The Circadian System of Crayfish: A Developmental Approach

MARÍA LUISA FANJUL-MOLES* AND JULIO PRIETO-SAGREDO
Institutional Affiliation: Lab. Neurofisiología Comparada, Departamento de Biología, Facultad de Ciencias, Universidad Nacional Autónoma de México, Mexico City 04510, Mexico

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ABSTRACT Adult crayfish exhibit a variety of overt circadian rhythms. However, the physiological mechanisms underlying the overt rhythms are controversial. Research has centered on two overt rhythms: the motor activity and the retinal sensitivity rhythms of the genus *Procambarus*. The present work reviews various studies undertaken to localize pacemakers and mechanisms of entrainment responsible for these two rhythms in adult organisms of this crustacean decapod. It also describes an ontogenetic approach to the problem by means of behavioral, electrophysiological, and neurochemical experiments. The results of this approach confirm previous models proposed for adult crayfish, based on a number of circadian pacemakers distributed in the central nervous system. However, the coupling of rhythmicity between these independent oscillators might be complex and dependent on the interaction between serotonin (5-HT), light, and the crustacean hyperglycemic hormone (CHH). The latter compound has, up until now, not been considered as an agent in the genesis and synchronization of the retinal sensitivity rhythm. *Microsc. Res. Tech. 60: 291–301, 2003.*

INTRODUCTION

The different species of freshwater crayfish are largely nocturnal and display a great variety of circadian rhythms controlled by periodic functions in the nervous system. Kalmus (1938) concluded that the eyestalk neurosecretory system was the source of control of crayfish activity rhythm. Welsh (1941) proposed that variations in the activity of nervous centers were responsible for the hormonal control of the rhythmic migration of retinal shielding pigments in this animal. Since these early works, a large body of experimental evidence has been gathered, which suggests that the central nervous and neuroendocrine systems control the generation and coordination of many behavioral and physiological circadian rhythms such as locomotor activity (for review see Page, 1981), retinal shielding pigment migration, electroretinogram (ERG) amplitude (for review see Aréchiga et al., 1993), heart rate (Bojsen et al., 1998; Hernández-Falcón and Ramón, 1998; Pollard and Larimer, 1977) as well as metabolic and endocrine functions (Aréchiga and Mena, 1975; Fingerman, 1955; Fingerman and Lowe, 1957; Kallen et al., 1990; Kleinholz, 1966; Rodríguez-Sosa et al., 1994). Recently, rhythms of melatonin (Balzer et al., 1997) and acetyl-*N*-transferase (Agapito et al., 1995), and various parameters of the glutathione circadian rhythms (Durán-Lizarraga et al., 2001) have been reported. However, analysis of the neural basis of circadian rhythmicity is quite limited, possibly because the localization and identification of the various components of this animal's circadian system, clock pacemakers, and entrainment, are controversial. Some of these aspects are reviewed here, focusing on the two rhythms controlled by restricted portions of the nervous system functioning as circadian pacemakers: the motor activity rhythm and the ERG amplitude rhythm.

MOTOR ACTIVITY RHYTHM

Circadian activity rhythm in adult crayfish has been extensively studied. Surgical interference experiments as well as ablation studies have been undertaken in an effort to localize pacemakers and mechanisms of entrainment responsible for this rhythm. In 1938, Kalmus reported that the removal of the eyestalk of crayfish *Potamobius* resulted in an increase in activity as well as an apparent loss of circadian activity rhythm. Other authors (Roberts, 1944; Schallek, 1942) confirmed these results in *Cambarus*. However, Page and Larimer (1975a) demonstrated in *Procambarus clarkii* the persistence of rhythmicity following eyestalk ablation, although neural isolation of thoracic motor centers from the supraesophageal ganglion by section of the circumesophageal connection always resulted in aperiodic behaviour. The above-mentioned authors proposed the supraesophageal ganglion as the origin of the locomotor activity rhythm. This ganglion is coupled to the thoracic locomotor centers via axons in the circumesophageal connectives. Controversially, Fuentes-Pardo and Inclán-Rubio (1981) reported persistence of this rhythm in decerebrate animals. There is scant information about the circadian photoreceptors and entrainment pathways of activity rhythm in crayfish. Page and Larimer (1972, 1976) demonstrated that the photoreceptors participating in the entrainment are

*Correspondence to: María Luisa Fanjul-Moles, Ap. Pos. 41-630, C.P. 11000, México, D.F. México. E-mail: mlfm@hp.fciencias.unam.mx

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not located in retina or caudal ganglion, but in the supraesophageal ganglion. Simultaneously, they proposed that the neurosecretory elements of the eyestalk are not required for the expression of activity rhythm, but could play some role in rhythm entrainment by light cycles. However, Fuentes-Pardo and Inclán-Rubio (1987) ascertained in *Procambarus bouvieri* the sixth abdominal ganglion as a locus of circadian photoreception.

ERG AMPLITUDE RHYTHM

The ERG amplitude rhythm is the result of day-night variations in the amplitude of the photoreceptor response to a light pulse of a standard intensity. It has been proposed (Aréchiga et al., 1993) that these variations depend on: (1) changes in the sensitivity of the photoreceptor, (2) the position of the absorbing proximal pigment granules (PP) or retinular pigment (Halberg and Elofsson, 1989) within the photoreceptor itself, and (3) the position of the absorbing distal pigment granules (DP). Both pigments are mainly ommochromes (Ghidalia, 1985) and they are the effectors of a photo-motor response to light that provides each ommatidium with its own regulating pupil (Rao, 1985; Stavegna, 1979). In darkness, the PP are retracted in the axonal end of the retinular cell, thus leaving the photoreceptor exposed to light. Under illumination, the PP are dispersed, shielding the photoreceptors from light. The distal pigment is located inside the distal pigment cells, also known as primary pigment cells (Rao, 1985). These cells are found around the crystalline cone with their processes extending apically towards the cornea cells and basally towards the basement membrane. These pigments are also retracted under darkness and they disperse under illumination.

