

# Circadian rhythm in melatonin release as a mechanism to reinforce the temporal organization of the circadian system in crayfish

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**Abstract** Melatonin (MEL) is a conserved molecule with respect to its synthesis pathway and functions. In crayfish, MEL content in eyestalks (Ey) increases at night under the photoperiod, and this indoleamine synchronizes the circadian rhythm of electroretinogram amplitude, which is expressed by retinas and controlled by the cerebroid ganglion (CG). The aim of this study was to determine whether MEL content in eyestalks and CG or circulating MEL in hemolymph (He) follows a circadian rhythm under a free-running condition; in addition, it was tested whether MEL might directly influence the spontaneous electrical activity of the CG. Crayfish were maintained under constant darkness and temperature, a condition suitable for studying the intrinsic properties of circadian systems. MEL was quantified in samples obtained from He, Ey, and CG by

means of an enzyme-linked immunosorbent assay, and the effect of exogenous MEL on CG spontaneous activity was evaluated by electrophysiological recording. Variation of MEL content in He, Ey, and CG followed a circadian rhythm that peaked at the same circadian time (CT). In addition, a single dose of MEL injected into the crayfish at different CTs reduced the level of spontaneous electrical activity in the CG. Results suggest that the circadian increase in MEL content directly affects the CG, reducing its spontaneous electrical activity, and that MEL might act as a periodical signal to reinforce the organization of the circadian system in crayfish.

**Keywords** Cerebroid ganglion · Circadian system · Crayfish · ERG rhythm · Melatonin · *Procambarus clarkii*

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## Introduction

*N*-acetyl-5-methoxytryptamine (melatonin [MEL]) is a highly conserved and ubiquitous molecule present from unicellular organisms to mammals that is released following a nocturnal circadian rhythm. In vertebrate species, circulating plasma MEL is synthesized principally in the pineal gland (Mizutani et al. 2016; Erren and Reiter 2015; Hardeland et al. 2011); however, it is also released from peripheral tissues following local dynamics (Huether 1993). MEL has been described as a pleiotropic, diffusible hormone across the plasma membrane that acts on different structures, binding specifically to several targets at the cellular level, such as heptahelical receptors (Dubocovich and Markowska 2005), nuclear receptors (Hardeland et al. 2011), as well as other intracellular proteins such as calmodulin (Benítez-King et al. 1993). MEL is involved in the modulation of different physiological functions, such as

firing rate or ionic currents of neurons from the suprachiasmatic nucleus (Liu et al. 1997) and hippocampus (Wan et al. 1999), and is additionally involved in the functioning of retinal cells (Baba et al. 2013; Sengupta et al. 2011). In particular, this indoleamine has been characterized as a non-photic synchronizer of the circadian rhythms in rodents (Redman et al. 1983).

MEL in crayfish presents similar features, as described previously, to those of vertebrate species; for instance, its content in eyestalks (Ey) follows a daily variation under photoperiodic conditions, increasing under darkness (Balzer et al. 1997). Regarding MEL actions, it increases the level of motor activity and modulates the concentration of several metabolites in hemolymph (He) (Tilden et al. 2003), as well as influences the process of neuritogenesis in neural cells isolated from Ey (Cary et al. 2012). In addition, this indoleamine induces the increase in electrophysiological response to the light of photoreceptors activating membrane receptors with pharmacological properties similar to MT2 (Mendoza-Vargas et al. 2009).

In crayfish, the role of MEL as a circadian modulator has been studied by the chronic recording of the electroretinogram (ERG); the amplitude of this response follows a circadian rhythm with a nocturnal peak. ERG rhythm is expressed by the reticular photoreceptors and controlled by the CG (Fanjul-Moles and Prieto-Sagredo 2003; Fuentes-Pardo and Hernández-Falcón 1993; Barrera-Mera and Block 1990). This rhythm under free-running (constant darkness and temperature) is synchronized by a daily injection of exogenous MEL, at the same external time during several consecutive days (Solís-Chagoyán et al. 2008) which suggest that MEL is a non-photic synchronizer in crayfish.

