The Journal of Experimental Biology 204, 1073–1081 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3124

DAILY VARIATIONS IN CRUSTACEAN HYPERGLYCAEMIC HORMONE AND SEROTONIN IMMUNOREACTIVITY DURING THE DEVELOPMENT OF CRAYFISH

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Accepted 15 December 2000; published on WWW 26 February 2001

Summary

The present study investigated changes in crustacean hyperglycaemic hormone (CHH) and serotonin (5-hydroxytryptamine, 5-HT) immunoreactivity in the retina and the X-organ/sinus gland complex (XO-SG) of the crayfish *Procambarus clarkii* at two developmental stages, post-embryonic stage two (PO2) and the juvenile stage, at three different times of day, under a photoperiod cycle of 12 h:12 h L:D, using qualitative and quantitative immunohistochemical methods. In the retina, CHH immunoreactivity is located in the tapetal cells, while 5-HT immunoreactivity is found in the retinular cells. In the XO-SG, CHH-immunoreactivity is localized to the CHH-producing cell perikarya and in their axons and endings in the sinus gland, while 5-HT immunoreactivity is restricted to axon endings branching into the perikarya of the CHH-producing cells. A stereological analysis demonstrates that the PO2 and juvenile stages show significant differences in the amount of the immunoreactive CHH and 5-HT material at the three selected time points, indicating daily and related changes in the levels of CHH and 5-HT in the XO-SG and the retina. Our findings therefore support the idea that daily rhythms in the secretory activity of the XO-SG complex affect the circadian sensitivity of the eye. Furthermore, the differences found between the PO2 and juvenile stages suggest that both CHH and 5-HT are key factors in the development of the circadian rhythm of retinal sensitivity.

Key words: crustacean, *Procambarus clarkii*, hyperglycaemic hormone, serotonin, crayfish, immunoreactivity, development.

Introduction

It has been proposed that serotonin (5-hydroxytryptamine; 5-HT) is a circadian modulator of the visual system of invertebrates (Nadakavukaren et al., 1986). In Crustacea, particularly in adult crayfish (*Procambarus clarkii*), the daily secretions of different neurohormones in the X-organ/sinus gland complex (XO-SG) seem to affect the circadian sensitivity of the eye (for a review, see Aréchiga et al., 1993). Interestingly, Kulkarni and Fingerman (Kulkarni and Fingerman, 1992) have suggested that there may be interactions between 5-HT, light and some neurohormones, such as red pigment dispersing hormone and distal retinal pigment light-adapting hormone. The crustacean hyperglycaemic hormone (CHH) is a well-studied hormone synthesized and released by the XO-SG (for a review, see Van Herp, 1998), and circadian changes in its secretion that correlate with a circadian blood glucose rhythm (Hamann, 1974) have been reported in different species of crayfish (Kallen et al., 1988). However, the role of this hormone in the mechanisms underlying the sensitivity of the circadian rhythm of the eye of *Procambarus clarkii* have not been explored, and we still lack data on possible interactions between CHH and 5-HT.

One approach to investigating this interaction is an ontogenetic study of the daily changes in levels of both hormones. Previous developmental studies on various crayfish circadian rhythms have shown that two particular stages of development, the second post-embryonic stage (PO2) and the juvenile stage, are key stages in the final maturation and expression of several circadian rhythms, e.g. the electroretinogram (ERG) circadian rhythm (Fanjul-Moles et al., 1987), the locomotor circadian rhythm (for a review, see Fanjul-Moles, 1998) and the 5-HT circadian rhythm (Castañón-Cervantes et al., 1999). Changes in the parameters of these rhythms, such as period, phase and amplitude, occur between the PO2 and juvenile stages and may be considered as maturation markers of the effectors responsible for the overt rhythm and of their coupling to the pacemakers. In the case of the ERG rhythm, these effectors are the photoreceptors in the eye, the sensitivity of which depends on the neurohormones released from the XO-SG (Fanjul-Moles et al., 1987). One of these neurohormones could be CHH, although there are no reports indicating its presence in the eyestalk of *Procambarus clarkii* during ontogeny. However Gorgels-Kallen and Meij (Gorgels-Kallen and Meij, 1985) have described an increase in

the number of CHH-immunopositive cells in the XO-SG complex during post-larval development of the crayfish *Astacus leptodactylus* and an increase in haemolymph glucose concentration in adult crayfish after injection of an extract of larval eyestalks. On the basis of these results, these authors proposed that a hyperglycaemic factor is present in the eyestalk of *Astacus leptodactylus* immediately after hatching, and that levels of this factor subsequently increase during development. Hence, the present study aimed to investigate (i) whether CHH and 5-HT are present in the major structures involved in the ERG circadian rhythm (the XO-SG complex and the retina) and (ii) whether the daily variations in 5-HT immunoreactivity, reported elsewhere for the retina (Escamilla-Chimal et al., 1998), also occur in the XO-SG complex and are related to daily variations in CHH immunoreactivity within the same structures. Differences between PO2 and juvenile crayfish may indicate that CHH and 5-HT are involved in the mechanisms underlying the ERG circadian rhythm. This study was performed in the two post-embryonic stages considered as essential in the development of crayfish, the PO2 and juvenile instars. It is between these two stages that the maturation of the overt circadian rhythm occurs.

