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Original article

Daily variations of immunoreactive melatonin in the visual system of crayfish

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In crustaceans, melatonin has been detected in the central nervous system and some other organs. The aim of this study was to analyze the melatonin content in the visual system of *Procambarus clarkii*, by means of radioimmunoassay, at different day-night phases. We have also studied the action of exogenous melatonin on the main properties of the electroretinogram (ERG) circadian rhythm. Experiments were conducted with 25 specimens maintained under controlled conditions of 16°C and 12 h of light alternating with 12 h of darkness. Eyes where cut in dim red light and shock frozen with liquid nitrogen and pulverized in a mortar until a homogenous powder was obtained. Melatonin was extracted with acetone, followed by centrifugation, diluted with an equal volume of aqua bidest to ensure freezing at –80°C for at least 90 min and lyophilization at the same temperature. Lyophilizates, after having been dissolved in RIA buffer, were used for determinations of melatonin. Long-term recordings of electrical responses to light (ERG) were obtained for 10 or more consecutive days. At the 5th day, a single dose of melatonin was injected and its effects on amplitude and period of the ERG circadian rhythm were measured. Melatonin concentrations differed considerably depending on the circadian time and attained a maximum during dark phase. Among the crustaceans, *Procambarus clarkii* represents the first case in which melatonin peaks during the night following the typical pattern known in the majority of organisms. After melatonin injection, period and amplitude of the ERG circadian rhythm were increased. This effect suggests the involvement of melatonin in the oscillators underlying the generation and expression of circadian rhythms in crayfish. (© Elsevier, Paris)

crayfish / melatonin / circadian rhythms / electroretinogram

BIOLOGICAL MODELS

Melatonin is a hormone secreted on a circadian basis by the vertebrate pineal gland, reaching elevated peripheral blood levels at night. However, it has been detected in all animal species studied so far, in a great variety of plants, algae and unicells (Balzer and Hardeland, 1996).

Among crustaceans, it has been found in the eyestalks of *Carcinus maenas* showing a constant concentration at both day and night, but with seasonal differences between May and November (Vivien-Roels and Pévet, 1986). In the eyestalks of the prawn *Macrobrachium rosenbergii*, daily variations of this indoleamine have been reported to peak during the photophase (Withyachumnarnkul *et al*, 1992). In the giant tiger shrimp, *Penaeus monodon*, melatonin was identified in the optic lobe, but diurnal variability has not been studied to date (Withyachumnarnkul *et al*, 1995).

Crayfish displays multiple physiological functions and behaviors that underlie endogenous rhythms of various frequencies (Sánchez and Fuentes-Pardo, 1977; Page, 1981; Beltz, 1988; Thurman, 1988; Fuentes-Pardo and Hernández-Falcón, 1993; Fuentes-Pardo *et al*, 1996). Circadian rhyth-

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micity has been reported for motor activity and for amplitude of electrical response to light (electroretinogram, ERG) by visual photoreceptors. When a crayfish is put under constant environmental conditions of temperature and illumination, the amplitude of the ERG shows an evident circadian profile (Fuentes-Pardo and Inclán-Rubio, 1981).

Although this circadian rhythm has been well characterized, up to date no information is available about the oscillators' organization underlying the circadian pacemaker as well as the overt rhythm. In consequence, the presence of melatonin and its putative role in circadian rhythmicity is an open problem in this species.

Under conditions of constant light or constant darkness, ERG oscillations persist but show differences in frequency and amplitude (Fuentes-Pardo and Inclán-Rubio, 1981). Since the rhythm of the ERG represents the best studied circadian oscillation in *Procambarus clarkii*, we analyzed the melatonin content in eyestalks by means of radioimmunoassay and the effect of this indoleamine on the main characteristics of the ERG rhythm.

The presence of melatonin in crayfish has been suggested (Dubbels and Elofsson, 1989). Daily variations of serotonin-N-acetyltransferase and melatonin content in a crayfish from Valladolid, Spain have been reported (Agapito *et al*, 1995). Their results differed from our own experience as discussed later. To date, immunocytochemical studies are scarce in crustaceans.

