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Research Report

Crayfish brain-protocerebrum and retina show serotonergic functional relationship

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ABSTRACT

The results from various studies have indicated possible functional relationships between crayfish electroretinogram (ERG) rhythmic amplitude changes and the serotonergic pathways projecting from the central brain through the optic neuropils to the eye, but to date, this functional interaction has not been proven. Here, in a set of experiments using an isolated eyestalk–brain preparation, we investigated whether there is a circadian input from the brain to retina that regulates this rhythm. We sought to determine whether the protocerebral bridge (PB) stimulation affects the ERG amplitude in accordance with the zeitgeber time (ZT) and whether 5-HT modulates the associate input. Our results showed that photic stimulation of retina produced changes in both the amplitude and the frequency of spontaneous electrical activity in the protocerebral neuropils. In addition, electrical stimulation of the medial protocerebrum, particularly the PB, produced statistically significant changes in the ERG that depended on both the time of day and the level of serotonin. This suggests that pathways between retina and PB seem to be serotonergic.

1. Introduction

In *Procambarus clarkii* crayfish, the electroretinogram (ERG) amplitude circadian rhythm is the result of day–night variations in the amplitude of the photoreceptor response to a light pulse of standard intensity and duration. These variations depend on (1) changes in the sensitivity of the photoreceptor, (2) the position of the absorbing proximal pigment granules (PP) or retinal pigment (Hallberg and Elofsson, 1989) within the photoreceptor itself, and (3) the position of the absorbing distal pigment granules (DP).

The results of various experiments (for review, see Fanjul-Moles and Prieto-Sagredo, 2003) indicate that the ERG amplitude of the circadian rhythms of a mutually entrained bilateral system of oscillators that are coupled and synchronized by putative pacemakers in the brain as well as their neural input to the eyestalk neurosecretory system is responsible for the humoral modulation of the retina. Larimer and

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Abbreviations: AMPN, anterior medial protocerebral neuropil; BP, brain protocerebrum; DC, deuterocerebral commissural interneurons; DP, distal pigment; ERG, electroretinogram; LEBP, light evoked brain potentials; PB, protocerebral bridge; PMPN, posterior medial protocerebral neuropil PP, proximal pigment; SBP, spontaneous brain potentials; ZT, zeitgeber time; MTS, methysergide; CPH, cyproheptadine; m-CPP, m-chlorophenilpiperazine

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Smith (1980) have proposed that the coupling information could be carried by neurosecretory fibers that extend from the brain to the optic lobes, i.e., the brain controls the optic lobe neurosecretions that are responsible for pigment movements. Some authors (Barrera-Mera and Block, 1990) have proposed the brain protocerebrum (BP) as the putative pacemaker. Although it has been reported that ERG rhythms persist for some days in isolated retina (Aréchiga and Rodríguez-Sosa, 1998), these authors have not proven the synchronization ability of this structure. Their preparation contained both photoreceptors and a set of neurons in the lamina ganglionaris, where the photoreceptors establish the first synapse of the visual pathway, but in this preparation the proposed circadian input from the brain to retina that regulates the rhythm of the ERG amplitude is absent. There is immunocytochemical and electrophysiological evidence of 5-HT in the vicinity of the photoreceptors and of the influence of 5-HT on the responsiveness of photoreceptors to light (Aréchiga, et al., 1990; Escamilla-Chimal et al., 1998; Escamilla-Chimal et al., 2001). A recent study conducted in our laboratory reported clock protein immunoreactivity in the retina, the optic neuropil and various brain clusters and protocerebral neuropils (Escamilla-Chimal et al., 2010), as well as circadian-induced c-Fos immunoreactivity in the protocerebral bridge (PB) (Granados-Dominguez et al., 2005), which is a V-shaped neuropil containing many large through-running axons (Sandeman et al., 1992). Extraretinal photoreceptors lying in protocerebral cluster 6 terminate in this neuropil (Sandeman et al., 1990; Sullivan et al., 2009). A network of 5-HT-immunoreactive neurons is associated with the PB, with axons projecting along the protocerebral tract to the optic ganglion (Sandeman et al., 1988). In the crayfish *Cherax destructor*, serotonin-immunoreactive fibers project from the protocerebral bridge to the X-organ then into the terminal medulla, the external medulla and the region of the sinus gland where different endocrine compounds controlling ERG circadian rhythms are secreted (for review, see Aréchiga and Rodríguez-Sosa, 2002; Fanjul-Moles and Prieto-Sagredo, 2003). Recently, the presence of the 5-$HT_{1\alpha}$ receptor in the retina, all of the eyestalk ganglia and various cell clusters of the brain in *P. clarkii* indicates different sites of the synaptic action of 5-HT in the crayfish nervous system (Spitzer et al., 2005, 2008).

Here, we investigate whether there is circadian input from the PB to the retina that regulates the rhythm of the ERG amplitude. We sought to determine whether PB stimulation would affect the ERG amplitude in accordance with zeitgeber time (ZT) and whether 5-HT modulates the associated input. We also investigated whether 5-HT agonists and antagonists would modify this effect.