Materials and methods

Animals and experimental design

Procambarus clarkii (Girard 1852) (24 animals), born and raised in our laboratory from different mothers, were maintained under identical light:dark cycles (L:D 12 h:12 h, lights on at 07:00 h) and temperature (20–22 °C). The animals were separated into two developmental groups according to previous criteria (Fanjul-Moles, 1998): 12 post-embryonic stage two instars (PO2; 10–14 days old) and 12 juveniles (2 months old). Four specimens of each group were selected at random at three times of day: 08:00, 15:00 and 20:00 h. At each time point, they were adapted to darkness for 30 min and then anaesthetized on ice. The optic lobes were dissected and fixed in 10 % formaldehyde in 0.1 mol l^{-1} phosphate buffer (PBS) for 12 h.

Histological procedures

The fixed material was routinely treated and embedded in Paraplast. Both eyestalks of each specimen were sectioned longitudinally (4 μm thick) with a calibrated microtome. Serial sections were collected, mounted on gelatin-coated glass slides and rehydrated. As a first approach to localizing CHH and 5-HT, one specimen of PO2 and one of the juvenile stage per time point were processed for immunohistochemistry according to the ABC method (Priestley, 1997) using one eyestalk to visualize each antigen. To localize CHH, sections were incubated for 18 h at 4 °C with the primary antiserum, a rabbit polyclonal anti-*Astacus*-CHH serum (Gorgels-Kallen and Van Herp, 1981) diluted 1:500 in PBS. They were then rinsed in PBS, incubated with a biotinylated anti-rabbit IgG for 20 min, labelled with alkaline phosphatase/streptavidin substrate complex (Biogenex), and finally rinsed in PBS and stained with Fast Red and Mayer's haematoxylin. The 5-HT-immunoreactive structures were visualized using the same ABC method, but these tissues were incubated in a polyclonal rabbit 5-HT serum (1:80 dilution) as described elsewhere (Escamilla-Chimal et al., 1998). These procedures were performed simultaneously in pairs of control tissues without the primary antibody. The sections were mounted in Vikel under glass coverslips. To localize the immunoreactive material, the cells and fibres of the retina and XO-SG were studied by light microscopy.

To quantify CHH and 5-HT immunoreactivity, a double immunofluorescence technique was applied to each eyestalk of the remaining 18 specimens. The sections were incubated for 18 h at 4 °C in monoclonal mouse anti-5-HT serum (Dako; dilution 1:10) and polyclonal rabbit anti-*Astacus*-CHH serum (dilution 1:500). To visualize the primary immunoreactions, the sections were incubated for 2 min at 59 °C and then for 6 h in goat anti-mouse FITC (Dako, dilution 1:10) and for 1 h in goat anti-rabbit Texas Red (Rockland, 1:50) at room temperature. The slices were preserved with fluorescent mounting medium (Dako). The specificity of the immunoreaction was tested using two methods: (i) control sections were treated in the same way, except that the primary antiserum was omitted; and (ii) sections were treated by preadsorption of the 5-HT antiserum with an excess of 5-HT/creatinine sulphate complex (Sigma; 500 μg ml^{-1}) or by preadsorption of the CHH antiserum with a crude extract of the sinus gland of *P. clarkii* (Kleinholtz et al., 1967).

Stereological image analysis and quantification

One series of at least three consecutive sections was selected from each eyestalk of all the PO2 stage and juvenile specimens and examined for double immunofluorescence. The first section was selected at random, and the second and third sections were subsequently taken with a sampling distance of five sections between them. The immunofluorescent double-labelled sections were studied with a Nikon epifluorescent microscope (Labophot 2). From each histological section, three video images of each of the structures described below were captured, using the ×40 objective, and digitized by means of a Hamamatsu, Argus 20 image processor system, relayed to a PC *via* a Buzz video capture system, captured with MGI Video Wave software and further analyzed stereologically using Sigma Scan Pro (Jandel, version 3.0). Total retinal area was divided into the regions shown in the inset of Fig. 1A and described elsewhere (Escamilla-Chimal et al., 1998): proximal (PR, basal membrane and retinal cells axons), medial (MR, tapetal cells, retinular cells and rhabdoms) or distal (DR, distal tip of rhabdoms, crystalline cone cells and cone roots). These three regions of the retina, as well as the sinus gland, the OX-SG tract and the medulla terminalis/X-organ (MT-XO) were assessed on coded eyestalk, with experimental conditions unknown to the observer. The volume fraction of green (5-HT) or red (CHH) fluorescent material in each of these structures was estimated using the point counting technique (Gundersen et al., 1988).

