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Research report

Modulatory effects of melatonin on behavior, hemolymph metabolites, and neurotransmitter release in crayfish

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Abstract

Melatonin affects a variety of circadian processes such as behavior and neurotransmitter release in vertebrates. Crayfish melatonin production occurs in the eyestalks, and the cycle of production may change seasonally. To date, however, melatonin's roles and mechanisms of action in crustacean physiology are unclear. We injected melatonin or saline into crayfish in scotophase and monitored activity and hemolymph glucose/lactate over 24 h in early spring. Crayfish were significantly more active in photophase versus the expected scotophase, and had concurrent glucose/lactate peaks. Melatonin reversed the activity pattern, causing a scotophase activity peak, but not the glucose/lactate patterns. This study was repeated in late summer, during which control activity and glucose/lactate levels were elevated in scotophase. Melatonin decreased the amplitude of scotophase activity and glucose/lactate, eliminating activity and glucose cycles. We also injected melatonin or saline at various times of day in early summer and monitored locomotor activity for 1 h. Controls had high activity at 1200 (mid-photophase) and 2100 h (early scotophase), and melatonin increased activity at 1200 h but decreased it at 2100 h. Melatonin also increased activity at 1500 h but not 1800 h (late photophase). Next, we examined the influence of melatonin on crayfish neurophysiology. Melatonin (10 μM) enhanced synaptic transmission at the neuromuscular junction (NMJ). The presynaptic action resulted in more vesicles being released during evoked stimulation. Our study indicates that melatonin may have a phylogenetically conserved role in the transduction of circadian information in invertebrates as in vertebrates. Behavioral and physiological effects may be mediated by modulation of central pathways, enhanced at the peripheral level via neuromodulation of the NMJ.

Theme: Neural basis of behavior
Topic: Biological rhythms and sleep

Keywords: Melatonin; Crayfish; Neuromuscular junction; Activity pattern; Locomotion; Metabolite

1. Introduction

The presence of melatonin in crustaceans is now well documented; however, the nature of its production and its roles in crustacean physiology and behavior have not been extensively investigated. Melatonin is found in high concentrations within the eyestalks, as is *N*-acetyltransferase, the rate-limiting enzyme in vertebrate melatonin production [51,59,60]. Vertebrates have a characteristic scotophase increase in melatonin production, and melatonin is involved in the entrainment of circadian rhythms of behavior and physiology [41,56]. Crustaceans, on the other hand, exhibit greater variability in melatonin production. The intertidal fiddler crab *Uca pugilator*, for example, may have a tide-associated melatonin cycle [50,51]. In the giant freshwater prawn (*Macrobrachium rosenbergii*), females showed a melatonin cycle with a photophase peak [59]. In addition, early-photophase melatonin levels were different between males and females, indicating possible sex differences in melatonin cycles [60]. In the crayfish *Procambarus clarkii*, melatonin levels were higher during photophase in one study [1] and in scotophase in another study [6].

A variety of influences of melatonin have been reported in crustaceans: In *U. pugilator*, melatonin increased the rate

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of limb regeneration in eyestalk-intact and -ablated crabs [50]; it had both hypo and hyperglycemic effects and altered glucose and lactate cycles [52]; and it influenced locomotor activity [53]. In *P. clarkii*, melatonin caused both dark-adapting [6] and light-adapting [45] responses in the visual system. Together, these studies indicate a variable pattern of production and a variable influence of melatonin that may relate to seasonal or other changes. These studies suggest a role of melatonin in modulating circadian, seasonal, and other functions in crustaceans. Melatonin's role in circadian behavioral rhythms in vertebrates [8,25,28,55] is mediated by neuromodulation of the nucleus accumbens [37] and suprachiasmatic nucleus [27]. Much less is known about melatonin and behavior in invertebrates. In the cricket (*Acheta domesticus*), melatonin may entrain circadian locomotor rhythms [61]; and in *U. pugilator*, its influence may be tide-based [53]. However, the mechanisms and locations of action are not known.