2. Results

2.1. *Effect of photic stimulation of the eye on the brain areas*

Photic stimulation of retina produced changes in both the amplitude and the frequency of the spontaneous electrical activity of the PB (Fig. 1). Both evoked and spontaneous field potentials were identified. We identified four types of electrical activity. (1) Power spectra recorded for 1 s showed a maximum frequency power at approximately 500 Hz, decreasing to the level of noise at approximately 1 Hz. Spike histograms showed that low-voltage (about 5–200 μV), high-frequency spikes were light sensitive, increasing or decreasing the spike rate in response to whether the lights were on or off. Some of the larger spikes (0.2–5 mV) did not respond to light (5 mV not shown). (2) Rhythmic, spontaneous high-amplitude spikes (500 μV) indicated action potentials with a similar interspike interval (100 ms). (3) Sometimes, the light stimulus elicited either bursts of activity that were sustained during all the luminous stimulus or higher amplitude evoked potentials (250 μV) in the PB. This activity appeared about 200 ms after the light was turned on, lasted about 50 ms and disappeared before lights off.(4) The light stimulus elicited long latency (190 ms) high amplitude evoked potentials (250 μV) in the PB. Fig. 1 shows some of the typical electrical activity recorded. Because of the variation in the electrical activity amplitude, we only analyzed the high-frequency spikes that were sensitive to light.

At ZT 3, the PB displayed a higher spontaneous brain potentials (SBP) frequency rate ($\overline{X}=52.6\pm5.4$) than the anterior medial protocerebral neuropil (AMPN) ($X=34.8\pm5.2$) and the posterior medial protocerebral neuropil (PMPN) ($\overline{X}=35.0\pm4.2$). At this time point, only PB and PMPN showed statistically significant effects on the spontaneous brain potentials resulting from retinal stimulation (PB $F=7.5$,$P<0.01$; PMPN $F=3.0$, $P<0.05$).

Fig. 2 and Table 1 (Supplementary material) show the significant differences between the pre- and poststimulation conditions; these differences were even more obvious in the PB ($P<0.01$). In the PB, the light stimulus evoked a 15% Hz increase. At night (ZT 17), the spontaneous frequency rate dramatically increased in all three protocerebral regions, PB, AMPN and PMPN. However, the spontaneous frequency, particularly at the PMPN showed a statistically significant decrease during light stimulation, increasing again after the light was turned off.

2.2. *ERG changes with electrical stimulation of PB*

Based on previous reports that proposed the PB as an important site of serotonergic projections (Sandeman et al., 1988), in the present work, the electrical stimuli were applied in the dorsal region of the PB. The animal's right-eye ERG was recorded. Each experiment lasted 180 min and was divided into three periods. During the first hour, either vehicle (saline) or 5-HT agonist or antagonist agents were applied, and the ERG was recorded. During the second period, an electrical stimulus was applied to the PB, and the ERG was recorded (see Experimental procedures). During the third period, the drugs were washed out and ERG was recorded.

The effect of electrical stimulation on the ERG amplitude was always higher at ZT 17 (25%) than at ZT 3 (2%) (Fig. 3A, B). At these time points, in both nonstimulated and stimulated conditions, an increase in the ERG amplitude was observed during the last 60 min. This demonstrates the persistence and even the improvement of our preparation throughout the experimental period. The one-way ANOVA revealed statistically significant differences in the ERG amplitude between