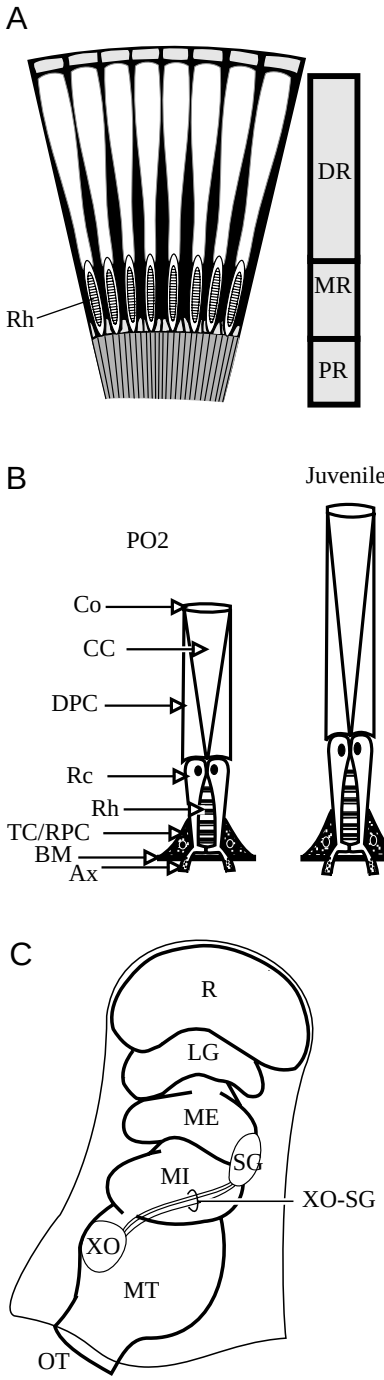

Fig. 1. (A) Schematic representation of a set of ommatidia and retinular cell axons. PR, proximal region; MR, medial region; DR, distal region; Rh, rhabdom. (B) Comparison of the ommatidia typical of post-embryonic stage two (PO2) and juvenile crayfish. Note the elongation of the dioptric system and the increase in size of the main rhabdom in juveniles. Co, cornea; CC, crystalline cone; DPC, distal pigment cell; RC, retinular cell; Rh, rhabdom; TC, tapetal cell; RPC, reflecting pigment cell; BM, basement membrane; Ax, retinular cell axon. (C) The eyestalk of a juvenile crayfish. R, retina; LG, lamina ganglionaris; ME, medulla externa; MI, medulla interna; MT, medulla terminalis; SG, sinus gland; XO, X-organ; XO-SG, X-organ-sinus gland tract; SG, sinus gland; OT, optic tract.

Briefly, the total area of each of the structures was defined as the reference space where the boundaries of the fluorescent regions of interest were outlined, and the volume fraction ($\hat{V}$v) of 5-HT and CHH immunoreactivity for each structure was estimated according to the method of Thompson (Thompson, 1930) using the equation:

$$\hat{V}\text{v}(Y,\text{ref}) = L_\text{p}(Y,\text{ref}) = P(Y)/P(\text{ref}),$$

where $P(Y)$ is the number of points contacting the phase of interest, P(ref) is the number of points hitting the reference space, L_p is the number of points defining the area of interest and (Y,ref) is the number of points defining the unit area of the section. Point counting is used to estimate areas, which are themselves estimators of volumes (Howard and Reed, 1998). In the present study, the volume fraction expresses the relative amount of CHH- or 5-HT-immunoreactive material in each of the structures. The criterion for selecting the immunoreactivity targets was a minimum ratio of background (black, 0 pixels) to green fluorescence (150 pixels) and red fluorescence (200 pixels). The results were verified using a Nikon confocal microscope (MRC 2100). Data are presented as the mean and the standard deviation (S.D.) of the immunofluorescent volume fraction, expressed as a percentage. For statistical analysis, analysis of variance (ANOVA) was followed by Scheffé *post-hoc* comparisons.

Results

Qualitative analysis

Light microscope localization revealed that CHH immunoreactivity was localized not only in the XO-SG complex but also in basally located tapetal cells of the retina (see Fig. 2). In both developmental stages, the immunopositive CHH perikarya in the eyestalk form a distinct group that lies at the latero-ventral side of the medulla terminalis (MT). The axons originating from these cells form an immunopositive tract that runs up to the sinus gland (SG), where CHH immunoreactivity is observed. Interestingly, only the basally located cells of the retina showed CHH immunoreactivity. This immunoreaction was specific because none of cell populations gave a signal in control incubations. Although no statistical test was performed for this qualitative light microscopic part of the study, we detected differences in the total areas of immunoreactivity between the PO2 and juvenile groups in the retina and XO-SG complex and in the number of cells in all the structures studied (not shown). However, in the two developmental stages, the general organization of both organs was similar. Fig. 1B,C shows a schematic representation of a typical ommatidium of the PO2 stage, of a juvenile crayfish and of the XO-SG complex in the eyestalk of the juvenile crayfish.

The locations of 5-HT-immunoreactive structures in PO2 and juvenile crayfish confirmed previous reports (Escamilla-Chimal et al., 1998). A positive reaction was present not only in several elements of the retina, namely in the crystalline cone and in the cytoplasm and axons of the retinular cells, but also

in the MT-XO, where 5-HT endings project to the periphery of the CHH perikarya. In contrast, the SG did not show 5-HT immunoreactivity in either stage or at any sampling time. The extent of 5-HT fluorescence varied according to the time of day.

Quantitative analysis

Volume fraction ($\hat{V}$v) estimation of green and red fluorescent material revealed cyclic differences in the amount of CHH- and 5-HT-immunoreactive material in the structures of the two developmental stages and in the relationship between the two, depending on the time of day. Figs 2A–F and 3A–F show confocal microscopic images of the tapetal cells, retinular cell axons and X-organ of the PO2 and juvenile crayfish at 08:00, 15:00 and 20:00 h.

Fig. 4 shows the cyclic changes in the relative amounts of 5-HT and CHH immunoreactivity in the retina and XO-SG complex of PO2 and juvenile crayfish at the three time points. Fig. 5 displays these data to provide a general overview.