The control of these photomechanical movements is under hormonal influence (Fernlund, 1971; Kleinholz, 1961), but regulated by light (Kleinholz, 1966; Rao, 1985). The proximal pigment migrates as a direct response to light and darkness (Frixione et al., 1979; Olivo and Larsen, 1978) and the distal pigment migrates by the reflex control of neurohormones from the optic peduncle, such as the distal pigment dispersing hormone (DPH) (Aréchiga et al., 1993) and the red pigment concentrating hormone (RPCH) (Garfias et al., 1995). There is evidence that movement of the proximal pigment could be controlled by the membrane potential of the retinular cells in some crustaceans (Ludolph et al., 1973) or by a serotonergic modulation in crayfish (Frixione and Hernández, 1989). It has long been known that the proximal pigment maintains a circadian rhythm of mechanical movement under constant darkness (DD) (Bennitt, 1932) whereas the distal pigment maintains a similar rhythm under both DD and constant light (LL) (Welsh, 1930). In the crayfish *Procambarus bouvieri* and *P. clarkii,* both distal and proximal retinal pigments display circadian rhythms of movement, but the reflecting pigment contained in the secondary pigment cells or tapetal cells, is stationary (Rao, 1985). The rhythmic movements of the former pigments are accompanied by a circadian variation of retinal sensitivity measurable as changes in the amplitude of the ERG (Aréchiga and Fuentes-Pardo, 1970). In this rhythm, the ERG amplitude is greatest when the pigments are in the dark-adapted position and lowest during the light-adapted state (Aréchiga and Wiersma, 1969). It is remarkable that to date, the ERG amplitude rhythm in crayfish has not been associated with other circadian rhythms in the cellular elements of the retina or the optic lobes, but only to these two retinal pigments.

However, it has been shown that virtually no structural component of the compound eye of an arthropod can be regarded as unchanging over the day:night cycle (Meyer-Rochow, 1999). In *P. clarkii,* there is a diurnally constant level of synthesis and assembly of new rhabdom constituents (Hafner and Tokarski, 1988). In an effort to localize pacemakers and entrainment pathways that control the elements that contribute to the circadian amplitude of the ERG rhythm, many experiments involving surgical and chemical lesions as well as ablation studies, implant and organ isolation have been undertaken in two species of crayfish *Procambarus bouvieri* and *Procambarus clarkii.* These studies, albeit contradictory, suggest that the ERG rhythm is controlled by neural and neuroendocrine mechanisms that depend on the brain and neurosecretory structures of the optic lobe (in particular the XO-SG), but there is an intrinsic oscillation that depends on retina. Briefly, after the removal of the brain, some authors report the suppression of the ERG amplitude and retinal shielding pigment rhythms (Aréchiga et al., 1973), while others report the persistence of both rhythms but at low amplitude (Barrera-Mera, 1976). After the complete section of the protocerebral tract the ERG is abolished (Page and Larimer, 1975b), but with low intensity stimuli a low level rhythm can be seen (Barrera-Mera, 1978). After eye explant to host, the grafted eye assumed the retinal screening pigment rhythm of the host (Aréchiga, 1977). Bisection of the brain at the midline eliminated the ERG amplitude rhythm from both eyes, but when the brain was split slightly off-center, the ERG rhythm of the eye associated with the larger half persisted (Page and Larimer, 1975b).

Organ culture of the eyestalk revealed the persistence of a low amplitude ERG rhythm without the oscillations of the retinal shielding pigments (Sánchez and Fuentes-Pardo, 1976). Recent reports mention the persistence of ERG rhythm in isolated preparations of retina and lamina kept in darkness (Aréchiga and Rodriguez Sosa, 1998). All experiments performed in intact animals showed that both eyes display phase-locked oscillations (Barrera-Mera, 1978; Page and Larimer, 1975b, 1976; Smith and Larimer, 1977) but maximum uncoupling occurs when a small lesion is placed in the center of the protocerebral tract (Larimer and Smith, 1980), after optic tract sectioning (Larimer and Smith, 1980), or in split brain preparations (Barrera-Mera, 1976). Since the rhythm in each eye persists, the above implies a mutually entrained bilateral system of oscillators. Barrera-Mera and Block (1990) demonstrated that an isolated protocerebrum-eyestalk complex exhibits phase-locked robust ERG rhythms that can be desynchronized after surgical bisection of the protocerebrum. The results of these experiments indicate there is a brain input to the optic lobe that controls the different effectors of the ERG circadian rhythm. Earlier, Larimer and Smith (1980) had proposed that the coupling information could be carried

out by neuron neurosecretory fibers coursing from the brain to the optic lobes, i.e., the brain controls the optic lobe neurosecretions responsible for the pigment movements. All of the above indicates that the putative pacemakers and their neural input to the optic lobes are required for the expression and the bilateral synchronization of the ERG. This axonal coupling could be provided by axons running from one side to the other of the SOG or by efferent fibers in the optic lobe. Some of these axons seem to contain serotonin (Aréchiga et al., 1990; Sandeman et al., 1988, 1990). A second pathway that contributes to the coupling and synchronization of this rhythm seems to be a hormonal pathway since proximal and distal pigments in the retina are responsive to such blood-borne agents as DPH, RPCH, and 5-HT. The presence of a circadian rhythm of DPH (Aréchiga and Mena, 1975) and RPCH (Rodriguez-Sosa et al., 1994) has been demonstrated in isolated eyestalk of *Procambarus clarkii.*

The fact that a circadian rhythm can be entrained by a light cycle demonstrates that the pacemakers have access to a photoreceptor. As in the case of the motor activity rhythm, Page and Larimer (1975b) showed that the ERG amplitude rhythm of *P. clarkii* is entrained by an extra-retinal and extra-caudal photoreceptor located in the supraesophageal ganglion. However, Fuentes-Pardo and Inclán-Rubio (1987) reported phase shifting of the ERG amplitude rhythm by local illumination of the sixth abdominal ganglion of *P. bouvieri.* Recently, Sandeman et al. (1990) by means of electrophysiological and immunohistochemical techniques demonstrated the presence of photoreceptors in the brain of the crayfish *Cherax destructor*. These brain extra-retinal photoreceptor axons end in the protocerebral bridge where serotonin immunoreactive fibers project to the external medulla and the region of the sinus gland (Sandeman et al., 1988). After this gland's surgical removal, disrupted ERG and pseudopupil (glow) circadian rhythms appeared in the crayfish *Procambarus clarkii* (Hernández–Falcón et al., 1987; Moreno-Sáenz et al., 1987). All the above suggests a direct serotonergic input from the brain photoreceptors to the circadian system. All of the studies reviewed so far seem to fit the conceptual model proposed by Larimer and Smith (1980). The circadian rhythm of retinal sensitivity is controlled by a pair of extraretinal photoreceptors and three pairs of oscillators (the retinular cells in the two eyes, the neurosecretory system in the two optic lobes, and a pair of putative brain pacemakers).