In order to obtain more insight into the role of MEL as a circadian signal in crayfish, it is necessary to determine whether the endogenous release of MEL maintains a circadian pattern in constant environmental condition and also to study the MEL action on the CG, the neural structure involved in the control of the ERG rhythm in this animal. Thus, the main purpose of this study was to measure the circulating concentration of MEL in He, as well as MEL content in Ey and CG during a daily cycle under free-running. In addition, the effect of MEL on the spontaneous electrical activity of the CG was evaluated at different circadian times (CT) to determine whether MEL might directly influence the activity of this ganglion.

## Materials and methods

Male adult crayfish *Procambarus clarkii* in intermolt stage were used for experimentation. They were collected from River Conchos, Chihuahua, Mexico. To acclimate animals

to laboratory conditions, they were maintained for 1 month in a room with controlled temperature at  $16 \pm 1$  °C, and under a photoperiodic regimen (LD 12:12), with lights turned on at 7:00 hours. Crayfish were fed twice a week with pellets for shrimps.

## Sampling of hemolymph and dissection of tissues

To obtain He samples and to dissect Ey and CG, 40 crayfish were distributed in 8 aquaria (5 animals/aquarium). Prior to sampling, animals were maintained during 8 consecutive days under controlled conditions: the former 3 days under photoperiodic regimen (LD 12:12), and the subsequent 5 days, under constant darkness (DD). By day 9 and also under constant darkness, 5 animals at 3-h intervals were anesthetized by packing them in ice for 15 min. Under dim red light, He (500 µL/animal) was collected with a 1-mL syringe from the right chelae, at the meropodite–carpopodite joint and placed in an Eppendorf tube with heparin to avoid coagulation. These samples were stored immediately at  $-70$  °C until MEL measurement was performed.

In addition, both Ey were cut with microscissors and the optic nerve was pulled with forceps to separate the tissue from the chitin layer, while the CG was isolated by cutting all of the peripheral nerves with microscissors. The pair of Ey and the CG obtained from each crayfish was processed separately. These tissues were washed with chilled Van Harveld solution (VH) and immediately placed in an Eppendorf tube with 800 µL of VH for their storage at  $-70$  °C. The composition of VH, modified by Miller and Glantz (2000), was as follows (in mM): NaCl 205; KCl 5.4; MgCl<sub>2</sub> 2.6; CaCl<sub>2</sub> 13.6, and HEPES 5; pH was adjusted to 7.5 with NaOH.

## Determination of circadian time of sampling

To obtain samples of He, Ey, and CG, crayfish were maintained under DD during 5 days. Therefore, to determine the CT of sampling, the circadian rhythm of the electroretinogram (ERG) amplitude was determined in other animals ( $n = 3$ ) maintained in a recording chamber as follow. These 3 crayfish were maintained under the same regimen of illumination as the animals from which samples were collected to measure MEL: 3 days under LD 12:12, and 5 days under DD. For ERG recording, the procedure described in detail by Fuentes-Pardo and Inclán-Rubio (1981) and Solís-Chagoyán et al. (2008) was followed. Briefly, crayfish were fixed to a clamp and partially immersed in a water tank covering the gills, but not the Ey. The chitin layer of Ey was fixed to the rostrum with dental cement, and a stainless steel electrode (5 µm at the tip) was implanted into the right Ey. The electrical response to light

pulses (ERG) was elicited by applying a light flash of 10  $\mu$ s of duration and 200 lx of intensity using a Grass PS-22 photostimulator every 30 min. ERG was amplified by a low-level Grass 7P122 DC amplifier, and the electrical signals were stored by means of an analog-to-digital transformer card Model MP101 (Biopac Systems) and AcqKnowledge ver. 8.1 software (Biopac Systems). The voltage of the HI component was considered as the ERG amplitude. HI is the amplitude of ERG subtracted from the baseline until maximal response (Fuentes-Pardo and Inclán-Rubio 1981). This amplitude was measured and plotted versus external time. In these graphs, correspondence was established between external time and CT, considering CT<sub>0</sub> as the moment of the cycle at which ERG amplitude reached 50% of maximal amplitude (Solís-Chagoyán et al. 2008; Fuentes-Pardo and Inclán-Rubio 1981).