Retina

In PO2 crayfish (see Figs 4, 5A), the stereological analysis revealed no statistical differences between 5-HT immunoreactivity in the retinular cells of the middle portions of the retina at 08:00 and 15:00 h ($MR\hat{V}$v, 16.82 % and 14.23 % respectively). Positive CHH immunoreactivity in the tapetal cells ($MR\hat{V}$v, 12.68 %) and positive 5-HT immunoreactivity in the crystalline cone (7.92 % of the distal retina) were observed at 15:00 h. At 20:00 h, 5-HT immunoreactivity persisted in the crystalline cone, accompanied by a peak in 5-HT immunofluorescence in the retinular cell axons ($PR\hat{V}$v, 91.71 %). At this time point, there

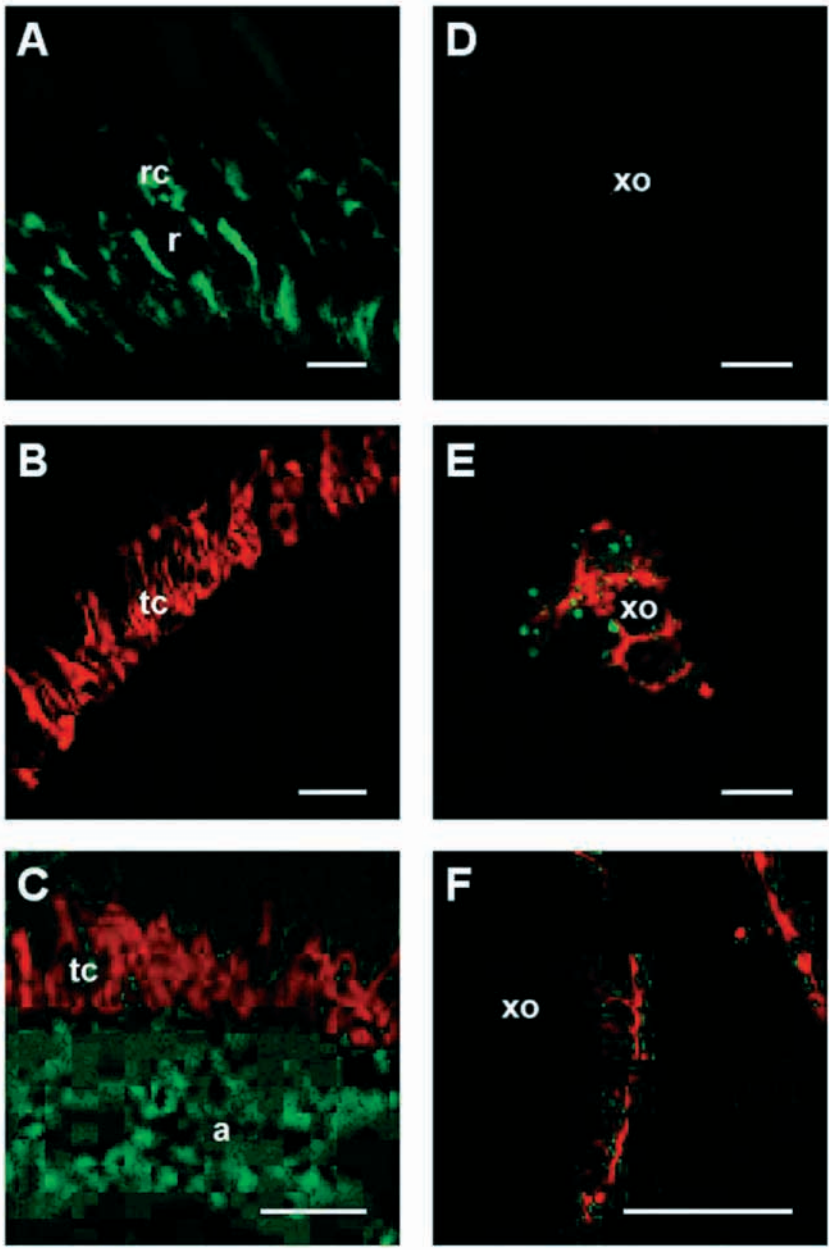

Fig. 2. The retina and X-organ/sinus gland complex of a post-embryonic stage two (PO2) crayfish showing daily changes in crustacean hyperglycaemic hormone (CHH) (red) and serotonin (5-HT) (green) immunoreactivity. (A) 08:00 h; retinular cell cytoplasm (rc) expressing 5-HT. Note that the rhabdoms (r) did not express immunoreactivity. (B) 15:00 h; tapetal cells (tc) expressing CHH. (C) 20:00 h; tapetal cells (tc) expressing CHH and retinular cell axons (a) expressing 5-HT. (D) The X-organ at 08:00 h (note the absence of immunoreactivity). XO, X-organ. (E) X-organ cells (xo) at 15:00 h expressing CHH, with nerve terminals expressing 5-HT. The yellow stain corresponds to sites where CHH and 5-HT co-occur. (F) X-organ cells (xo) at 20:00 h expressing CHH. Scale bars, 19.5 μm.

was an almost twofold increase in the volume fraction of CHH fluorescence in the medial retina, localized to the tapetal cells (28.17 %).

These values were reversed in juveniles (Figs 4, 5B). A maximal level of CHH immunofluorescence in the tapetal cells of the medial retina ($\hat{V}$v, 9.17 %) was accompanied by intermediate values of 5-HT immunofluorescence in the retinular cells axons (*PR*$\hat{V}$v, 33.04 %) and slight 5-HT immunoreactivity in the crystalline cone (*DR*$\hat{V}$v, 1.49 %) at 08:00 h. At 15:00 h, the amount of 5-HT-immunoreactive material was higher in the crystalline cone (*DR*$\hat{V}$v, 18.33 %) and was maximal in the cytoplasm and axons of the retinular cells (*MR*$\hat{V}$v, 75 %). At 20:00 h, fluorescence was still detectable in the axons of the retinular cells; in the proximal retina, the relative amount was greatly reduced ($\hat{V}$v, 18.53 %). Meanwhile, the tapetal cells again showed slight fluorescence in the CHH-immunoreactive cell bodies (*MR*$\hat{V}$v, 3.24 % of medial retina).