AN ONTOGENETIC APPROACH TO THE STUDY OF BOTH RHYTHMS

Although the general model described above is valuable, the neural mechanisms reported to be involved in the expression and synchronization of the activity and ERG amplitude circadian rhythms of crayfish are sometimes controversial. One way of elucidating them is by studying these two rhythms during the different stages of post-embryonic development, when functions underlying the rhythms begin to show some temporal organization that eventually has fully circadian characteristics. For the expression of an overt circadian rhythm, it is necessary that both the anatomical and physiological frameworks of the circadian system have reached maturity and established a functional relationship with their constituting elements (Davis, 1981). Hence, during ontogeny changes can be observed in the main components of the circadian system: pacemakers, pathways for the overt rhythm, and elements participating in the entrainment. All of the above may be analyzed by the developing parameters of the rhythm under study. The period, phase, relative amplitude, and oscillation level of the activity and ERG amplitude rhythms, as well as their abilities to synchronize to light, are some of the markers that will change during development and can contribute to the knowledge of the mechanisms involved in the temporal organization of crayfish.

Therefore, we approached the study of the motor activity and ERG amplitude circadian rhythms focusing on (1) the temporal development of clock pacemakers, (2) the temporal development of clock entrainment and synchronization.

BEHAVIORAL AND ELECTROPHYSIOLOGICAL EXPERIMENTS

Adult *Procambarus clarkii* reproduce three times during the 12 months of the year in the laboratory where light, temperature, and water pH are controlled. All animals used in the experiments were born in the laboratory from field-collected animals and acclimatized and mated there in controlled light:dark conditions and temperature. In the period from fertilization to hatching, the eggs are attached to the mother. Once hatched, the first postembryonic stage (PO1) organisms are attached to the inside of the mother's chorion. Their large cephalothorax is filled with egg yolk and their eyes are sessile. Antenna and antennula are curved posteriorly. Telson and uropods are not yet differentiated into separate appendages. Twelve days later, at laboratory temperature (22°C), moult occurs and the second postembryonic stage (PO2) emerges. These organisms are larger and with less yolk stored in the cephalothorax. Their eyes are stalked and the telson and uropods are differentiated, they are able to move freely around the mother, but they are not particularly active. PO2 stage lasts about 15 days, then the organism moults again resulting in a miniature young, morphologically very similar to the adult. It is highly mobile and accepts food (Escamilla-Chimal et al., 1998). The young crayfish keeps moulting at least eight more times during the first year, the age at which it attains sexual maturity (Castañón-Cervantes et al., 1995). The activity and the ERG amplitude rhythms of crayfish at all the above-mentioned ages are difficult to evaluate. The endocrine changes due to growing and the light sensitivity changes occurring in retina and neural structures, mask both rhythms. The activity rhythm, when it is evident, is neither clear nor robust, requiring quantitative statistical methods to be evaluated.

We used the methods described below to study the development of the components controlling circadian activity and ERG amplitude rhythms. To study the activity rhythm, all animals, divided into groups according to age and number of moults, were placed individually and unrestrained in small double-compartment aquaria made of black acrylic plastic. One of the compartments was a tunnel simulating a burrow and

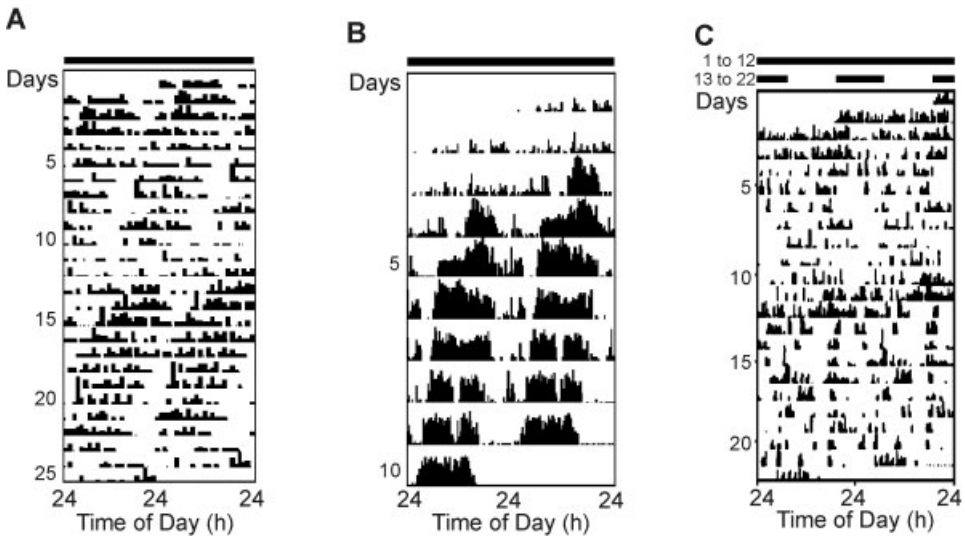

Fig. 1. Characteristics of the free running and entrained activity rhythm of young crayfish during development. **A:** Double plotted activity record of one of the youngest animals. Solid bars indicate the amount of movement. **B:** Activity record of a juvenile crayfish 130 days old. **C:** Activity rhythm of a 140-day-old entrained to LD 12:12 cycles. Note the bimodal activity rhythm consisting of a burst of activity during lights on and longer burst associated with lights "off."

the other compartment was a wide chamber. Light was provided by a neon lamp with monochromatic filters when necessary and controlled by a programmable timer. Motor activity was monitored with a motor recording activity system reported elsewhere (Fanjul-Moles et al., 1996). All data were collapsed in bins, plotted in actograms, and quantitatively analyzed. To characterize the ERG amplitude rhythm, test animals grouped by the mentioned developmental stages were fastened with wax and individually housed in chambers under controlled temperature and darkness, and retinal potentials to white or monochromatic light were recorded for 10 or more days. The crayfish eyestalk was immobilized and a steel microelectrode was implanted through the cornea, to record the ERG (Fanjul-Moles et al., 1987). Retinal potentials relayed to a pre-amplifier were registered with a polygraph (Grass model 7 or 79) for five or more days. A photostimulator delivered a test light flash of fixed intensity every 3 min. The resulting ERG was relayed to a personal computer and its amplitude measured and plotted vs. time to analyze its oscillations. To accomplish the above-mentioned objectives, the young crayfish activity and ERG amplitude rhythms were analyzed under various experimental protocols.