MATERIALS AND METHODS

Experiments were conducted in adult crayfish, *Procambarus clarkii*, of either sex, from Chihuahua, Mexico. Once the animals had arrived in laboratory, they were placed into oxygenated tap water, fed three times a week and kept under controlled conditions of temperature (16°C) and light (12 h of light alternating with 12 h of darkness, *ie* LD 12:12; photophase between 07.00 am and 19.00 pm). Light intensity on the water surface was approximately 100 Lux. Animals were randomly sampled. Experiments were performed during the 24-h cycle. At different circadian times, animals were anesthetized by cold, decapitated, and their eyestalks removed with fine scissors under dim red light. Eyestalks were shock frozen in liquid nitrogen and pulverized in a mortar until an homogeneous powder was obtained. Melatonin extraction was performed with cold acetone followed by centrifugation, dilution with an equal volume of aqua bidest to ensure freezing at –80°C for at least 90 min and lyophilization at the same temperature. Lyophilizates were dissolved in 300 µL RIA buffer (tricine 0.1 M, pH 5.5); 200 µL of diluted melatonin antibody (purchased from Stockgrand Ltd, UK) were added to the sample, and the mixture was incubated for 15 min at room temperature; 100 µL of [^{3}H] melatonin (specific activity 86 Ci/mmol, Amersham, Braunschweig, Germany) were added and

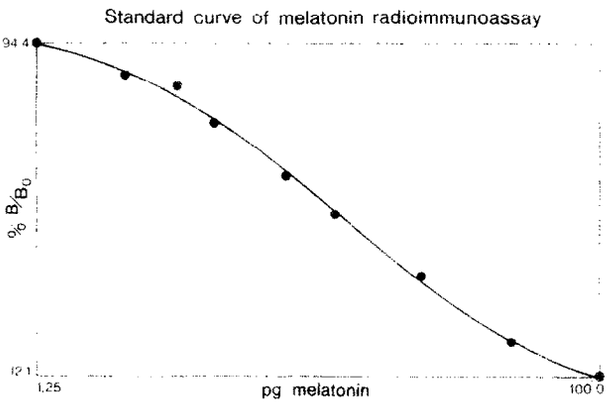

Fig 1. Binding profile of melatonin standards in an assay run. Closed circles, observed means of standard triplicates.

the mixture was incubated at 4°C for 18 h. Bound and free ligands were separated by incubation with 500 µL of dextran-coated charcoal for 30 min at 4°C and centrifuged at 1500 *g*, 4°C for 15 min. Thereafter, 800 µL supernate were aliquoted into scintillation vials containing 7.2 mL of the scintillation mixture and incubated 1 h before counting. The standard curve was simultaneously determined by the same procedure using 0 to 600 pg melatonin (fig 1). Accuracy and specificity of the assay were verified according to criteria of serial dilution, parallelity, and cross-reactivity with various tryptophan metabolites. The detection limit of RIA was 10 pg/mL. The specificity of the antiserum has been tested several times by different groups (Webley *et al*, 1985; this paper, table I).

Long-term ERG recordings were performed under constant temperature (16°C) and darkness for 10 or more days. Crayfish were fixed by a cork attached to the carapace and immersed into an aquarium with aerated tap water covering their gills. A brief flash light (10 µm, 180 Lux) was delivered by a photostimulator 3 min each. A semimicroelectrode (5 µm at tip) was impaled into the cornea and connected to a DC preamplifier. HI and HII ERG components were recorded, but only HI amplitude was considered in the analysis. A single dose of melatonin (100 µL, 10^{-4} M), diluted in saline solution for crayfish, was injected into the basis of the first pair of ambulatory legs at different circadian times. Periodogram analysis was made before and after the melatonin administration.

RESULTS AND DISCUSSION

Melatonin immunoreactivity was detected in all samples assayed. The concentration ranged from 30 to 1813 pg/eyestalk, depending on the time of removal. Highest levels were measured during the dark phase showing a pronounced peak at CT 20; the minimum was around CT 8 (fig 2).

The radioimmunoassays clearly show the presence of melatonin in *Procambarus clarkii*. Moreover, the quantities of indoleamine measured varied with the time of the day exhibiting a maximum during

Table I. Cross-reactivity of tryptophan and tryptophan metabolites tested in the present study*.

Substance	*Cross-reactivity (%)*
Tryptophan	< 0.0000003
Hydroxytryptophan	< 0.001
Methoxytryptophan	< 0.001
Tryptamine	< 0.001
Tryptophol	< 0.001
Hydroxytryptamine	< 0.001
Hydroxytryptophol	< 0.001
Hydroxyindole acetic acid	< 0.001
Indole acetic acid	< 0.001
Methoxytryptamine	< 0.001
Methoxyindole acetic acid	< 0.001
Indole propyl acid	< 0.001
Indole lactic acid	< 0.001
Kynuramin	< 0.001
Kynurenin	< 0.001
Harmine	< 0.001
Pinoline	< 0.001
Methoxytryptophol	< 0.002
N-acetylserotonin	< 0.03
N-acetyltryptophan	< 0.1
Hydroxymelatonin	< 0.33

*The IC_{50} of the corresponding compounds was determined and cross-reactivity is expressed as percentage of melatonin binding to the antibody (IC_{50} for melatonin is 45 pg/tube). Data from Pöggeler (1992).