X-organ/sinus gland complex

As shown in Fig. 4 and in Fig. 5C, PO2 animals showed a maximal relative amount of CHH immunoreactivity in the SG ($\hat{V}$v, 53 %), a small amount of CHH in the XO-SG tract ($\hat{V}$v, 11.89 %) and no reaction at the level of the perikarya at 08:00 h. Levels in the SG and XO-SG tract decreased at 15:00 h, when the SG expressed only 27.7 % and the XO-SG tract was CHH-immunonegative. At this time of day, the XO was slightly immunopositive ($\hat{V}$v, 1.99 %). At 20:00 h, the average relative amount of CHH was unchanged in the XO ($\hat{V}$v, 2.18 %), but this was accompanied by higher levels of CHH in the XO-SG tract ($\hat{V}$v, 16.29 %); there was no CHH-immunoreactive material in the SG. We found a small relative

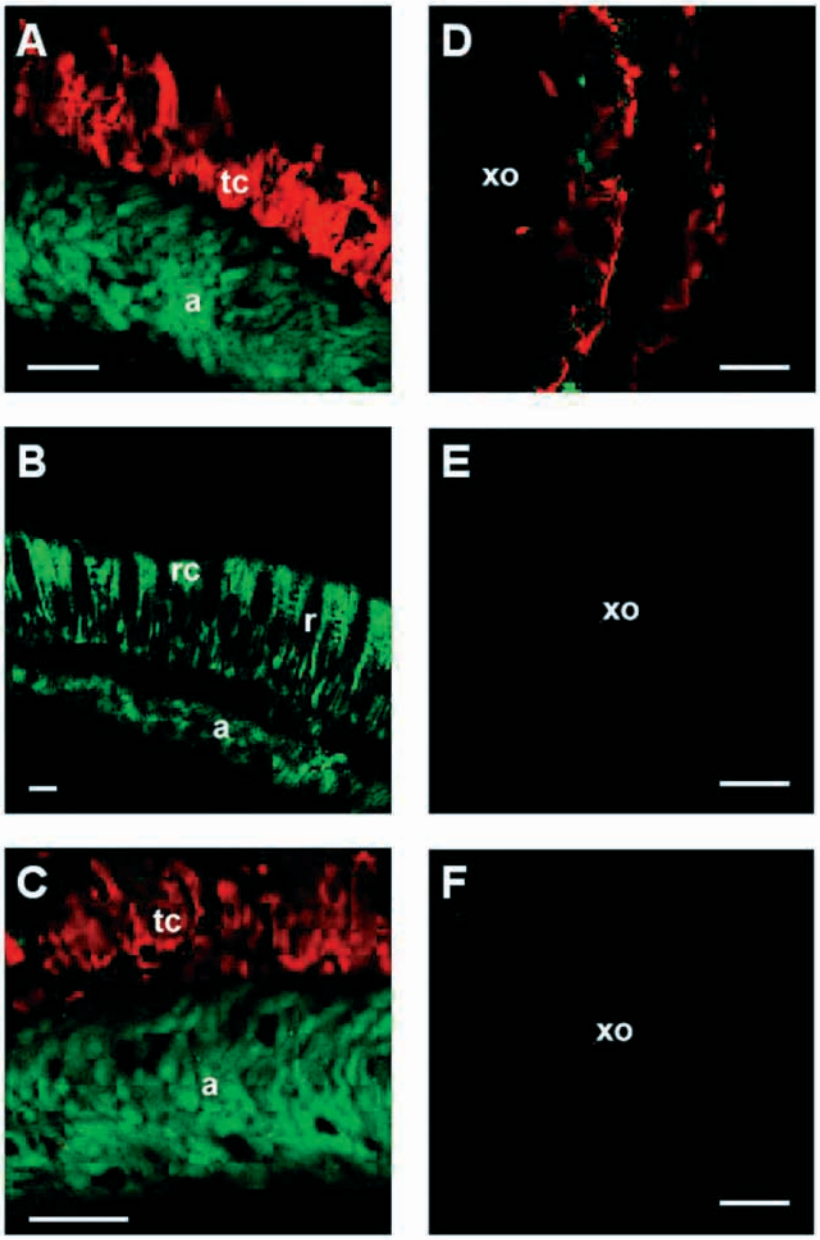

Fig. 3. The eyestalk of a juvenile crayfish showing daily changes in serotonin (5-HT) and crustacean hyperglycaemic hormone (CHH) immunoreactivity. (A) The retina at 08:00 h; tapetal cells (tc) expressing CHH and retinular cell axons (a) expressing 5-HT. (B) The retina at 15:00 h; retinular cell cytoplasm (rc) and axons (a) expressing 5-HT. Note that the rhabdoms (r) expressed no immunoreactivity. (C) The middle retina at 20:00 h; tapetal cells (tc) expressing CHH and retinular cell axons (a) expressing 5-HT. (D) The X-organ (xo) at 08:00 h; CHH cells expressing the hormone are surrounded by axon terminals containing 5-HT-immunoreactive material. (E,F) The X-organ at 15:00 (E) and 20:00 h (F) showing the absence of immunoreactivity. Scale bars, 19.5 μm.