Development of the Clock Pacemaker. We recorded the activity rhythm of unrestrained animals from four different age groups, from 10 days, when they start to move freely, to 20 weeks old. The animals were maintained under constant darkness (DD) and temperature (Fanjul-Moles et al., 1996). The activity of animals in all age groups varied between rhythmic and aperiodic patterns. The free-running rhythm of the crayfish may come and go due to changes in the internal environment, such as those related to moulting. Activity of 52 animals aged between 10 and 140 days maintained under constant conditions was analyzed, based on the rhythmicity criterion established at 0.01 confidence level in the periodogram (Sokolove and Bushnell, 1978). Only 50% of youngest animals showed a circadian rhythm. This percentage increased with increasing age of the animals until reaching 90% in the oldest (Fanjul-Moles et al., 1996; fig. 5). The average estimated period of rhythmic animals was not significantly different among groups, ranging from $\tau = 25.0 \pm 2.1$ h in youngest animals to $\tau = 24.3 \pm 1$ h in the eldest. The activity/rest ratio (α/ρ) diminished from 2.12 to 1.3, respectively. The unrestrained crayfish under constant conditions exhibited a unimodal motor activity from the first post-embryonic stages on, indicating the presence of a functional pacemaker from hatching (Fig. 1A). The presence of the rhythm as well as its parameters, i.e., period, level of activity, and phase, seem to require some time to become established (Fig. 1B). Changes in activity and in period suggest the maturation of neural and endocrine elements of the brain and eyestalk proposed for the control of this rhythm (Page and Larimer, 1975a). The changes in the probability of occurrence of the rhythm as well as changes found in its parameters indicate the clock is present, but is not expressed because the coupling with the motor activity requires different maturation times in different organisms.

Ultradian periodical changes in ERG amplitude (Fanjul-Moles et al., 1987) under constant darkness could be ascertained from the first day after hatching, when the young were detached from their mothers. However, from this age up to 30 days old the crayfish showed no overt circadian rhythm in ERG amplitude or in shielding pigment reflex, assayed by the glow area. From 30 to 150 days, the free-running ERG amplitude rhythm attains a period stability of around 24 hours (Fig. 2A,B). Simultaneously, the complex parameters of the rhythms, α/ρ ratio, and night/day amplitude ratio tend to the adult rhythm values (Fig. 2C). This indicates that the coupling strength between the pacemaker and the neural and endocrine pathways to the retinal effectors, depends largely on the maturity of the nervous and endocrine systems and the retina. The fact that activity and ERG amplitude rhythms appear at different ages and that their amplitude increases in different ways (Fanjul-Moles et al., 1987, 1996) does not necessarily mean that they are controlled by different pacemakers. These differences may reflect when

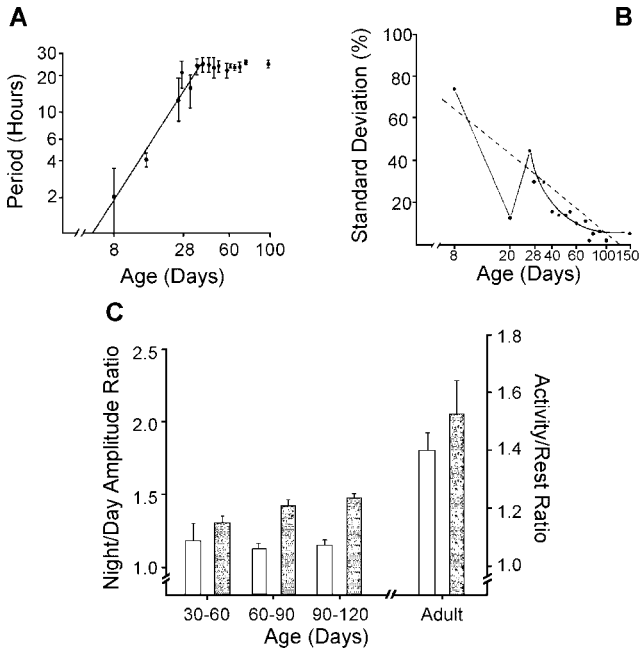

Fig. 2. **A:** Logarithmic relationship between crayfish age and the ERG amplitude rhythm period value. Note that the ERG rhythm of the younger animals shows short ultradian period values. The rhythm's period value enlarges throughout the crayfish maturation to attain circadian values in the elder animals. Each point represents the average of five animals. The bars represent the standard deviation (SD). **B:** Changes between crayfish age and the percentage of standard deviation. Note the decreasing values of this parameter associated with the animal's growth, demonstrating the progressive ERG circadian rhythm stability. **C:** Changes in the circadian parameters of the ERG rhythm during development. Note the progressive increase in these parameter (see text). Modified from Fanjul-Moles et al., 1987, with permission of the publisher.

and how fast the overt functions are plugged into central pacemakers. Both rhythms have different effectors, which are complex in the case of the ERG rhythm. The different elements constituting the afferent and efferent pathways to the pacemakers as well as the effectors of the rhythms may have different times of maturation. But an interesting fact is that both rhythms seem to attain stability at the same age, about 140 days.

Development of the Entrainment Mechanisms. To investigate the temporal course of the entrainment mechanisms, two different kinds of experiment were conducted: (1) the parametric and non-parametric effect of white light on the activity rhythm by means of protocols consisting of complete and skeleton photoperiod (Fanjul-Moles et al., 1996, 1998) and (2) the effect exerted by a single 15-min bright light pulse on the free-running ERG rhythm analyzed by means of a phase-response curve (PRC) (Fuentes-Pardo et al., 1992).

Complete light-dark 12:12 cycles. Each experiment began by recording the activity pattern of an animal under constant darkness for at least 10 days to search for possible changes in the exogenous and endogenous nature of this rhythm related to the L/D transition. Then the animals were transferred to LD 12:12 cycles for another 10 days. Animals of different ages could be active in either the dark or the light period. Generally, after the L/D or, the D/L transition, a burst of activity of variable duration occurred. Only the older animals showed a clearer bimodal activity rhythm consisting of a brief burst of activity during "lights on" and a longer burst of activity associated with "lights off" (Fig. 1C). In these experiments, we used relatively high illumination due to the low sensitivity of the crayfish's eye during the early developmental stages (Fanjul-Moles and Fuentes-Pardo, 1988). But this high level illumination might have masked the rhythm in some cases. Some animals ceased their activity after the L/D transition, remaining in the burrow during the dark period of post-entrainment. This made it difficult to assess the entrainment mechanisms in some experiments. However, circular statistical analysis revealed that the rhythms of younger crayfish tend to set to LD, clustering their maximal activity phase mainly in the photophase, and those of older crayfish showed a bimodal distribution of maximal activity phases associated with lights "off" and "on" (Fanjul-Moles et al., 1996, fig. 10).

Skeleton Photoperiods. These experiments were carried out to investigate the non-parametric effect of light on the activity rhythm of the postembryonic stages of crayfish, and to elucidate the development of the mechanisms of entrainment, trying to rule out the masking effects of light. In a first experiment (Fanjul-Moles et al., 1998), young crayfish separated in different groups according to age and moult, were individually monitored with a motor activity recording system as described above, in constant photic conditions. For all groups, three trials with different skeleton photoperiod (SP) imitating complete LD 8:16, LD 12:12, and LD 20:4 were conducted, each followed by exposure to a period of constant darkness. The activity rhythm and its entrainment were evaluated. All ages showed a circadian rhythm able to synchronize to the different SP. This capability increases with age. The activity rhythm of the older crayfish was synchronized by the light pulse corresponding to the dusk, shifting their activity onset close to sunset as expected for a nocturnal species.