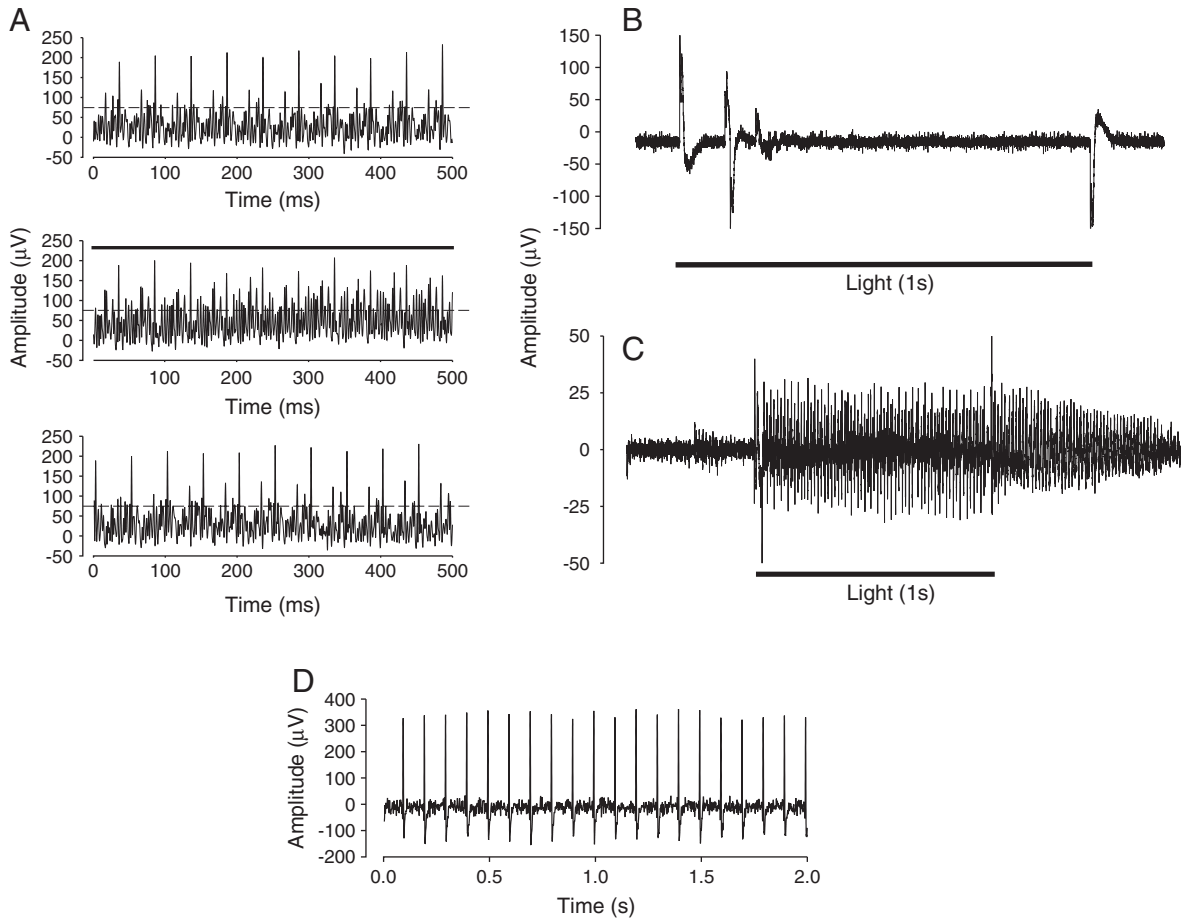

Fig. 1 – Typical extracellular electrical patterns recorded from PB. A) low-voltage (5–200 μV), light sensitive high-frequency spikes increased amplitude (not measured) and spike rate (from 72 to 128 Hz) in response to light on, while some of the larger spikes (200 μV) did not show frequency changes in response to light. B) The light stimulus elicited long latency (190 ms) high amplitude evoked potentials (250 μV) in the PB. C) The light stimulus elicited a burst of activity after a delay of 50 ms. This was sustained during the photic stimulus and decreased after it was turned off. D) Rhythmic, spontaneous high-amplitude spikes (500 μV) showing action potentials with a similar interspike interval (100 ms).

the electrically stimulated and nonstimulated PB at ZT 17 ($F=4.2$, $P<0.01$).

2.3. *Drug effects*

Exogenous serotonin application produced a slightly greater increase in ERG amplitude than vehicle control stimulation at ZT 03 and ZT 17 (Fig. 3C and D). However, the drug application plus stimulation provoked a slight decrease in the magnitude of the ERG at ZT 03 and a statistically significant increase at ZT 17 ($P<0.05$) compared with the control stimulation. Both effects were reversed by methysergide. Under either stimulated or nonstimulated conditions, this drug increased the ERG amplitude at ZT 03 and produced a decrease at ZT 17 (Fig. 3C and D). The wash did not produce any changes in the effect of serotonin on the ERG but exaggerated the effect of methysergide at ZT 03 and ZT 17 ($P<0.05$) (Fig. 3C and D). When we compared the effects of serotonin on ERG amplitude with those produced by the antagonist cyproheptadine at ZT 03, we did not find any statistically significant effect (Fig. 3E) compared with either the control or the serotonin condition. However, at ZT 17, even though there was no difference compared to the control in either the stimulated or nonstimulated experiments, cyproheptadine decreased the ERG amplitude when compared with the serotonin-stimulated or nonstimulated experiments. Although the wash did not produce changes in the effect of 5-HT on the ERG, under nonstimulated conditions, the cyproheptadine wash produced a statistically significant decrease in the ERG amplitude (Fig. 3F).

3. Discussion

As shown in Fig. 2, after 24 h, the crayfish brain maintained *in vitro* displayed an extracellular endogenous discharge characterized by low-amplitude burst periods with oscillations that indicated a firing rate related to the time of day. This rate varied from average values of 40 Hz at ZT 3 to 75 Hz at ZT 17 and varied for the different protocerebral regions explored. The PB displayed a higher spike rate during the subjective day than the anterior and posterior medial neuropils. However, during the subjective night, the firing rate increased in all regions, particularly in the AMPN and the PMPN, which showed significant statistical differences (see Fig. 2).