### Melatonin determination by ELISA

Measurement of MEL concentration was performed using an enzyme-linked immunosorbent assay (ELISA) commercial kit designed to quantify MEL in samples of human saliva with a standard range of 0.6–25 pg/mL (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Therefore, prior to measuring MEL in homogenates of crayfish tissues, the efficiency of MEL extraction employing octadecyl-bonded silica gel columns (JT Baker) was determined by adding tritiated melatonin ([<sup>3</sup>H]-MEL) (PerkinElmer) to homogenized tissues. In this regard, Ey and CG were homogenized with an ultrasonic homogenizer and centrifuged at 12,000 rpm for 5 min at 7 °C, discarding the pellet. [<sup>3</sup>H]-MEL was then added to 100  $\mu$ L of the supernatant mixed with ultrapure water at a ratio of 1:2, and the diluted samples were added to the extraction columns suctioned with a vacuum pump. [<sup>3</sup>H]-MEL was eluted with methanol, which was evaporated with a SavantDNA120 Model Concentrator (Thermo Scientific), and then [<sup>3</sup>H]-MEL was reconstituted with the ELISA kit buffers and measured utilizing a liquid scintillation counter (PerkinElmer). Extraction efficiency was determined in quadruplicate by means of tissue, considering 100% as counts-per-minute detected in tubes with [<sup>3</sup>H]-MEL added directly to the ELISA kit buffer and the scintillation liquid.

After confirming that silica gel columns comprise a suitable strategy for extracting MEL from tissue homogenates, the concentration of this indoleamine was quantified following the instructions described by the ELISA kit supplier (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Briefly, two Ey or the CG obtained from each crayfish was homogenized separately to detect MEL. 100  $\mu$ L of homogenates was diluted with ultrapure water at a 1:2 ratio. Similarly, 100  $\mu$ L of He was diluted with water

at the same ratio. MEL concentration was determined with a microplate reader (ThermoScientific) at 540 nm. Each sample was assayed in triplicate, and the value of MEL concentration per CT was determined with samples obtained from five crayfish. The data of MEL concentration were divided by tissue (2 in the case of Ey) or reported in pg/mL in the case of He. Data of MEL concentration among CT were compared with Kruskal–Wallis test followed by post hoc Wilcoxon test pairwise comparisons using the software GraphPad Prism for Windows Version 7.02.

### Recording of the spontaneous electrical activity of the cerebroid ganglion

To determine the effect of exogenous MEL (Sigma Chemical Co.) on the electrical activity of the CG, this hormone (1  $\mu$ M, 100  $\mu$ L) or the vehicle solution was injected into the animals through a stainless steel needle implanted near the crayfish cardiac cavity under dim red light, as described by Solís-Chagoyán et al. (2008, 2012). MEL was dissolved in dimethyl sulfoxide (DMSO) and diluted in VH solution. On the other hand, the vehicle was composed of DMSO diluted in VH without MEL. Four crayfish per CT were injected with MEL and four with vehicle. These animals were subjected to the same regimen as the crayfish from which the samples were obtained: 3 days under LO 12:12 and 5 days under DD. On day 9, MEL was injected under dim red light at CT<sub>4</sub>, CT<sub>12</sub>, CT<sub>18</sub>, and CT<sub>22</sub>, and the effect of this indoleamine was determined by electrophysiological recording of CG spontaneous electrical activity as described in the next paragraph. To establish correspondence between external time of injection and CT, the ERG circadian rhythms were plotted similarly, as detailed in “[Determination of circadian time of sampling](#)” section, to determine the CT of the sampling.