The ability of this rhythm to synchronize to SP from the earliest stages of development indicates an early development of the circadian sensitivity to the light, suggesting that the photo-entrainment in crayfish is mediated by extraretinal photoreceptors. Other experiments demonstrated that the activity rhythm of young crayfish deprived of retina and lamina synchronizes to blue and red monochromatic light SP with advances and delays (Miranda-Anaya and Fanjul-Moles, 1997). This confirmed the extra-retinal nature of this rhythm photoentrainment.

ERG amplitude rhythm phase-response curve. Light synchronization of the ERG rhythms seems to be a complex mechanism (Fuentes-Pardo et al., 1992). Due to the ultradian nature of the ERG rhythm from hatching to 30 days of age, these mechanisms could be explored in animals older than one month. In a first group composed of crayfish aged from 4 to 8 weeks, the light pulse induced advances or delays independently of the circadian time (CT) at which the pulse was applied. In a second group composed of crayfish from 8 to 20 weeks old, the light stimulus induced phase advances or delays that were dependent on the CT. The

above was associated with the diurnal habits of the crayfish of the first group, suggesting that the young crayfish have not yet fully developed the subjective night and day.

However, this conclusion seems controversial because, as stated above, experiments on activity rhythm entrainment revealed that this rhythm is able to entrain to different SP depending on the animal's subjective time. On the other hand, young crayfish are able to synchronize their ERG rhythm in response to monochromatic light and the period of the rhythm depends on the wavelength of the light stimulus (Fanjul-Moles et al., 1992), and caudal monochromatic stimulation produces a phase shifting of the rhythm (Bernal-Moreno et al., 1996). In an effort to understand the ontogeny of circadian rhythms in crayfish, some mathematical models on rhythm genesis and entrainment have been developed (Fuentes-Pardo and Lara-Aparicio, 1995; Fuentes-Pardo et al., 2001). However, these models are sometimes reductionist, bypassing important physiological evidence such as masking. Masking and entrainment have been proposed as flexible and not preserved characters of the circadian system. During the post-embryonic development of crayfish, both characteristics are present, appearing at different times. This fact, which deserves further research, may lead to misinterpretations. Hence all interpretations brought out by behavioral experiments should be analyzed in view of cellular and biochemical evidence.

NEUROCHEMICAL EXPERIMENTS

The circadian system incorporates three main functional divisions: input, pacemaker, and output. The final output of the ERG amplitude rhythm is the retina. In general, with the exception of one report (Aréchiga and Rodríguez-Sosa, 1998), crayfish retina appears not to function as a circadian generator. It receives the circadian signal from central neural and endocrine oscillations. Hence, the ERG ultradian oscillations reported for this rhythm may be due to the maturation of the retinal structures—photoreceptors, pigment accessory cells, and dioptric system (crystalline cone and tract)—and to their modulation by the neural and endocrine processes.

Young crayfish (PO1 and PO2) are devoid of a retinal pigment shielding reflex (Fanjul-Moles et al., 1987). Between the first, second, and third instar of crayfish, changes in proximal and distal pigment density and differentiation of the rhabdoms, retinular cells, and crystalline cones are spectacular. By the second instar, there is a continuous elongation of the crystalline tracts, the rhabdoms, and increased pigmentation (Hafner et al., 1982, 1991; Hafner and Tokarski, 1998). At the third instar, these authors describe further changes during the juvenile stage, including differentiation of the cellular elements, increased pigmentation and its separation into discrete retinal locations. Furthermore, the crayfish eye seems to change from an apposition to a superposition one along these three instars as reported for other species (Land, 1996). The absorbing pigment in the retinular cells is the first to appear (Elofsson, 1969); the reflecting one is the last (Halberg and Elofsson, 1989).

Therefore, the photomotor reflex shown by the circadian rhythms in the glow and the ERG amplitude are absent in the young probably because: (1) proximal and distal pigments are not fully differentiated or are not responsive to the DPH; (2) the neuroendocrine mechanisms involving the complete maturation and the control of the *medulla terminalis* X-organ-sinus gland complex (XO-SG) and thus the circadian control of DPH secretion (Aréchiga and Mena, 1975; Aréchiga et al., 1985) with the concomitant circadian movement of the distal pigment, are not functional until the crayfish is older than 30 days. This last proposition is feasible because in some crustaceans the SG is not recognized until the third stage after hatching (Pyle, 1943). In the crayfish *Astacus leptodactylus*, this complex has been detected as functional immediately after hatching (Strolenberg, 1979), but synthetic activity seems to increase throughout development (Gorgels-Kallen and Meij, 1985). In *Procambarus clarkii,* unpublished histological data from our laboratory reveal the presence of XO-SG since the PO1 instar, but there is no photomotor reflex until the third instar when the retinal elements are fully differentiated. However, in all probability the full maturation of the neuroendocrine system will be accomplished when the crayfish attains sexual maturity. Young crayfish growth seems to fit the simple model of moult control proposed by Passano (1953): the eyestalk neurosecretory tissues are a source of moult-inhibiting hormone (MIH) that, when released from SG, negatively regulates the synthesis of the moulting hormones (ecdysteroids) by the Y-organs. The duration of this cycle increases with the juvenile age, probably due to the increasing titers of MIH as a consequence of the maturation of the XO-SG complex. Overt expression of the ERG rhythm could involve the maturation of a central serotonergic system for the neural modulation of the ERG rhythm. In adult *Cherax destructor*, serotonin immunoreactive fibers project from the protocerebral bridge to the external medulla and to the sinus-gland (Sandeman et al., 1990). The medulla terminalis of the crayfish *Pacifastacus leniusculus* contains several 5-HT immunoreactive perykaria and fibers leading to the brain (Elofsson, 1983) where the X-organ neurosecretory cells receive their input (Andrew and Saleudin, 1978; Andrew, 1983). In *P. clarkii*, serotonin-containing efferent fibers reach the retina (Aréchiga et al., 1990). In *Cherax destructor,* all components of the serotonergic system seem present in the juvenile organisms (Sandeman and Sandeman, 1990).