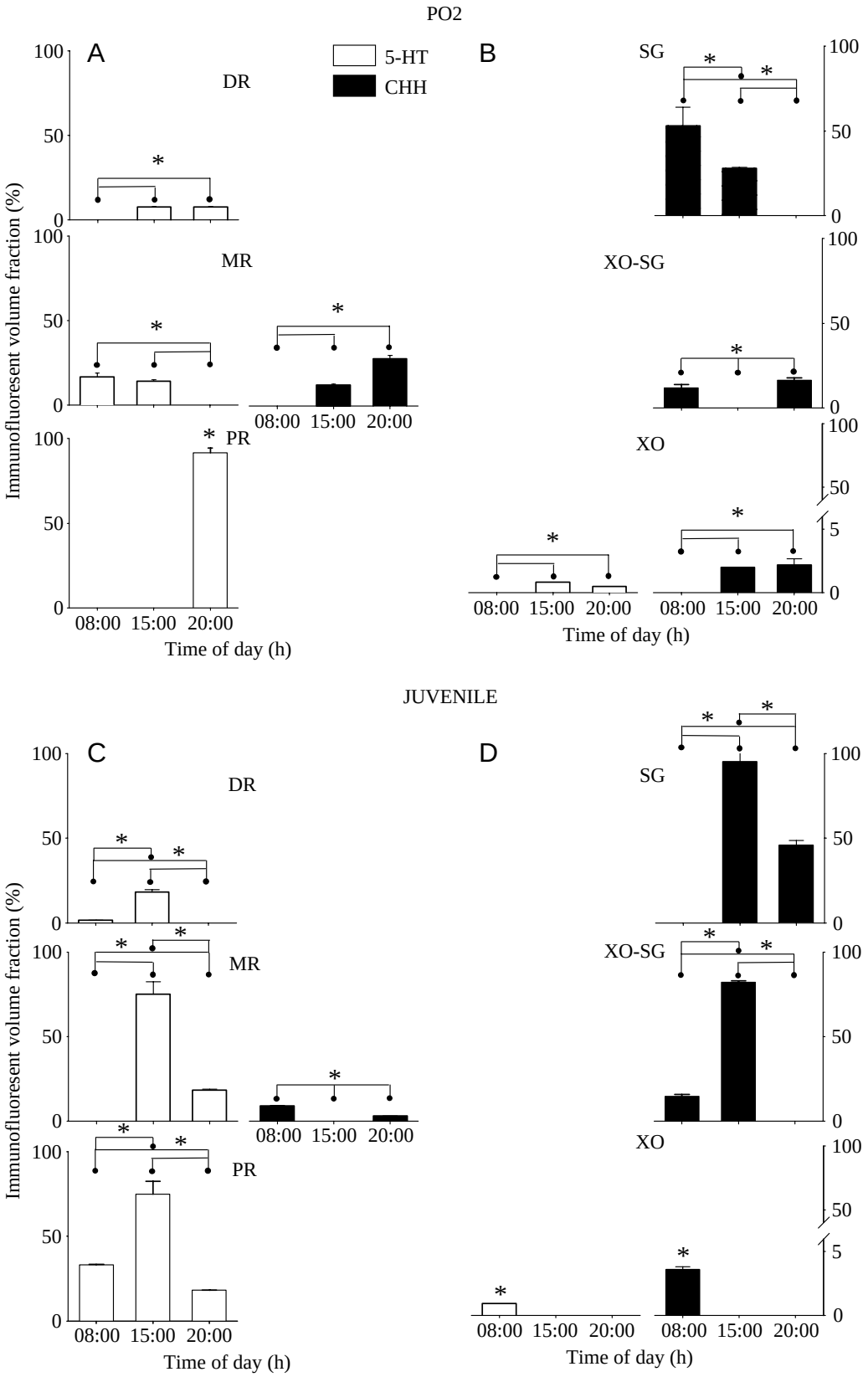

Fig. 4. Relationship between the time of day and serotonin (5-HT; open columns) and crustacean hyperglycaemic hormone (CHH; filled columns) immunoreactivity as a fraction of the total volume of the proximal (PR), medial (MR) and distal (DR) retina, and of the volume of the sinus gland (SG), the X-organ/sinus gland tract (XO-SG) and the X-organ (XO) of post-embryonic stage two (PO2) (A,B) and juvenile (C,D) crayfish. Values are means ± S.D. (N=9). Asterisks indicate significant difference (P<0.05). See text for further explanation.

amount of 5-HT immunoreactivity in the XO at 15:00 h ($\hat{V}$v, 0.82 %) and 20:00 h ($\hat{V}$v, 0.51 %), simultaneous with CHH immunoreactivity (Fig. 2E,F).

The situation was reversed in juveniles (see Figs 4, 5D). At 08:00 h, CHH fluorescence in the XO ($\hat{V}$v, 3.58 %) was accompanied by some fluorescence in the XO-SG tract ($\hat{V}$v,