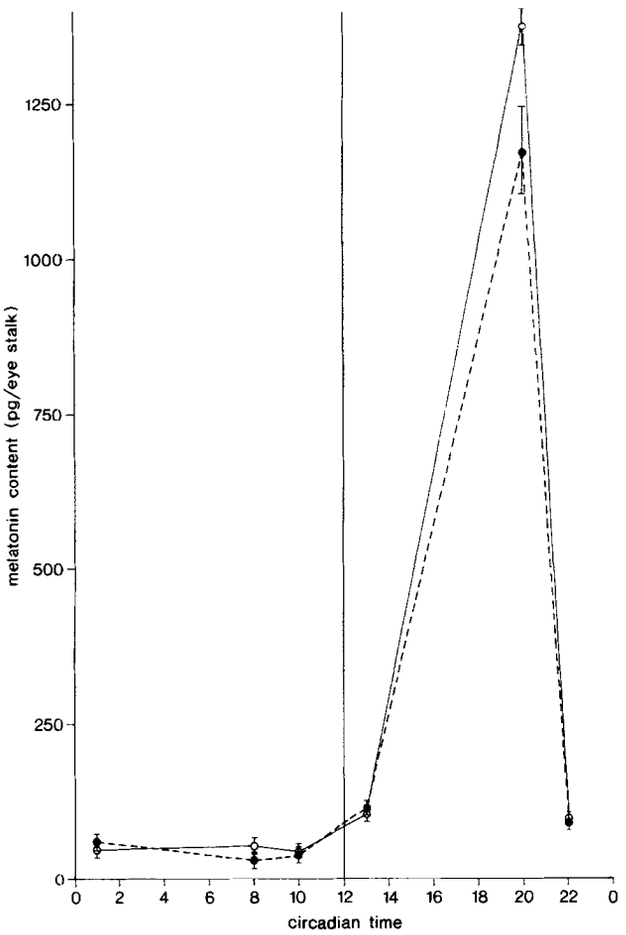

Fig 2. Daily rhythm profile of immunoreactive melatonin in *Procabarus clarkii* eyestalks. Values represent the means ± SE of two series of six independent measurements each. Photophase from 0 and 12 h.

the dark phase and were characterized by a high amplitude. Among crustaceans, *Procambarus clarkii* represents the first case in which melatonin peaks during the night, thus showing the typical pattern followed by the majority of organisms studied. The contradictory results of Agapito *et al* (1995), showing a day maximum of this indoleamine in the crayfish of Spain, can be a consequence of following aspects: first, the species of *Procambarus clarkii* from Spain and Mexico have two different patterns of melatonin production; second, measurements have been performed using antibodies of distinct specificity, one of them reacting also with other indoleamines and causing interference. Compared to our melatonin values, those of Agapito and collaborators show lower amplitude; this fact could speak for such an interference. In addition, they measured melatonin from the eyes of crayfish directly, without extraction, and finally, no statements concerning validation of the method used are given. The activity patterns of serotonin-*N*-acetyltransferase (SNAT) they measured in *Procambarus clarkii* do not account for the melatonin production they observed; we have to keep in mind that, although the activity of this enzyme has shown repeatedly to be an index of melatonin production in some mammals and birds, generalizations as to assume that SNAT is also the key enzyme of melatonin production in crayfish have yet to be proven. In another arthropod, *Drosophila*, it has been shown not to be the case. In this fly hydroxyindole-O-methyltransferase (HIOMT) actually represents the key enzyme of melatonin synthesis (Callebert *et al*, 1991).

Regarding the site of melatonin production in crayfish, the X-organ/sinus-gland system is a strong candidate. The presence of pinealocyte-like cells inside the X-organ has been reported (Elofsson and Lake, 1971), and the axis protocerebrum X-organ/sinus-gland has not only been identified as the major structure involved in hormonal synthesis in crayfish, but it represents the most plausible site for the generation and expression of ERG circadian rhythmicity (Fuentes-Pardo and Hernández-Falcón, 1993; Lara-Aparicio *et al*, 1993).