The problem of neural modulation during development was approached by: (1) investigating daily changes in 5-HT and tryptophan-hydroxylase (TPH) in the final output of the rhythm, the retina (Escamilla-Chimal et al., 1998), and (2) investigating developmental circadian changes in 5-HT concentration in two structures proposed to contain pacemakers in *P. clarkii*: the SOG and the eyestalk (Castañón-Cervantes et al., 1999). On the one hand, results of the first work confirm the structural changes reported elsewhere in crayfish during development (Hafner et al., 1982). From PO2 on, crayfish showed statistically significant retinal 5-HT and TPH changes related to time of day. 5-HT was found mainly in the retinular cells and the crystalline cones and TPH in retinular and accessory cells. Maximal TPH immunoreactivity always precedes 5-HT immunoreactivity, suggesting that the retina is a

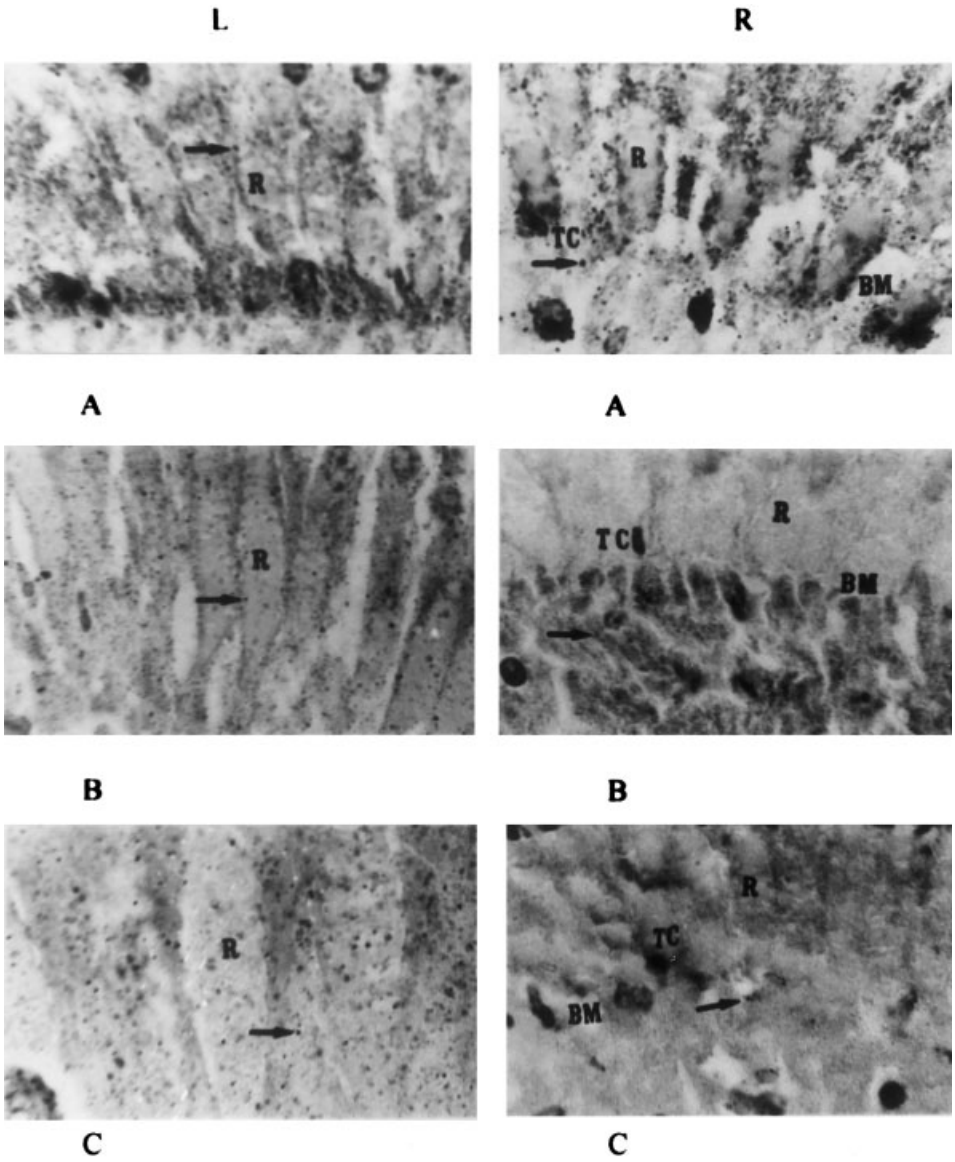

Fig. 3. **Left:** 5-HT-like immunoreactivity (arrow) along the development of crayfish in the middle region of retina at 1500 h. **A:** PO1, 1,268×; **B:** PO2 1,332×; **C:** juvenile organism 1,227×. Note the changes in size and characteristics of rhabdoms throughout the development. 5-HT-like immunoreactive granules seem to be located in rhabdoms and retinular cells. **Right:** TPH-like immunoreactivity in the middle region of retina of a PO2 crayfish at three different hours of day. **A:** 0800 h, 1,042×; **B:** 1500 h, 990×; **C:** 2000 h, 850×. Note the TPH-like immunoreactive granules (arrow) inside the tapetal and retinular cells. Note the changes in the number of TPH-like immunoreactive granules and in structure among the three times of day. R, rhabdoms; TC, Tapetal cells and their nucleus; BM, basement membrane. Reproduced from Escamilla-Chimal et al., 1998, with permission of the publisher.

possible locus of serotonin synthesis from the PO2 to juvenile instar (Fig. 3). Interestingly, daily retinal structural changes that may be related to 5-HT immunoreactivity were found in PO1, PO2, and juvenile stages of this species. These findings suggest a circadian serotonin regulation of rhythmic size changes as reported for insects (Meinertzhagen and Pyza, 1996, 1999).

On the other hand, both the cerebral ganglion and the eyestalk of *P. clarkii* showed circadian rhythms in 5-HT content, determined by reverse-phase HPLC, during development (Castañón-Cervantes et al., 1999). As development advances, pulsatile variations with a period value of about 9 to12 h are superimposed on the circadian component. These 5-HT circadian changes seem to be correlated with the endogenous and exogenous peaks of the activity rhythm and reflect changes in the activity of brain serotonergic neurons involved in patterns of crayfish behavior. The release of 5-HT in response to electrical stimulation of the eyestalk (Rodríguez–Sosa et al., 1997) together with changes in the electrical activity of the serotonergic neurons in the brain have been reported in different species of adult crayfish (Sandeman and Sandeman, 1994; Sandeman et al., 1995). Electrolytic lesion in the protocerebrum where serotonergic neurons project (Sandeman et al., 1995) caused changes in the circadian rhythm of activity in the juvenile *P. clarkii* (Fig. 4). Interestingly, the 5-HT pulsatile variations appearing in the latest stages of development both in the brain and eyestalk (Castañón-Cervantes et al., 1999, figs. 3 and 4) could be related to brain regulation of the complex mechanisms underlying the ERG rhythm. This pulsatile relationship suggests period variations in target-organ (i.e., eyestalk-brain) response, as proposed for some neuroendocrine rhythms in mammals (Haus et al., 1998).