Recording of spontaneous electrical activity was performed following the procedure described by Solís-Chagoyán et al. (2012). Briefly, in unrestrained animals, a silver chloride electrode was implanted through the ventral surface of the cephalothorax below the CG and fixed with dental cement. The extracellular spontaneous electrical activity of the CG was recorded by Model P511 AC preamplifiers (Grass Technologies) with filters passing from 3 Hz to 5 kHz. Electrical activity was digitized by a Model MI100 analogical-to-digital transformer card (Biopac Systems) and employing the AcqKnowledge ver. 8.1 software program (Biopac Systems).

To quantify the effect of the MEL injection on CG electrical activity, the number of potentials recorded in 1 s was determined in quadruplicate at 4-min intervals during 40 min and plotted versus time. Prior to application of the

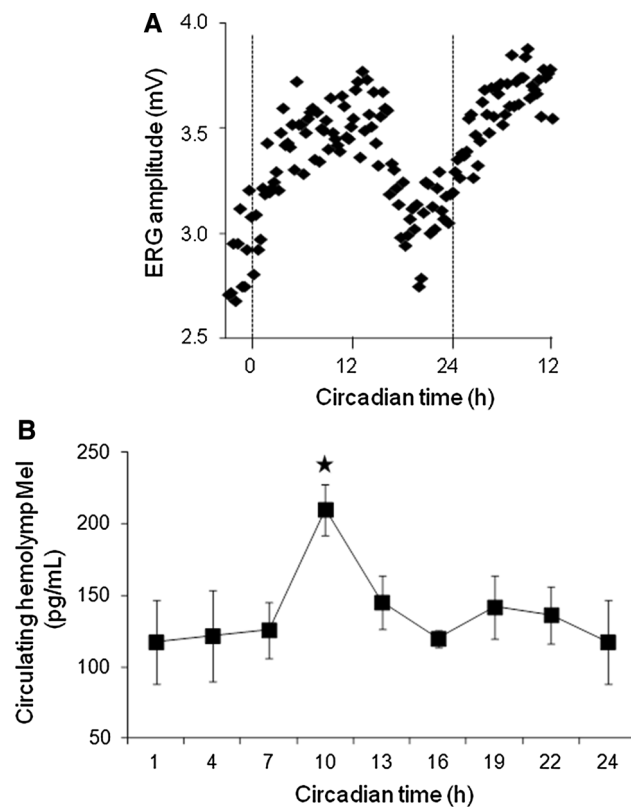
MEL injection, a baseline was established and each crayfish was injected at 22 min. Data were standardized considering 100% as maximal number of potentials obtained during baseline prior to MEL injection. Data in percentages were transformed with the arcsin function and compared by Kruskal–Wallis test followed by post hoc Wilcoxon test pairwise comparisons.

## Results

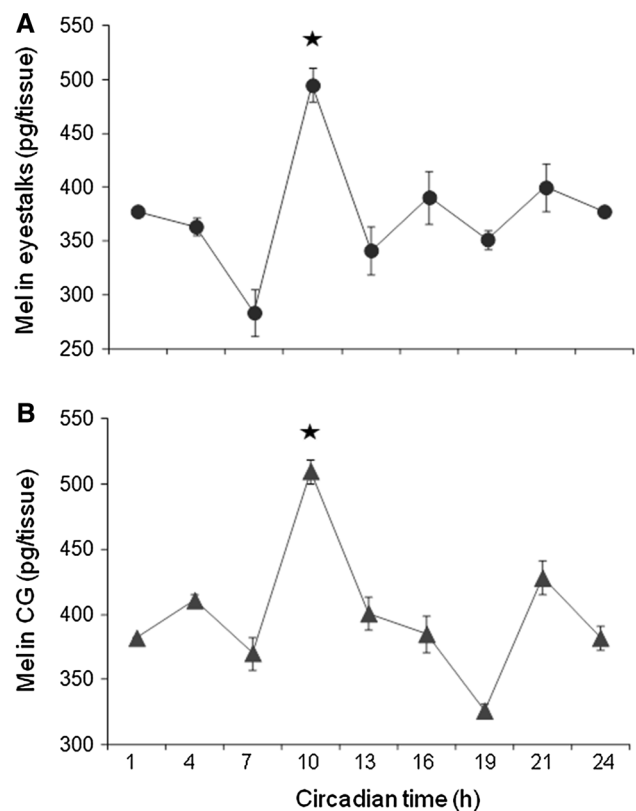
To confirm the usefulness of the ELISA commercial kit for measuring MEL extracted from crayfish tissues, the efficiency of the extraction procedure was quantified. In homogenates of Ey or CG ( $n = 4$ ), efficiency was  $96.5 \pm 2.1$  and  $94.3 \pm 3.2\%$ , respectively, and  $97.4 \pm 2.0\%$  of initial  $[3H]$ -MEL added to He samples was extracted with the columns. To control the CT of the sampling, ERG amplitude was chronically recorded in