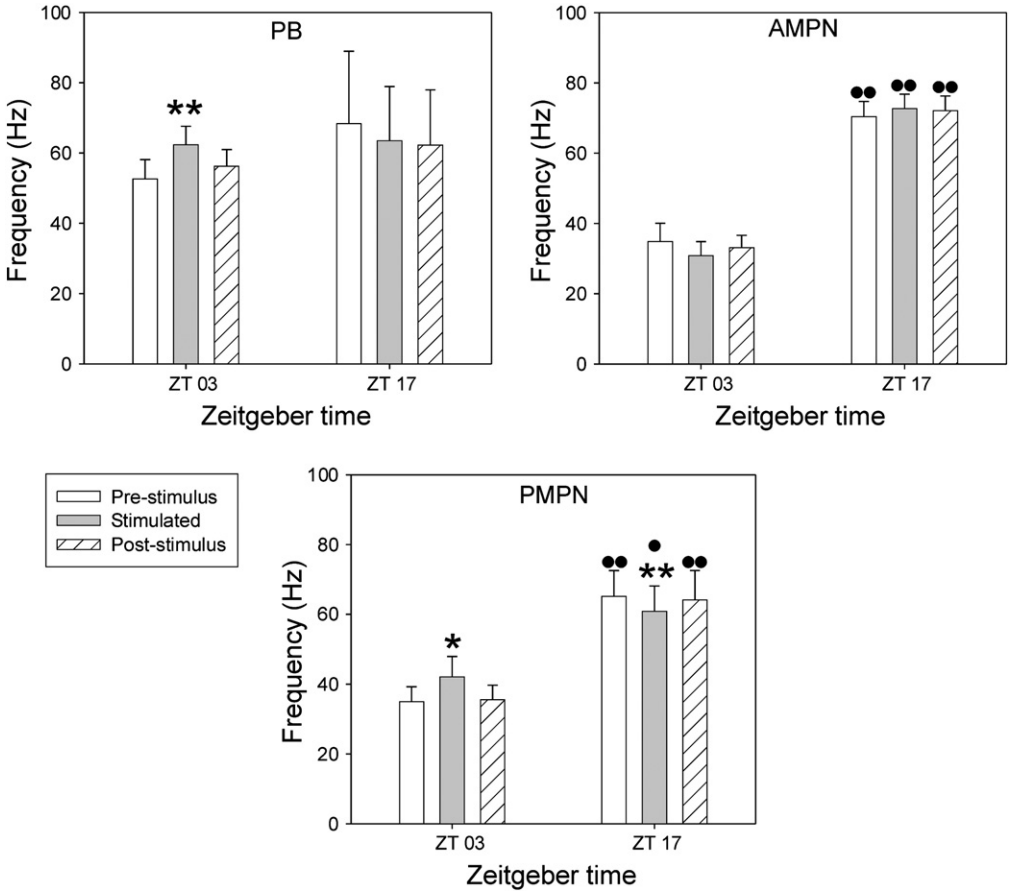

Fig. 2 – Spike frequency analysis of the brain activity elicited by photic stimulation of retina (see Experimental procedures). Recordings were obtained just before, during and after a 1-second light stimulus applied to one eye. The results are shown for two different recording times: ZT 03 and ZT 17. Statistically significant differences between the stimulated versus the pre- or poststimulated condition are shown with $^{*}P<0.05$ or $^{**}P<0.01$. The differences between ZT 03 and ZT 17 values are indicated by $\bullet P<0.01$ or $\bullet\bullet P<0.001$. Each bar represents ±standard error, $n=7$ animals. See text for explanation.

We determined the spontaneous spike amplitude and frequency during both night and day. The results of this work are consistent with previous reports performed in unrestrained crayfish (Hernández-Falcón et al., 1999), and they suggest a daily electrical activity rhythm in the dorsal protocerebrum. Eye illumination evoked a 20% increase in firing rate in the PB ZT 03 but not at ZT 17 (Fig. 2A). This region has been reported as the projection site for many axons of the lateral protocerebrum and the optic neuropils, as well as for branches of class 2 deutocerebral commissural interneurons (DC). The DC axons of the class 2 neurons are small and project into the dorsal part of the anterior lobe of the medial protocerebrum and not to the central body (Sandeman et al., 1995). This area of the protocerebrum receives projections from lateral protocerebrum and optic neuropils, from the "sustaining fibers" and "dimming fibers," which are tonically firing light-sensitive neurons that increase or decrease their firing frequency depending on the intensity of light falling on the eyes (Kirk et al., 1983; Wiersman and Yamaguchi, 1966, 1967; Yamaguchi and Ohtsuka, 1973).

Sandeman et al. (1995) worked with intracellular physiological recordings from DC2 neurons and showed them to be sensitive primarily to the intensity of light falling on the eyes. These neurons were often tonically active, with unitary potentials superimposed on continuous subthreshold synaptic activity. Our recordings seem to correspond to unitary extracellular activity in these neurons because the frequency of the tonic discharge increased and the activity was sustained for the duration of the light pulse (Fig. 1). As in the case of type 2 CD neurons, our recordings are characterized by a long latency of 50–200 ms.

3.1. *Stimulation of the protocerebrum and drugs*

These results showed a clear effect of PB stimulation on the ERG amplitude at night (ZT 17). This effect was not observed during the day. Based on ablation and dissection experiments, some authors have proposed the medial protocerebrum as a pacemaker responsible for the ERG circadian rhythm (Barrera-Mera and Block, 1990). Recently distinct clock neurons, as demanded by a pacemaker, have been identified in this brain area and particularly in the PB (Escamilla-Chimal et al., 2010). Here, we show that the ERG amplitude increased after PB stimulation. The PB comprises axonal extraretinal photoreceptors responsible for rhythm activity synchronization (Sullivan et al., 2009) that contact serotonin-IR fibers (Sandeman et al., 1988;