Dispersing pigment hormone and RPCH are neuropeptides secreted by the XO-SG complex whose

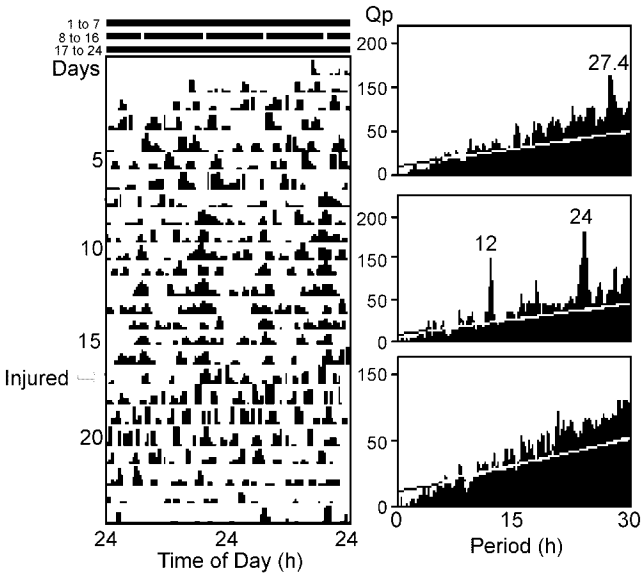

Fig. 4. Actogram of a juvenile crayfish under different experimental conditions, accompanied by statistical analysis. Days 1–7 show a free running activity rhythm under constant darkness. The statistical analysis (periodogram) at right depicts a significant circadian rhythm accompanied by ultradian bouts of activity. From day 8 to 16, the crayfish was placed under a skeleton photoperiod mimicking a LD 12:12.and it displayed an entrained activity rhythm (τ = 24 h). From day 17 to 24, after surgically interfering protocerebrum medial structures, the crayfish was placed again in DD; note the disruption of the activity rhythm The actogram and associated periodogram (right) show the arrhythmic pattern (see text). Modified from Fanjul-Moles (1998) with permission of the publisher.

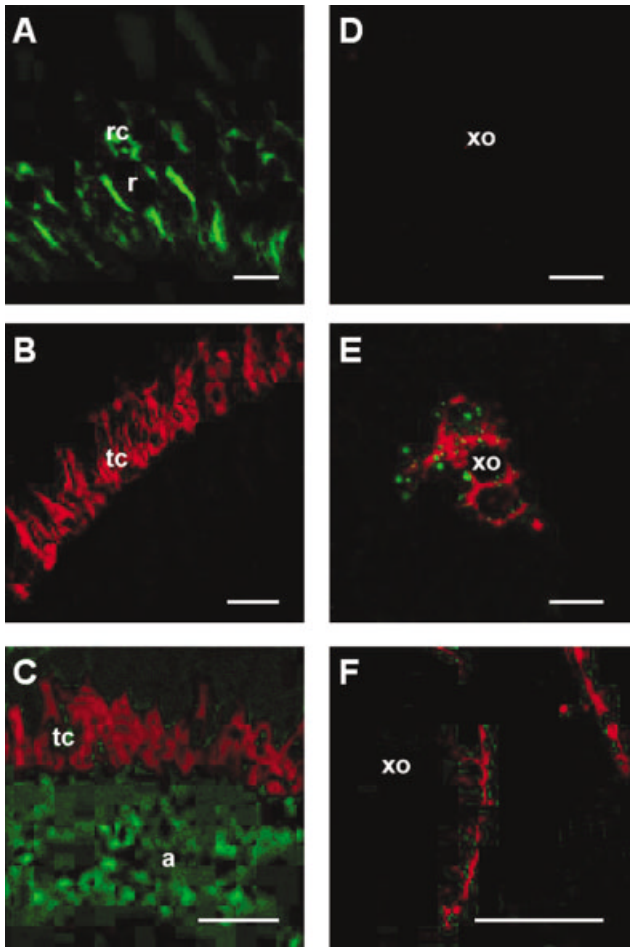

Fig. 5. Photomicrographs of double immunolabeled sections of the retina and X-organ-sinus gland complex of a PO2 crayfish (CHH in red and 5-HT in green) showing daily changes. **A:** 0800 h, retinular cell cytoplasm (rc) expressing 5-HT. Note that the rhabdom (r) did not express immunoreactivity. **B:** 1500 h, tapetal cells expressing CHH. **C:** 2000 h, tapetal cells expressing CHH and retinular cells axons (a) expressing 5-HT. **D:** The X-organ (xo) at 0800 h. Neither CHH-like nor 5-HT-like immunoreactivity is observed. **E:** X-organ cells at 1500 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 at 2000 h expressing CHH. All scales bars = 19.5 μm. Reproduced from Escamilla-Chimal et al. (2001) with permission of the publisher. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rhythms can be modulated by 5-HT, determining the ERG final output. Indeed, it cannot be ruled out that other neuropeptides that have been localized in the eyestalk of different crustacean species (for review see Fingerman, 1997; Van Herp, 1998) affect the amplitude and rhythm of the ERG. The effect of different neuropeptides on the ERG of *Orconectes limosus* has been described (Gaus and Stieve, 1992).

Recently Escamilla-Chimal et al. (2001) demonstrated developmental cyclic differences in the presence and density of the crustacean hyperglycaemic hormone (CHH) immunoreactivity in *P. clarkii*. The CHH was found in the tapetal cells of the retina and in the axons and terminals of the XO-SG tract and SG. The above was correlated with 5-HT immunoreactivity in the retinular cells and in nerve terminal branching in the CHH-population of the MT-XO (Figs. 5 and 6). These changes, summarized in Figure 7, support the idea that rhythms of the XO-SG complex secretory activity involving CHH circadian changes, affect the circadian sensitivity of the eye. Furthermore, the differences found between the PO2 and juvenile stages suggests that both CHH and 5-HT are key factors in the development of the circadian rhythm. The SG of the PO2 instar of *P. clarkii* shows its highest level of CHH immunoreactivity at 0800 h, followed by a subsequent reduction in the level at 1500 h, indicating a release of CHH after the onset of light. The CHH and 5-HT found in the XO-MT suggests that the 5-HT stimulus of CHH synthesis occurs during the afternoon (between 1500 and 2000 h), i.e., the phenomenon is reversed with respect to the juveniles. The above indicates a phase reversal during the CHH maturation rhythm similar to that reported for ERG and motor activity rhythms and in coincidence with the crayfish changing from a diurnal to nocturnal species (Fanjul-Moles et al., 1987, 1996).