three crayfish and measured to plot the ERG circadian rhythm. Both animals from which samples were obtained and animals employed to determine the CT were maintained under the same environmental conditions. CT0 in ERG rhythm was considered as the time during which the amplitude reached 50%, and the maximal values of the ERG response occurred between CT6 and CT10 (Fig. 1a). Regarding circulating MEL in He, the concentration increased with statistical significance at CT10 and followed a clear circadian variation (Fig. 1b). These results suggest that under constant darkness and temperature, the increase in MEL released into the circulatory system coincided with the circadian increase in the ERG amplitude.

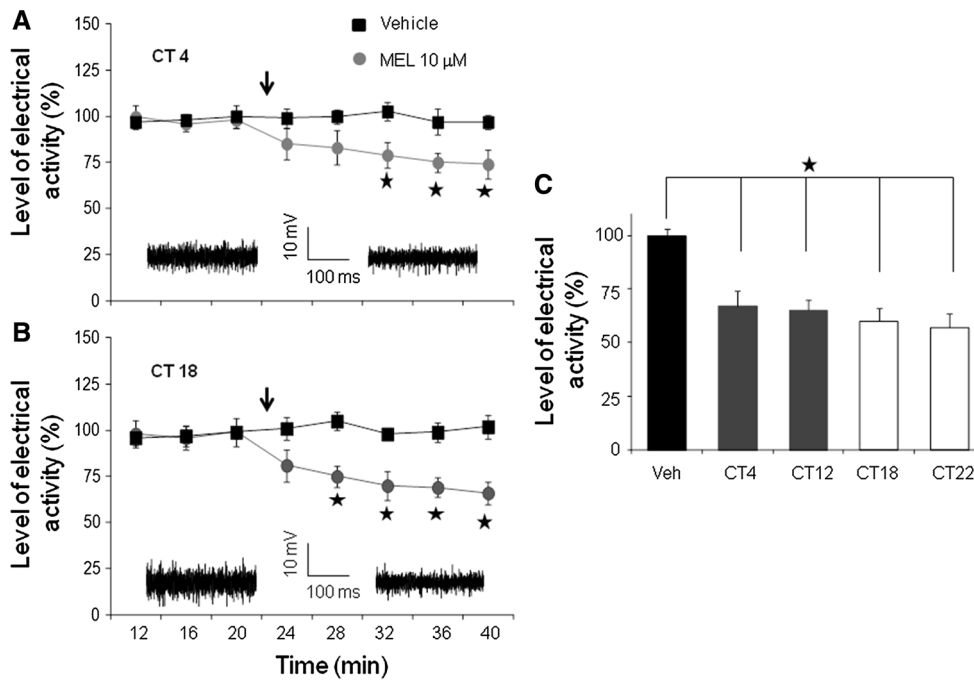
In addition, MEL extracted from homogenates of Ey (Fig. 2a) and CG was determined (Fig. 2b). In these neuronal tissues, MEL concentration also increased with statistical significance at CT10. These data suggest that MEL