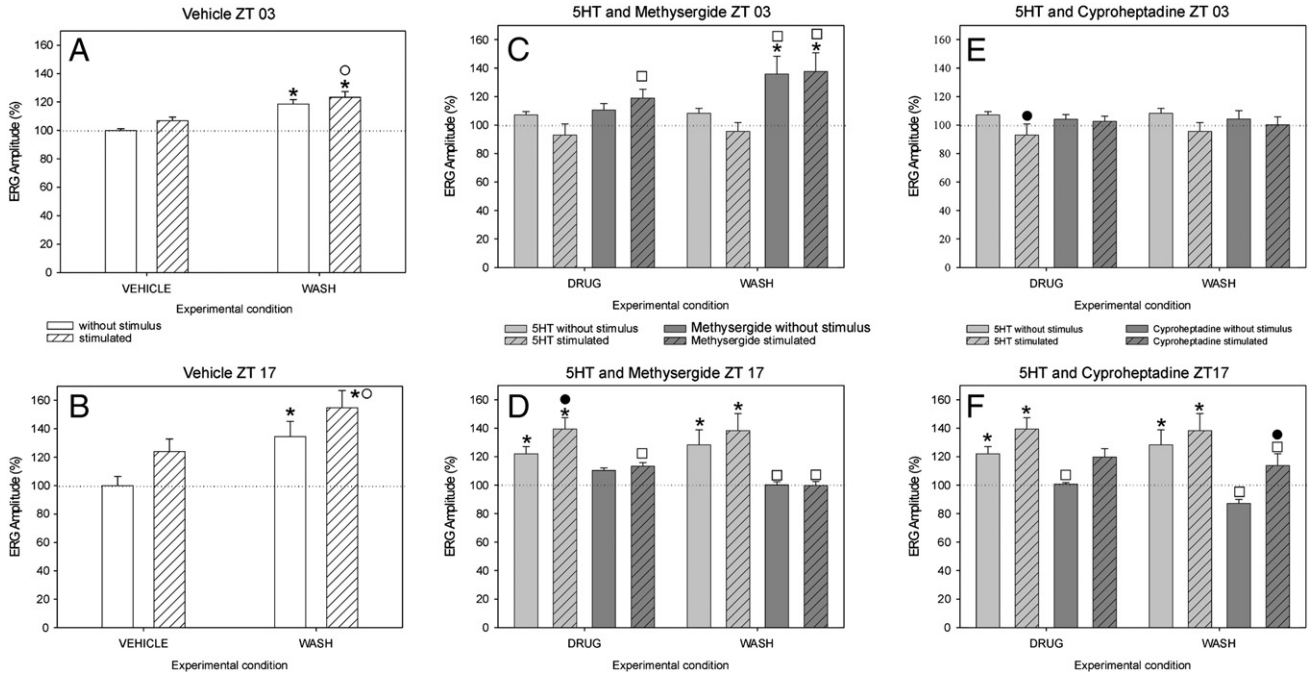

Fig. 3 – PB electrical and chemical stimulation. A,B: control experiments using the vehicle (Van Harreveld–Hepes). The ERG amplitude values are percentages of the ERG amplitude data obtained during the first hour of recording (first white bar on the upper panel). The dotted lines indicate the 100% value. The ERG data are presented for both experimental conditions. This figure shows ERG obtained in nonstimulated and stimulated experiments as well as the vehicle application and the wash (first and second hours of recording). The upper panel depicts data obtained at ZT 03, while the lower panel depicts results obtained at ZT 17. Statistically significant differences ($P<0.01$) between means are shown using the following keys: *, differences vs. control; •, differences between stimulated vs. nonstimulated; , drug vs. wash times. $n=5$ animals. In each case, the symbol represents the comparison between corresponding experimental conditions. See text for explanation. F values for repeated-measures ANOVA: ZT 03: $F=8.43$, $P<0.01$; ZT 17: $F=4.2$, $P<0.01$. C,D: effects of exogenous 5-HT and methysergide on the ERG amplitude of the isolated eye–brain preparations. The average ERG amplitude values are percentages of control values of both experiments. The control values are marked at the 100% level with a dotted line. Both nonstimulated and stimulated conditions are shown for comparison. Upper panel: ZT 03, lower panel: ZT 17. Statistically significant differences between means are marked using the same symbols as in A and B plus □ for differences between the 5-HT and methysergide. Each bar represents the mean ± standard error, $n=5$ animals. F values for repeated-measures ANOVA: ZT 03: $F=4.84$, $P<0.01$; ZT 17: $F=5.52$, $P<0.01$. E, F: the effects of cyproheptamide and 5-HT on the ERG amplitude of the isolated eye–brain preparations. The average ERG amplitude values are percentages of control values during the "drug" and "wash" phases of the experiment. The control value is marked at 100% with a dotted line. Both nonstimulated and stimulated conditions are shown for comparison. Upper panel: ZT 03, lower panel: ZT 17. Statistically significant differences between means are marked using the same symbols as above. In each panel bars represent the mean ± standard error, $n=5$ animals. See text for explanation. F values for repeated-measures ANOVA: ZT 03: $F=3.9$, $P<0.05$; ZT 17: $F=5.73$, $P<0.01$.