The presence of CHH and TPH immunoreactivity in pigmented accessory retinal cells, such as the tapetal cells, and the increasing evidence that the reflecting pigment is only a part of this cell's function, whose main functions seem to provide metabolic support to retinular cells (Meyer-Rochow, 1999) as proven in insects (Saravelos and Tsacopoulos, 1995), open new per-

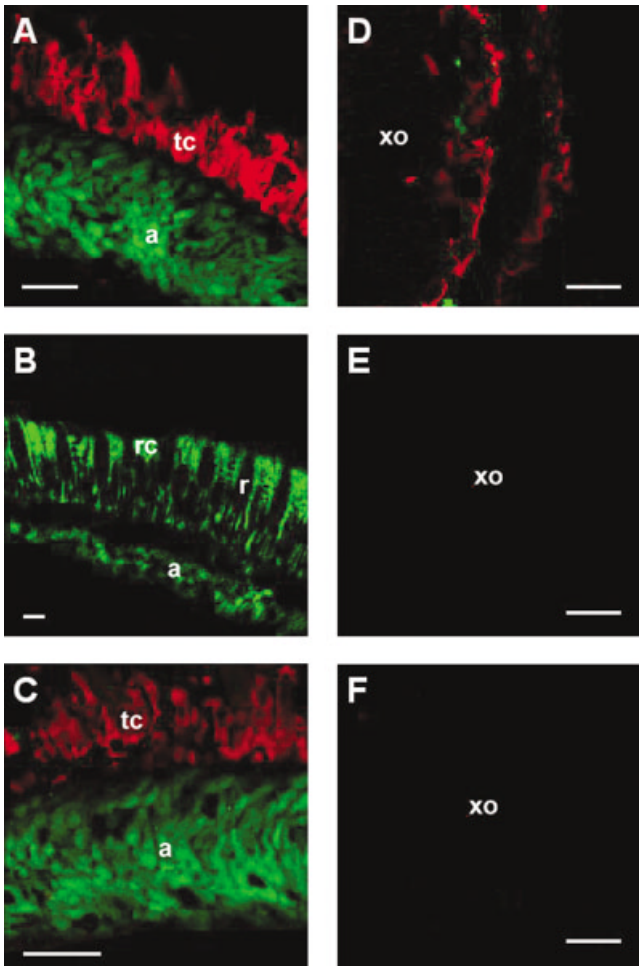

Fig. 6. The eyestalk of a juvenile crayfish showing daily changes in 5-HT (green) and CHH (red) immunoreactivity. **A:** The retina at 0800 h express CHH in tapetal cells (tc) and 5-HT in retinular cell axons (a). **B:** The retina at 1500 h, the retinular cell cytoplasm (rc) and axons expressing 5-HT. **C:** The middle retina at 2000 h express CHH in tapetal cell and 5-HT in retinular cell axons. **D:** The X-organ (xo) at 0800 h, cells expressing CHH are surrounded by axon terminals containing 5-HT immunoreactive material. **E,F:** The X-organ at 1500 and 2000 h, respectively, did not show any immunoreactivity. All scales bars = 19.5 μm. Reproduced from Escamilla-Chimal et al. (2001) with permission of the publisher. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

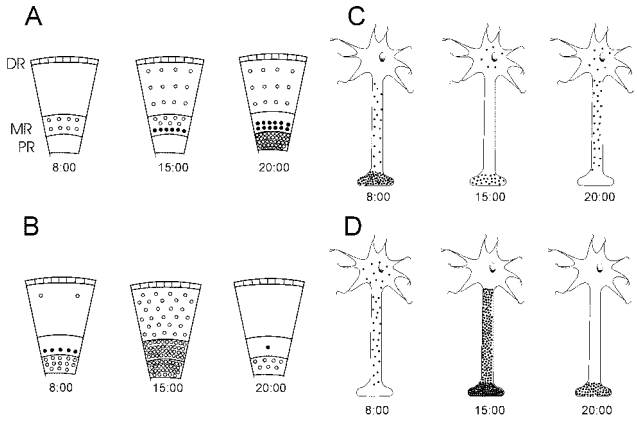

Fig. 7. Overview of the daily changes in CHH and 5-HT immunoreactivity. **A:** The retina of PO2 stage crayfish. **B:** The retina of juvenile stage. **C:** The X-organ/sinus gland (XO-SG) complex of a PO2 stage crayfish. **D:** XO-SG complex of juvenile crayfish. 5-HT (open circles), CHH (closed circles). The density of the symbols represents the degree of immunoreactivity. DR, distal retina; MR, medial retina; PR, proximal retina. Reproduced from Escamilla-Chimal et al. (2001) with permission of the publisher.

spectives for the crayfish retina studies. On the one hand, data from our laboratory suggest that isolated retinal and optic lobe cells pooled from juvenile and adult *P. clarkii* secrete CHH (Escamilla-Chimal et al., 2002). This secretion depends on 5-HT. These findings suggest retina is a new locus of neuropeptide synthesis that could contribute to the daily changes in ERG amplitude.

On the other hand, Aréchiga and Rodríguez-Sosa (1998) have revealed PER immunoreactivity in retinal photoreceptors as well as in glial cells of isolated retina and lamina complex of adult *P. clarkii*. The *per* expression in a structure is considered proof of its involvement in the circadian clock, if this expression is rhythmic (for review see Allada et al., 2001). This fact needs to be proved, but these experiments reinforce and bring out again the old proposition of a retinal circadian oscillator.

In the last decade, our knowledge of structure and function of the decapod neuropeptides has increased dramatically. Microanalysis and molecular genetics techniques have lead to new findings on crustacean endocrinology.. One of the most important was the demonstration that several of the neurohormones are highly related in structure. An interesting discovery was that neuropeptides involved in the control of moulting (MIH) and gonadal development are related to CHH, together forming a group known as the "CHH family" (for review see Webster, 1998; Van Herp, 1998). As mentioned above, during the moult cycle of crayfish, dramatic changes in their activity mask the activity and ERG amplitude rhythms and may lead to a misinterpretation of the mechanisms of genesis and entrainment. In the near future, new technical tools will contribute to the analysis of molecular and cellular changes leading to fuller understanding of the circadian behaviour of this decapod. It will be interesting to revise the old models and propositions, particularly in relation to a possible efferent neural control of the circadian rhythms of retina as has been proven in other arthropods (Barlow et al., 1980; Kass and Barlow, 1992; Ruta et al., 1998). The developmental approach seems to be valid for further research.

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