**Fig. 1** Circulating melatonin (MEL) in hemolymph (He) follows a circadian rhythm under free-running conditions. MEL was measured with an enzyme-linked immunosorbent assay (ELISA) designed to detect this hormone in human saliva. **a** the electroretinogram (ERG) circadian rhythm recorded to determine the correspondence between external time of sampling and circadian time (CT). The graph in **b** shows the concentration of MEL measured in He samples obtained from crayfish every 3 h. Mean and standard error (SE) were plotted, and the asterisk represents  $p < 0.05$



**Fig. 2** Circadian rhythm in the melatonin (MEL) concentration extracted from the neuronal tissues of crayfish maintained under free-running conditions. MEL concentration was determined from extracts of eyestalks (Ey) or cerebroid ganglion (CG). Crayfish were anesthetized to dissect tissues every 3 h. The pair of Ey and CG obtained from each crayfish was homogenized separately to extract MEL. This indoleamine was extracted from homogenates using silica gel columns and measured with an enzyme-linked immunosorbent assay. Correspondence between external time of sampling and circadian time was determined recording the electroretinogram (ERG) circadian rhythm. Mean and standard error (SE) were plotted, and the asterisk represents  $p < 0.05$



**Fig. 3** Exogenous melatonin (MEL) injected at different circadian times (CT) induced a reduction in the level of electrical activity of the cerebroid ganglion (CG) in crayfish. MEL was injected through a stainless steel needle implanted near the crayfish cardiac cavity, and its effect on CG activity was determined by recording the spontaneous potentials of this neuronal structure. **a** and **b** Reduction in the number of potentials elicited by the MEL injection at CT4 and CT18. The lower traces are representative examples of spontaneous potentials

recorded before the MEL injection and at the end of the experiments. Correspondence between external time of injection and CT was established measuring electroretinogram (ERG) circadian rhythm in other crayfish maintained under the same environmental conditions. *Arrows* in **a** and **b** depict the moment at which the injection of MEL was applied. **c** The comparison between the MEL effect obtained at different CT. Mean and standard error (SE) were plotted, and the *asterisk* represents  $p < 0.05$

extracts quantified in the neuronal tissues related to the control and expression of the ERG circadian rhythm, peaked at the same CT with respect to circulating MEL.

Furthermore, to test the effect of exogenous MEL on CG spontaneous electrical activity, an injection of the hormone was applied to crayfish at CT4, CT12, CT18, and CT22. MEL injections elicited a significant reduction in the number of spontaneous potentials recorded from CG in all CT with respect to vehicles (Figs. 3a, b); however, statistical differences were not detected on comparing the effect among the four CT (Fig. 3c). These results suggest that the increase in MEL concentration induced a reduction in the level of CG electrical activity.