1990). This finding confirms the proposition that these fibers may convey circadian information via serotonergic inputs to the retina via X organ-sinus gland (Sandeman et al., 1990). The enhancing effects of 5-HT on the ERG amplitude after 5-HT application into an isolated eyestalk or retina affect the position of PP in the retinal photoreceptors and increase the conductivity of photoreceptor membranes (Aréchiga et al., 1990). These modulatory effects seem to depend on circadian changes in concentration, as observed for one of the two 5-HT receptor types established, the $5\text{-HT}_{1\text{crust}}$ or $5\text{HT}_{1\alpha}$ (Spitzer et al., 2008). This receptor concentration in the retina-lamina ganglionaris is lowest at the end of the dark phase and reaches its highest concentration at the end of the light phase (Calderón-Rosete et al., 2006). This could explain the enhancing effect of the PB stimulation on the ERG found at ZT 03 but not at ZT 17, although our data show an evident increase of ERG values in the ERG amplitude at night. However, the topical application of 5-HT plus PB stimulation produced an opposite effect: there was an increase in amplitude of the ERG after 5-HT and stimulation at night but a slight decrease during the day. Cyproheptadine and methysergide seem to antagonize both effects because, during PB stimulation, both drugs act antagonistically to exogenous 5-HT with respect to ERG amplitude. It is therefore possible that both drugs antagonize the effect of serotonergic and electrical PB stimulation on the ERG. The enhancing effects of both 5-HT and stimulation on the ERG amplitude at this hour could be due to higher-brain serotonin-driven stimulation of the retina or lamina because of the circadian increase in brain serotonin levels, as has been

reported elsewhere (Castañón-Cervantes et al., 1999; Wildt et al., 2004). These results likely contribute to the increase in ERG amplitude observed at night.

3.2. *Pharmacological considerations*

The antagonist drugs used in this study were chosen based on the results of previous functional studies in crayfish. Cyproheptadine (CPH) and methysergide (MTS) have both been shown to block the effects of serotonin on the ERG response and on the rate of dark adaptation of the PP of *P. clarkii* photoreceptors (Aréchiga et al., 1990; Frixione and Hernández, 1989). MTS also blocks both the ability of 5-HT to modulate electrical activity in the X-organ-sinus gland of crayfish (Alvarado-Alvarez et al., 2000; Sáenz et al., 1997) and the secretion of crustacean hyperglycemic hormone (Lee et al., 2000, 2001). In addition, CPH acts as an antagonist of the serotonergic control of glycemic levels in the crayfish *Astacus leptodactilus* (Lorenzon et al., 2004). These authors reported that CPH alone raised hemolymph glucose relative to the control, but when CPH was co-injected with 5-HT, it was able to inhibit the 5-HT-induced hyperglycemic effect.

In crustaceans, at least five 5-HT receptors have been predicted, but only two have been cloned and characterized thus far; these are homologous to the vertebrate receptor subtypes 1A and 2B. Using specific antibodies, the crayfish 5-HT_1 receptor has been localized to the brain and ventral nerve cord, whereas the 5-HT_2 receptor has been localized to the stomatogastric system. Recently, experiments using RT-PCR have demonstrated that mRNAs of 5-HT receptors homologous to mammalian subtypes 1A and 2B are expressed in *P. clarkii* brain areas (Zhang et al., 2011).

In this study, we used CPH and MTS as 5-HT_1 and 5-HT_2 receptor antagonists. This choice was based on previous reports demonstrating the efficacy of both drugs in crayfish and on additional studies revealing the presence of the 5-HT_1 receptor in the retina and lamina of *P. clarkii* (Calderón-Rosete et al., 2006) despite the fact that both subtypes1A and 2B (referred for crustacean as 5-$HT_{1\alpha}$ and 5-$HT_{2\beta}$) are expressed in the eyestalk and brain (Zhang et al., 2011). These drugs are considered to be non-selective antagonists in both vertebrates and invertebrates, and they were able to block the physiological effects of 5-HT on both the ERG responses and the retraction of the PP of *P. clarkii* photoreceptors (Alvarado-Alvarez et al., 2000; Frixione and Hernández, 1989). As a result, Frixione and Hernández (1989) have proposed that a 5-HT-induced enhancement of Na^+/K^+-ATPase activity leads to Na^+ extrusion through the membrane, which decreases the cytoplasmic Na^+ (and thus Ca^{2+}) in crayfish photoreceptors; this process causes PP aggregation to the dark-adapted position.

In other decapods, such as the crab *Sesarma cinerum*, ERG responses and pigment granule movements returned to normal after EGTA–Ringer's solution was replaced with a medium containing normal levels of calcium. These results suggest that the centripetal migration of pigment granules in crustacean photoreceptors requires calcium (King-Smith and Cronin, 1996); a similar requirement has also been proposed for crayfish photoreceptors. The antagonistic effects of CPH on calcium-mediated contraction, membrane electrical events and calcium influx in some species are well known (Lowe et al., 1981). In mammals, CPH blocks constitutively active 5-HT_2c receptors; this blockage eliminates calcium currents and muscle spasms (Murray et al., 2010). However, in locusts, CPH inhibits the 5-HT-mediated relaxation of the locust midgut circular muscle (Molaei and Lange, 2003). Additionally, MTS has been associated with an antagonism of the 5-$HT_{2\beta}$ receptor. Because the 5-HT2 family of receptors activates phospholipase C, causing the release of intracellular Ca^{2+}, we believe that the results reported here could be related to the antagonistic effect of these drugs on 5-HT. The ability of serotonin to enhance transmitter release at the crayfish neuromuscular junction has been reported to be caused by an increase in intracellular calcium levels resulting from the activation of an IP_3 cascade (Dixon and Atwood, 1989; Tabor and Cooper, 2002).