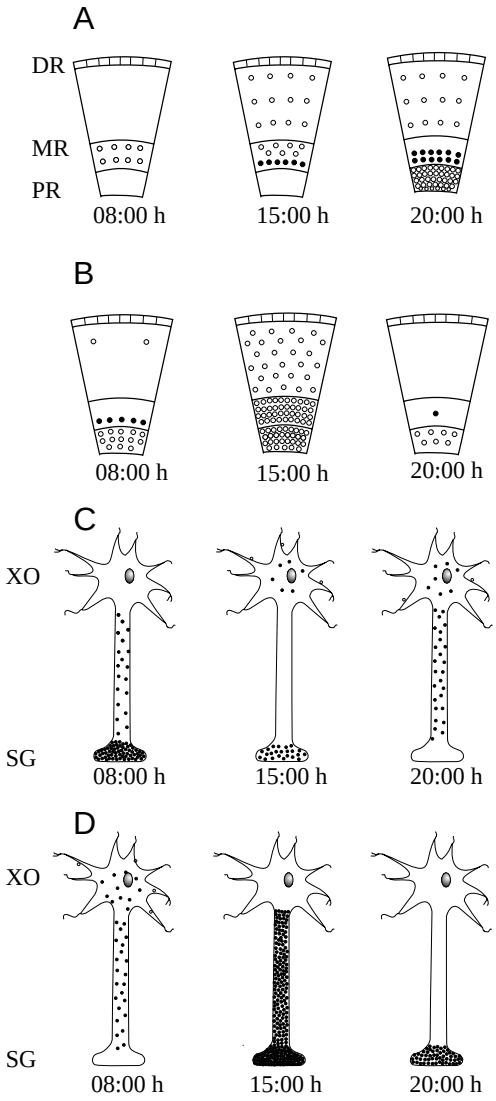

Fig. 5. Overview of the daily changes in crustacean hyperglycaemic hormone (CHH) and serotonin (5-HT) immunoreactivity. (A) The retina of post-embryonic stage two (PO2) crayfish, (B) the retina of the juvenile stage, (C) the X-organ/sinus gland (XO-SG) complex of the PO2 stage, (D) the XO-SG complex of the juvenile stage. (○) 5-HT, (●) CHH. The density of the symbols represents the degree of immunoreactivity. DR, distal retina; MR, medial retina; PR, proximal retina.

14.7 %) and no immunoreactivity in the SG. Relative amounts of fluorescence in the SG and XO-SG tract reached maximal values ($\hat{V}$v, 95.12 % and 83.36 %, respectively) at 15:00 h and decreased again to 45.9 % and 0 %, respectively, at 20:00 h. Confocal microscopy demonstrated 5-HT-immunoreactive fibres ($\hat{V}$v, 0.93 %) in the XO only at 08:00 h (Fig. 3D).

Statistical analysis revealed significant differences between the amounts of 5-HT immunoreactivity at the three time points in the different structures of PO2 (F=1819, P<0.01) and juvenile (F=249, P<0.05) crayfish and between the amounts of CHH-immunoreactive material at the three time points in PO2 (F=74.4, P<0.05) and juvenile (F=122.6, P<0.05) crayfish. The statistical comparisons from the Scheffé contrast test are shown in Fig. 4.

Discussion

The present study enabled us to visualize and quantify (i) CHH immunoreactivity in the basally located tapetal cells and 5-HT immunoreactivity in the retinular cells of the retina; (ii) the presence of 5-HT-immunoreactive nerve terminals branching into the CHH-immunoreactive cells of the MT-XO; and (iii) CHH immunoreactivity in the axons and terminals in the XO-SG tract and the SG. Stereological quantification of the immunofluorescent material in the respective regions in PO2 and juvenile developmental instars demonstrated cyclic differences in the presence and density of immunoreactivity for CHH and 5-HT, which exhibited maxima 12 h and 6 h out of phase, respectively. Although only three times of day were examined, this phenomenon may be an indication of the involvement of CHH and 5-HT in the mechanisms responsible of the maturation of the ERG circadian rhythm.

The amplitude of the ERG of juvenile crayfish varies in a circadian manner, reaching its highest values at night. However, during the early stages of development (PO2), the ERG shows ultradian oscillations that are superimposed on a circadian cycle, with maximal values during the day; i.e. at this stage, the crayfish behaves like a diurnal animal (Fanjul-Moles et al., 1987; Fanjul-Moles, 1998). Castañón-Cervantes et al. (Castañón-Cervantes et al., 1999) demonstrated a clear unimodal circadian rhythm for 5-HT concentration in the eyestalk of PO2 crayfish; this rhythm became bimodal in juveniles. When these rhythms were determined under 12 h:12 h L:D cycles, the maximal phase of the rhythm in the PO2 stage was at 20:00 h; the juveniles showed a maximum at 04:00 h and a second peak at 12:00 h. The findings of this study were interpreted as cyclic changes in the activity of serotonergic neurons in the brain related to the motor activity patterns of crayfish and as a brain serotonergic modulation of the XO-SG determining the circadian rhythm of the ERG. The cyclic changes in 5-HT levels reported here confirm the above results. Furthermore, *in situ* serotonergic structures are revealed that may be involved in the expression of the overt ERG circadian rhythm, showing quantitative differences between the PO2 and juvenile crayfish. The maturation of these structures could be involved in the physiological processes determining the coupling between the pacemaker and the effectors of the expression of this rhythm.

Gorgels-Kallen and Van Herp (Gorgels-Kallen and Van Herp, 1981) and Gorgels-Kallen and Meij (Gorgels-Kallen and Meij, 1985) have reported the presence of CHH immunoreactivity using light and fluorescence microscopy in adult and post-larval crayfish (*Astacus leptodactylus*). They described CHH immunoreactivity in a group of neurosecretory cells in the MT-XO, in fibres forming part of the XO-SG tract and in the axon terminals making up the SG in that crayfish species. The present study confirms these findings in another species of crayfish, *Procambarus clarkii*, during postembryonic development.

However, this is the first study to our knowledge that reports the presence of CHH immunoreactivity in accessory cells of the retina, the reflecting pigments cells. These cells are sometimes referred to as secondary pigment cells or tapetal cells and, in adult crayfish, they are located in a single layer around the ommatidia (Rao, 1985). Previous studies on crayfish development (Hafner et al., 1982; Escamilla-Chimal et al., 1998) indicate that these cells are neither clearly differentiated nor located in the retina before the PO2 developmental stage. However, in this instar, their location is similar to that in adults. There is mounting evidence that reflecting light is only one of the functions of tapetal cells and that pigment and retinular cell(s) are metabolically linked, the former subserving the latter (for a review, see Meyer-Rochow, 1999).

