bars at the bottom of each panel indicate the illumination conditions (open, light; closed, dark). Values are means ± S.D. (*N*=3). The corresponding western blot is shown at the top of each panel. Each lane represents the time points of sample collection. D-CRY is the stained control peptide. (A–C) Changes in the daily pattern of CRY abundance in the eyestalk, showing no significant circadian rhythms. (D–F) Changes in the rhythmic pattern of CRY abundance in the brain of *P. clarkii*: when the organisms changed from LD to DD, a clear circadian pattern was observed. Note that CRY abundance rhythm mean (mesor) increases under continuous darkness, especially in the eyestalk. The data for B,C,E,F were obtained from tissues taken from animals maintained in the dark for 24 and 72 h, respectively. A and D show data obtained from tissues form animals maintained in LD. Relative CRY abundance is indicated by the relative average intensity of the immunoreactive area of the band.

Table 1. *Single cosinor analysis of CRY abundance (% area) in* P. clarkii *eyestalk and brain*

	Period (h)	Mesor	Amplitude	PR (%)	Acrophase (h)
Eyestalk					
LD	24	50±6	16±9	12.8	22±1
DD 24 h	12	89±2	15±3	74.3	1±0.2
DD 72 h	24	71±4	11±6	27.3	22±2
Brain					
LD	24	66±5	21±7*	31.8	18±1
DD 24 h	25	70±2	17±3*	57.7	2.08±0.5
DD 72 h	11.5	81±2	19±3*	76.0	10±0.2

Mesor, arithmetical mean of the adjusted rhythm; PR, percentage of rhythmicity; L, light; D, dark.

Values are mean ± S.D.

*Amplitude differs significantly from zero: *P<0.05.

For an explanation of cosinor analysis, see Materials and methods.

shifting to the late subjective night and adjusting to statistically significant unimodal and bimodal rhythmic oscillations with a period value equal to 25 h and 11.5 h, respectively. After darkness, as shown in Table 1, the mesor of the rhythm increases to 70±2 and 81±2, respectively. Interestingly, when the crayfish changes from LD to DD the brain rhythm's mesor value increment is about half the value of the eyestalk rhythm (22% and 48%, respectively). Cosinor analysis detected that in the three experimental conditions the brain showed statistically significant circadian and bimodal rhythms but the eyestalk did not show any statistically significant rhythm (Table 1).

Discussion

The presence of cryptochromes in the putative central circadian pacemakers of crayfish was investigated. We discovered CRY immunoreactive cells in the lateral protocerebrum of the eyestalk and in the median protocerebrum of the brain. Two clusters of immunoreactive cell bodies (Fig. 2) are present in *P. clarkii*, one located in the caudal region of the hemiellipsoid body in the MT-HB complex, and the other along the anterior margin of the cerebral ganglion, in some of the anterior median cell cluster of the protocerebrum, described elsewhere in *Cherax destructor* (Sandeman et al., 1988).

This latter cluster of cells could be associated with the extraretinal brain photoreceptor reported elsewhere in the crayfish *C. destructor* (Sandeman et al., 1990). These photoreceptor cells are reported to specifically bind to a rhodopsin-antibody, showing maximal response at 540 nm. The findings of the present study suggest that the cryptochromes located in different cells of this cluster, together with this photoreceptor, may contribute to the wide spectral response of the circadian system of *P. clarkii* (400–700 nm) shown elsewhere (Fanjul-Moles et al., 1992). The activity rhythm of this species is able to entrain to blue and red monochromatic light in the absence of retina and lamina (Miranda-Anaya and Fanjul-Moles, 1997). This extraretinal synchronization is probably mediated by brain photoreceptors; the cryptochromes should be responsible for the blue spectrum of the circadian response to light. Unexpectedly, some of the lateral protocerebral neurons, those basal to the hemiellipsoid body, showed a CRY immunoreaction. These neurons apparently communicate with the medulla terminalis through a neurite (Fig. 2A) and seem to correspond to the interneurons described elsewhere (McKinzie et al., 2003; Mellon, 2003). In *P. clarkii* these cells are multimodal sensory neurons that receive sensory input of distinct sensory systems, among them a photic pathway from the ipsilateral eye (Mellon, 2000). Our results suggest that cryptochromes could be elements of the light input to the clock of crayfish, as has been proposed for other species (Stanewsky et al., 1998; Emery et al., 1998).

The results of the biochemical and immunocytochemical analyses performed under 12:12 LD conditions in the current work are in agreement. The immunoreaction found at the four time points tested, 11:00 h, 19:00 h, 23:00 h and 03:00 h, coincides with the relative abundance of CRY at these times, determined biochemically (Figs 3, 4). There is no statistical difference in levels in the eyestalk or the brain between 11:00 h and 23:00 h, but there was a significant difference between 19:00 h and 03:00 h, and the maximal CRY imunoreaction and abundance in both structures is semi-phase-locked, showing a mirror-image relationship (Fig. 2). The biochemical results of this study demonstrate daily and circadian changes in the CRY relative abundance in the eyestalk and the brain, respectively (Table 1). In the brain, these rhythmic changes appear to be endogenously driven, since they continue to run freely after 72 h of darkness, changing phase and period and running with statistically significant circadian periods in LD and DD conditions. However, the abundance of CRY in the eyestalk showed a non-statistically significant daily rhythm under LD, which was dampened 24 and 72 h after darkness, and revealed no statistically significant circadian rhythms. This indicates that the daily oscillation could be due to a masking effect of the LD cycle. The significance of the rhythms, the mirror image of their phases, as well as the effect of light on CRY abundance in eyestalk and brain, raise the possibility that this protein has a dual function: one in the MT-HB, acting as a photopigment able to absorb light and translating that information to the master oscillator, and the other in the median protocerebrum, proposed by some authors as a master oscillator of crayfish (Barrera-Mera and Block, 1990), where it participates in the rhythm-generating mechanisms. Both possibilities exist, as demonstrated in flies and mice, two species in which both genetic and molecular circadian mechanisms are well documented. In *Drosophila*, the latest evidence suggests that CRY is a photopigment that acts in the entrainment pathway of the clock in the brain, and also a protein that participates in the circadian rhythm-generating process of the compound eye and peripheral body tissues (Stanewsky, 2003). In mice most evidence indicates that CRY is only involved in the clock rhythmic generation, but it has

recently been proposed that, as with insect cryptochrome, mammalian CRYs function pleiotropically in circadian rhythm generation, photic entrainment and behavioral responses such as masking (Van Gelder et al., 2002). In crayfish, and generally in crustaceans, our knowledge on the genetic and molecular mechanisms underlying the circadian clock is scant, although the conserved nature of the clock genes could lead us to presume that all groups share similar genetic proprieties. Hence, knowledge of the molecular and physiological features of circadian mechanisms in different species will help us to understand the biological perception of time.

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