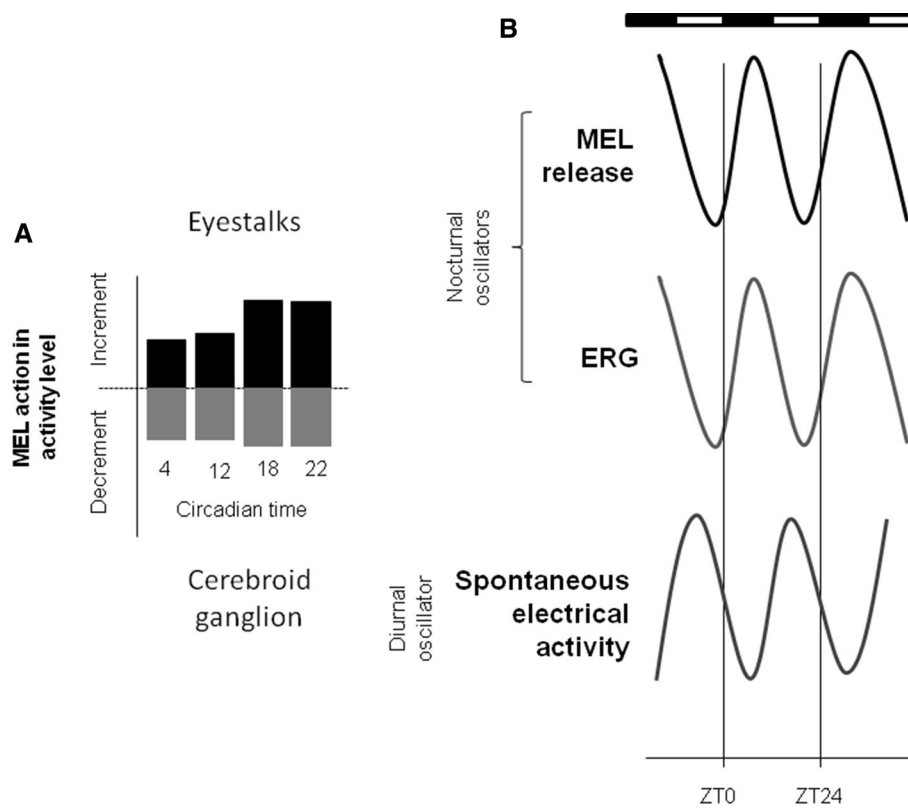
**Discussion**

In the present study, the results showed that circulating MEL in He or MEL content in Ey and CG changed following a circadian rhythm that peaked at the same CT. We also found that exogenous MEL injected into crayfish elicited a decrease in the number of spontaneous potentials recorded from the CG. Our results support the hypothesis that the circadian increase in MEL concentration is

detected by the CG to reinforce the temporal organization of the circadian system in crayfish.

Prior to use of the ELISA kit designed to measure MEL in human saliva samples, we confirmed the efficiency of the procedure for extracting MEL from homogenates of Ey or CG and diluted He. In this regard, the quantity of MEL extracted utilizing silica gel columns was nearly 100%, suggesting that the endogenous MEL was adequately obtained from homogenates to bind with ELISA antibodies. Our results indicated that in crayfish maintained under constant darkness and temperature (free-running condition), circulating MEL in He, as well as MEL content in homogenates, followed a circadian rhythm. These results suggest that in crayfish, the MEL concentration fluctuates in a circadian fashion, as occurs in other invertebrates such as marine zooplankton (Tosches et al. 2014), the echinoderm *Echinaster brasiliensis* (Peres et al. 2014), the nematode *Caenorhabditis elegans* (Migliori et al. 2012), the crab *Neohelice granulata* (Maciel et al. 2008), or in vertebrate species (Erren and Reiter 2015; Cazaméa-Catalan et al. 2014). This might imply that oscillatory synthesis of MEL in crayfish also comprises endogenous property of their circadian system.





**Fig. 4** Melatonin (MEL) induced opposite effects in the activity of diurnal or nocturnal circadian oscillators in crayfish. **a** Depicted a schematic representation of the MEL-induced increase in the electrophysiological response to light of retinal photoreceptors at different circadian times (CT) (modified from Mendoza-Vargas et al. 2009), as well as the MEL-induced decrease in the number of spontaneous potentials recorded from the cerebroid ganglion (CG) at the same CT (this work). In both cases, correspondence between external time of stimulation and CT was established by recording the

electroretinogram (ERG) circadian rhythm. **b** Schematic representations of the circadian rhythm found in the response to light pulses of retinal photoreceptors (nocturnal oscillator) (modified from Moreno-Sáenz et al. 1992), in the level of electrical activity recorded from CG (diurnal oscillator) [modified from Hernández and Fuentes-Pardo (2001)], and their phase relationship with the circadian rhythm of MEL release under photoperiod (modified from Balzer et al. 1997). In **b**, the *upper bar* represents the photophase and scotophase of the photoperiodic regimen