However, the effects of these drugs on the 5-HT receptors of crustaceans have not been fully studied thus far, and the current pharmacological evidence is scarce and sometimes contradictory.

In a recent pharmacological study, Spitzer et al. (2008) after having identified and cloned the 5-HT1a and 5-HT2 receptors in the lobster *Panulirus interruptus* (Clark et al., 2004; Sosa et al., 2004) obtained full-length clones for the same two receptors from the swamp crayfish *P. clarkii* and expressed them in a heterologous system to determine their second messenger interactions and pharmacological profiles. In *P. clarkii*, 5-HT activation of the 5-$HT_{2\beta}$ receptor resulted in increased intracellular levels of inositol phosphates (IP), the activation of protein kinase C (PKC) and no change in cAMP levels. Meanwhile, 5-HT activation of the 5-$HT_{1\alpha}$ receptor resulted in the inhibition of cAMP by blocking the forskolin-stimulated accumulation of cAMP.

The drugs m-chlorophenilpiperazine (m-CPP) and quipazine are 5-$HT_{1\alpha}$ agonists that do not affect 5-$HT_{2\beta}$. Conversely, methiothepin and cinanserin are antagonists of 5-$HT_{2\beta}$ but have no effect on 5-$HT_{1\alpha}$. A comparison of these two receptors with their orthologs from the California spiny lobster revealed a conservation of the protein structure, signaling and pharmacology. The protein domains involved in G-protein interactions are conserved between the two crustacean receptors and between other characterized arthropod and mammalian 5-HT receptors. Interestingly, in spite of previous functional reports, these authors found that MTS exhibited agonist activity at the crustacean 5-$HT_{2\beta}$ and 5-$HT_{1\alpha}$ receptors. This drug is a functional antagonist of both *Drosophila* 5-HT-dro receptor (Witz et al., 1990) and vertebrate 5-HT_2 receptors, but it is also an agonist of some vertebrate 5-HT_1 receptors. Other drugs, such as m-CPP act as non-selective agonists at vertebrate 5-HT_1 and 5-HT_2-family receptors but do not act through any 5-HT receptor to depress neuronal function in invertebrates (e.g., crayfish and *Drosophila*). Instead, m-CPP likely acts to decrease sodium influx through voltage-gated sodium channels present in both motor and primary sensory neurons in invertebrates (Sparks et al., 2003). The serotonergic effect of this drug may be related to its ability to reverse the direction of the 5-HT transporter, which therefore increases the concentration of serotonin.

Although our results seem to show clearly the 5-HT antagonist effects of MTS and CPH further experiments using 5-$HT_{1\alpha}$- and 5-$HT_{2\beta}$-specific agonist and antagonist drugs in crayfish used elsewhere as quipazine or methiothepin

melsyate (Spitzer et al., 2008; Zhang et al., 2011) will be useful to support our findings.

4. Experimental procedures

4.1. Experimental animals

The experiments were performed on adult crayfish (*P. clarkii*; $n=60$) weighing 25–30 g, in the intermolt state. The animals were obtained in Rio, Conchos, Delicias Chihuahua; they were maintained indoors in aerated tanks containing 5–10 animals at 18–20 °C and were fed twice a week, *ad libitum* with vegetables. All animals were maintained in a 12:12 hour, light/dark cycle, with lights on and off at 07:00 and 19:00, respectively.

We used an isolated eyestalk–brain preparation that provides access to the dorsal surface of the brain while keeping the eyes intact (modified from Sandeman and Sandeman, 1998). In this preparation, the esophageal connectives were severed, and the crayfish head was constantly perfused with Van Harreveld–Hepes solution. Neural activity was stable for at least 4 h.

4.2. Surgical procedures

The animals were anesthetized with ice for at least 30 min. They were then decapitated, and muscles and connective tissue were lifted to expose the brain. The brain sheath was carefully removed with glass manipulators, and the brain was exposed. The eyestalk–brain preparation, pinned dorsal side up, was placed on a Sylgard-lined recording chamber, which was filled with Van Harreveld solution (197.77 mM NaCl, 5.37 mM KCl, 13.51 mM CaCl2, 2.63 mM MgCl2) and 10.0 mM Hepes (Sigma, St. Louis, MO) at 20 °C±0.1 °C and pH 7.4. From that moment, the preparation was kept in constant darkness (DD) and at a constant temperature (20 °C±0.1 °C) throughout the experiment. The chamber was constantly perfused with Van Harreveld–Hepes solution at 9 ml/h.

4.3. Recording and stimulation procedures

4.3.1. Brain recording and eye´s photic stimulation

The experiments began at either ZT 03 or ZT 17 (external time 10:00 and 24:00). The extracellular activity of the brain was recorded at three different sites: PB and the anterior and medial protocerebral neuropils (AMPN and PMPM). The recordings were obtained using a tungsten electrode (1 MΩ WPI). An Ag–AgCl electrode was used as a reference in the nearby connective tissue. The recording electrodes were placed through the three protocerebral neuropils using a Nikon SZU1 microscope. Both electrodes were connected to a high-impedance probe (GRASS Mod HIP511GA). The signal was amplified by a Grass P511 AC amplifier and relayed to an Axon Instrument 1200 digitizer, then sent to a personal computer with the help of the AxoScope program (vs. 9) to be analyzed with Clampfit software (Version 10.2).