Another possibility regarding the site of melatonin production involves the retinular cells of the compound eye. Daily melatonin production in invertebrate photoreceptor cells has been reported in *Locusta migratoria* showing also a night peak (Vivien-Roels and Pévet, 1993). In vertebrates, par-

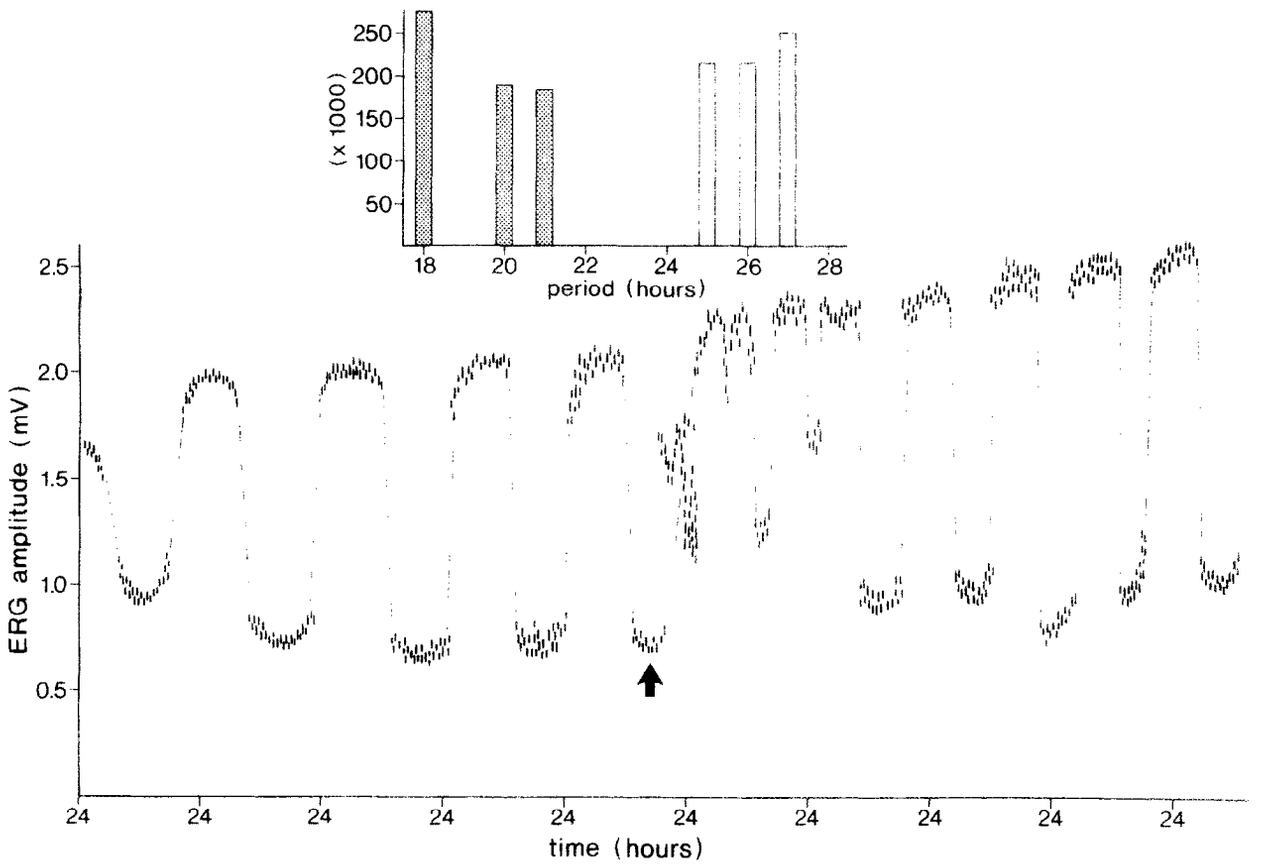

Fig 3. Circadian rhythm in the amplitude of the ERG recorded from the compound eye of crayfish. The arrow points to the time of melatonin addition. Note the changes in both period and amplitude of the circadian oscillation which persist until the end of the experiment. Ordinates, ERG voltage; abscissa, external time. The insert shows the main circadian periods before (white bars) and after (black bars) melatonin administration.

ticularly in hamster, lizard and lamprey, circadian production of melatonin in cultured retina has recently been demonstrated (Tosini and Menaker, 1996), suggesting that all organized vertebrate photoreceptor structures are able to synthesize melatonin under the control of circadian oscillators.

Our results revealed a clear action of exogenous melatonin on both endogenous period length (τ) and amplitude of the ERG circadian rhythm. Shortening of τ and enhancement of amplitude were more conspicuous when the hormone was applied during the subjective day (fig 3). When melatonin was given during subjective night (data not shown), its effect was similar but lesser, suggesting that melatonin acts on circadian oscillators by accelerating them.

When considering the cellular substrate of the ERG rhythmicity at the retinal level, the amplitude of photoreceptor and the relative position of accessory pigments are the main elements to be taken into account. It seems plausible to propose that melatonin acts on the gain of photoreceptors themselves, the movements of accessory pigments toward the 'dark-adaptation position' (Fernlund, 1976) or both of these factors. Consequently, melatonin seems to allow the photoreceptor cell to reach its maximal responsiveness to light, namely the 'dark-adaptation state'. This is in line with the increase in the ERG voltage observed after melatonin injection (fig 3).

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