The relationship between the cyclic changes in 5-HT and CHH immunofluorescence occurs at the same time of day in the retinular cell axons and the tapetal cells, but in the case of PO2 crayfish this relationship occurs only at 20:00 (Fig. 5A). This finding led us to conclude that this phenomenon must be related to the maturation of the daily sensitivity cycle of the eye. Interestingly, in the present work, CHH immunoreactivity in the tapetal cells and in the MT-XO of PO2 and juvenile instars occurs at the same time of day, but 12 h out of phase (Fig. 5C,D). Even though, to our knowledge, there are no reports of neural communication between the retina accessory cells and the XO-SG complex, a humoral communication between these structures is possible. In decapod crustaceans, the basement membrane seems to be an incomplete barrier to the haemolymph (Odselius and Elofsson, 1981). These authors proposed that one function of the cellular portion of the basement membrane is to satisfy the nutritional and hormonal requirements of the accessory cells of the retina. Hence, it is possible that CHH produced by the XO and bypassing this barrier could enter the tapetal cells and serve a different metabolic function related to the eye's sensitivity. However, an alternative and most interesting hypothesis should not be excluded. It is possible that the tapetal cells could be a supplementary locus for CHH synthesis. In spite of the fact that the tapetal cells are not considered to be neural cells in crayfish, as in other decapods, these cells must emanate from the medial proliferation zone where the ommatidia, the lamina ganglionaris and the medulla externa originate. During differentiation of the ommatidia, the proximal group of cells, in contact with the lamina ganglionaris, gives rise to sensory cells and the reflecting pigment (Elofsson, 1969). New neural and paraneural sources of CHH apart from the eyestalk neurosecretory tissues have recently been reported (Chang et al., 1999; Chung et al., 1999). Hence, our proposition seems plausible and deserves further experiments to validate it.

In previous studies on a different species of crayfish, a correlation was reported between the daily changes in the secretory dynamics of the CHH-producing cells in the MT-XO and haemolymph glucose levels (Gorgels-Kallen and Voorter, 1985). These studies indicated that the accumulation of CHH peptidergic material in the CHH-producing cells in adult *Astacus leptodactylus* is stimulated 2 h before the beginning of darkness, resulting in a transfer of CHH granules into the axons of the XO-SG tract. According to these authors, these granules reach the axon terminals in the sinus gland after the onset of darkness, when a strong release of CHH occurs into the haemolymph. Although, in the present study, only three times of day were examined, there seems to be agreement between all the above data and our findings in *Procambarus clarkii* juveniles. At this developmental stage, the only peak of immunofluorescence in the CHH-producing cells of the MT-XO, accompanied by slight CHH immunoreactivity in the XO-SG tract, was at 08:00 h, 1 h after lights-on. MT-XO CHH immunoreactivity subsequently disappeared, but the SG and the XO-SG tract showed their highest values of CHH immunoreactivity (see Figs 4, 5D), indicating a transfer of CHH to the SG terminals. Later, at 20:00 h, 1 h after the onset of darkness, the MT-XO and the XO-SG tract showed no CHH immunoreactivity and immunoreactivity in the SG decreased from 95.1 to 45.92 %, suggesting the previously described release of CHH into the haemolymph. Serotonin-like immunoreactive somata and fibres have been localized in the medulla terminalis (Eloffson, 1983; Sandeman et al., 1988), where the X-organ neurosecretory cells receive their synaptic input (Andrew and Saleuddin, 1978; Andrew, 1983). In addition, ultrastructural studies have demonstrated serotonergic synaptic structures on the axonal ramifications of the CHH-producing cells in the X-organ of crayfish *Astacus leptodactylus* (Van Herp and Kallen, 1991). Furthermore, physiological experiments in which 5-HT was injected produced a release of CHH from the SG axon terminals in several species of crustacean (Strolenberg and Van Herp, 1977; Martin, 1978; Kallen, 1988). Hence, one can postulate, as already proposed (Gorgels-Kallen and Voorter, 1985), that 5-HT may induce the transfer of CHH granules from the MT-XO to the SG *via* synaptic modulation. As summarized in Fig. 5C, the SG of the PO2 instar of *P. clarkii* has its highest level of CHH immunoreactivity at 08:00 h, and this is followed by a subsequent reduction in the level at 15:00 h, indicating a release of CHH after the onset of light. Moreover, the CHH and 5-HT immunoreactivity found in the XO-MT suggests that the 5-HT stimulus to the synthesis of CHH occurs during the afternoon (between 15:00 and 20:00 h), i.e. the situation is reversed with respect to that in juveniles (08:00 h, Fig. 4). These findings may indicate a phase reversal during the maturation of the CHH rhythm similar to that reported for the ERG and motor activity rhythms, and in coincidence with the crayfish changing from diurnal to nocturnal behaviour. The present results need further physiological experiments to clarify the role of CHH in the mechanisms responsible for the genesis of the sensitivity rhythm of the crayfish eye.

The authors are greatly indebted to Arturo Hernández-Cruz and Roman Vidal-Tamayo for their technical assistance in confocal microscopy. We also thank Julio Prieto-Sagredo for his technical help in the laboratory and in preparing the figures. Isabel Pérez-Montfort corrected the final version of the manuscript. This work is part of the doctoral thesis of Elsa G. Escamilla-Chimal and was partially supported by PAPIT IN 209397 and CONACYT 31858-N.

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