The spontaneous brain potentials (SBPs) were recorded for 1 s before and after luminous stimuli (25 lx and 1 s) produced by an L.E.D. lamp controlled by an S-48 Grass stimulator. The device was connected directly to the animal's right eye through an isolated black pipe, and the light evoked potentials (LEBP) produced on the SBPs were recorded. Then, the PB, AMPN and PMPN spontaneous and evoked activity levels were recorded 1 min before, during and after the photic stimulation. To test the position of the electrode, the tips were then blackened by dipping them first in polylysine and then in India ink (Sandeman et al., 1995). At the end of the experiment, the brains were dissected and fixed in Bouin's fixative at 4 °C for 12 h and embedded in 3.0% low-melting-point agarose (Invitrogen, Carlsbad, CA, USA) dissolved in 0.1 M PBS. The agar block was glued with cyanoacrylate onto the plate of a Motorized Advance Vibroslice (NVSLM1, WPI, Sarasota, Florida, USA) and 50 μm-thick sagittal slices were prepared using a vibrating-blade microtome. These slices were ultimately used to determine the appropriate electrode position (see Supplementary material).

4.4. Electrical stimulation of the protocerebral brain

Electrical stimuli (3 V, 2 ms) were applied on the dorsal surface of the PB through a monopolar tungsten electrode (1 MΩ) connected to an isolated GRASS S48 stimulator via a stimulus isolation unit (GRASS SIU5). Simultaneously, in order to record the ERG, a photostimulator (GRASS PS33) delivered a fixed pulse of light to the retina. Both the electrical and photic stimuli were synchronized by the S48 stimulator. A volley consisting of eight photic stimuli and four electrical stimuli (3 V, 2 ms) 7 s apart was created. The ERG produced was recorded every 10 min for 120 min. The signals, amplified by a Grass P511 amplifier, were transmitted to a digitizer (Axon Instruments, Axopatch 1200), and the data were recorded by a PC with AxoScope software (vs. 9) for analysis with Clampfit software (10.2).

4.5. ERG recording

For the ERG recording, a tungsten electrode (1 MΩ) was inserted into the cornea; an Ag–AgCl electrode was used as reference in the bath. A Grass PS33 photostimulator delivered a fixed test light flash (1500 lx, 20 μs) directly to the retina by means of a narrow black pipe. The pipe was always placed at the same distance from the eye (0.5 cm) and provided a 52 lx-intensity light pulse measured at the surface of the eye. The ERG evoked by the light flash was relayed to an AC preamplifier (Grass P511) and stored in a personal computer for analysis.

4.6. Application of serotonin agonist and antagonist agents

The effects of exogenous 5-HT and two 5-HT receptor antagonists, methysergide and cyproheptadine, were explored with the topical application at the protocerebrum, at the same concentrations used for crayfish elsewhere (Aréchiga et al., 1990). Before the experiment, the tissue was incubated with an agonist, antagonist or Van Harreveld–Hepes solution for 1 h without brain stimulation. Meanwhile, the ERG was simultaneously recorded every 10 min. During the following hour, PB stimulation was started in the presence of these treatments. During the third hour, the perfusion system was restarted, and the substances were washed out.

4.7. Chemicals

The chemicals used included the following: serotonin (5-hydroxytryptamine hydrochloride, Sigma Aldrich, México) 0.1 mM (Aréchiga et al., 1990), methysergide (methysergide maleate, Sigma Aldrich) 100 μM, cyproheptadine (cyproheptadine-hydrochloride, Sigma-Aldrich) 12 μM and Van Harreveld–Hepes saline solution.

4.8. Data analysis

4.8.1. Photic stimulation of the retina

The Clampfit software enabled discrimination of the SBPs based on amplitude. The digital discrimination window was first set for the light-evoked discharge so that the window correctly discriminated the SBPs only. This was done for ZT 3 and ZT 17. Frequency histograms were created for spans of 1 min before, during and after the light stimulus at each recording time (control, stimulation and poststimulation). The average frequency (Hz) and the standard deviation of the SBP and LEBP were calculated for each time point at the three brain sites. Each time recording for each protocerebral region at ZT 03 and ZT 17 was plotted against the frequency values. A repeated-measures ANOVA (dependent on time data) was computed for each plot.

4.8.2. Stimulation of the protocerebral bridge

ERG obtained throughout the 120 min of the experiment was captured with the AxoScope software and analyzed with PClamp software. The raw ERG amplitude values obtained without PB stimulation, with PB stimulation, and poststimulation and/or wash were analyzed using Excel 2003 software. All time points for each ZT were divided into three groups: control (nonstimulated), stimulated, and poststimulated and/or washed. ZT 03 and ZT 17 ERG amplitude values obtained with the vehicle, 5-HT, both antagonists, and nonstimulated or stimulated conditions were subjected to within-group comparisons using the one-way Student's *t* test. To visualize the data for each condition, the ERG amplitude was averaged and expressed as the mean ± s.e.m. The measurements were normalized to compare the quantitative changes in the ERG amplitude to the control levels.

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