In vertebrate and invertebrate species, MEL synthesis takes place in several tissues, including retina and the central nervous system (CNS) (Bembenek et al. 2005; Bubenik 2002; Tosini and Menaker 1996; Arnoult et al. 1994). We found that in *P. clarkii*, MEL concentration in circulating He or in extracts obtained from neuronal tissues peaked at the same CT. It is unknown the synthesis site of MEL in crayfish; however, it has been suggested that this hormone might be released from the Ey (Sainath et al. 2013; Balzer et al. 1997). With our data, it is not possible to deduce whether MEL synthesis occurs simultaneously at several sites, which is highly possible; although, our results led to the assumption that MEL is released rhythmically in free-running, maintaining the same phase relationship in two key structures involved in the expression and control of circadian change in ERG amplitude, the Ey and the CG (Fanjul-Moles and Prieto-Sagredo 2003; Fuentes-Pardo and Hernández-Falcón 1993; Barrera-Mera and Block 1990). It also opens the possibility of proposing that the increase in circulatory He MEL might be detected at the

same CT by other central and peripheral structures that oscillate in rhythmic fashion, such as the heart (Hernandez-Falcon and Ramon 1998), the midgut gland (Durán-Lizarraga et al. 2001), or photoreceptor neurons from the terminal abdominal ganglion (Rodríguez-Sosa et al. 2008).

With respect to MEL function in *P. clarkii*, previous reports have demonstrated that MEL enhances sensitivity to stimulation with light pulses of retinal photoreceptors at different CT, binding to MT2-like membrane receptors (Mendoza-Vargas et al. 2009; Solís-Chagoyán et al. 2008). Results obtained in this study showed that exogenous MEL reduced the number of spontaneous electrical potentials recorded from CG at 4 different CT. As depicted in the schematic representations included in Fig. 4a, MEL exerted the opposite action on neurons from the CG regarding retinal cells. This MEL effect in contrary directions has also been reported in other species (Wan et al. 1999). A plausible interpretation of these results is that the circadian increase in endogenous MEL might modulate the functioning of the neuronal structures involved in the

expression and control of the ERG circadian rhythm; therefore, the duo of retinas and CG might be influenced by the cycle of the released hormone in order to reinforce their circadian coupling.

In this regard, in *P. clarkii* maintained under a photoperiodic regimen, the MEL level increases under darkness (Balzer et al. 1997); in addition, at scotophase, reticular response to light is high (Moreno-Sáenz et al. 1992), whereas the level of spontaneous activity recorded from the CG is low (Solís-Chagoyán et al. 2012; Hernández and Fuentes-Pardo 2001) (Fig. 4b). According to this pattern, data obtained in this study suggest that application of exogenous MEL mimics the activity level in CG (valley in scotophase) and in reticular cells (peak at scotophase) (Mendoza-Vargas et al. 2009). This is in agreement with the phase relationship found between ERG rhythm and the rhythm of release of MEL under the free-running condition. Taken together, these results suggest that a role of MEL in crayfish might be to act as a periodical signal that reinforces the temporal organization of nocturnal and diurnal circadian oscillators in crayfish.

In the circadian systems of vertebrate or invertebrate species, oscillator structures expressing diurnal or nocturnal functions coexist, and their phase relationship is stable. For example, in mammals with diurnal behavior, circadian release of MEL from pinealocytes increases at night, whereas plasma leptin released from adiposities follows a circadian rhythm with a diurnal peak (Kumar et al. 2015). In the case of crayfish, an animal with nocturnal behavior, some of their rhythmic functions peaked at night, i.e., glucose levels in He (Kallen et al. 1988); however, some other functions peaked during the day, i.e., release of the pigment dispersing hormone from the sinus gland (Hernández-Falcón et al. 1987). Our results are in agreement with the idea that MEL signaling might comprise a possible mechanism for organizing the temporal order (phase relationship) of circadian oscillators whose fluctuations are out-of-phase, such as reticular cells and neurons from CG in *P. clarkii*.

In summary, the data obtained in the present study support the hypothesis that pacemaker-controlled release of MEL temporally organizes the circadian system in crayfish. In this regard, circulating MEL apparently reaches central and peripheral circadian oscillators at the same CT in order to modulate their functioning. Additional progress will require anatomical identification of sites of MEL synthesis and the physiological action of MEL on other central and peripheral targets that exhibit circadian periodicity.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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