In *P. clarkii*, the subject of our investigation, locomotor activity, displays a circadian rhythm: locomotor activity increases in scotophase and persists in constant conditions [5,23]. The mechanism of control of locomotor rhythmicity has not been clearly established in crustaceans. The x-organ/sinus gland complex, located within the eyestalk, releases neurodepressing hormone (NDH) which suppresses locomotor activity [3,4,26]. The entrainment of locomotor activity to photoperiod persists in the absence of the eyestalks, and the supraesophageal ganglion (SOG) has been identified as a component of the circadian pacemaker system [35,36]. A variety of other nervous system locations may also be involved in the crayfish circadian system [5]. Hemolymph glucose levels are also rhythmic: an increase occurs during scotophase in nocturnally active *Orconectes limosus*. This increase is concomitant with the peak of the eyestalk x-organ-produced crustacean hyperglycemic hormone (CHH) [31]. Lactate, a metabolic endproduct of activities and stresses [22], may also serve as a marker of locomotor activity. In the first part of our study, we examined the influence of melatonin on locomotor activity and on glucose and lactate patterns in *P. clarkii.*

The neuromodulator serotonin (5-HT), a precursor of melatonin, is also known to alter locomotor behavior in crayfish [49] and has been shown to increase in titer within the hemolymph during locomotor activity in crabs [44]. It is established that 5-HT enhances the following in crayfish: heart rate [32], neurotransmitter release at neuromuscular junctions (NMJ) of skeletal muscles [19,46], the drive of primary sensory neurons [14], and intrinsic CNS drive of motor neurons [47]. Since recent evidence showed that melatonin also causes an increase in the frequency of cardiac activity [54] as well as being correlated with locomotor activity, we used similar sensory and skeletal NMJ preparations as previously used for studies investigating 5-HT to compare the actions of melatonin.

2. Methods

2.1. Animals

All experiments were performed using *P. clarkii*, measuring 4–6 cm in body length (Atchafalaya Biological Supply, Raceland, LA, or Fruge's Cajun Crawfish, Branch, LA).

2.2. Activity and metabolites

For the activity and glucose/lactate studies, animals were held in aerated tanks and were fed daily at random times to prevent entrainment to food availability. They were acclimated to a 12L:12D photoperiod with lights on at 0700 h and off at 1900 h; the temperature was held at a constant 20 °C. Activity monitoring: male crayfish were placed in $30 \times 40 \times 15$ cm opaque Plexiglas™ boxes divided into water-tight lanes, 30 cm (length) $\times$ 8 cm (width) $\times$ 15 cm (height), with opaque dividers. Each lane received aerated, conditioned tap water to a depth of 13 cm, and one crayfish was placed into each lane; melatonin-treated alternated with saline-treated crayfish. The bottom of the tanks had gridlines placed 2 cm apart along the 30-cm length of each lane for quantification of locomotor activity. The activity monitoring room was a constant 20 ± 1 °C, with the same 12L:12D photoperiod as the acclimation conditions; light was provided by broad-spectrum fluorescent bulbs with a light intensity of 422 lx at the water surface. Sony 560× Digital8 Nightshot cameras were suspended from a ceiling rack such that each camera's field of view encompassed one box, and these cameras were connected to VCRs placed outside of the monitoring room.

Activity studies were conducted in early spring (March), early summer (June), and late summer (August); glucose and lactate levels were measured in early spring and late summer only. In the early spring and late summer studies, 100 μl of a melatonin solution [melatonin (Sigma) was dissolved in ethanol and diluted in crayfish saline [57] for final ethanol concentration of 1%] was injected into the base of a walking leg such that the final melatonin concentration in crayfish was 1 μg g^{-1} (4.3 nmol g^{-1}). Controls received 100 μl of saline with 1% ethanol; all injections were at 1900 h, at the onset of scotophase. Activity was monitored at the same time in 10 control and 10 experimental animals over a 24-h period, and different animals were used between early spring and late summer studies. We recorded activity for a 1-h period every 3 h over 24 h. The early summer (June) activity study was conducted similarly to the other two studies; however, melatonin or saline was injected into 10 crayfish each at 1200, 1500, 1800, and 2100 h, with different crayfish monitored for 1-h intervals (total: 80 animals). All activity data are reported as linear distance moved per hour.

For the glucose/lactate studies, crayfish were injected with melatonin ($n = 12$) or saline ($n = 12$) as described

previously, at 1900 h, with separate groups used in the early spring versus late summer studies. Hemolymph (50 μl) was collected from each animal at the base of a walking leg every 3 h over a 24-h period. Glucose and lactate levels were measured with a YSI 2300 STAT Plus glucose and lactate analyzer (YSI, Yellow Springs, OH); validation of this instrument for crustacean hemolymph has been previously determined [52].

2.3. Skeletal muscle preparations

All experiments were conducted using the opener muscle or the extensor muscle of the first walking legs. Preparations were dissected according to standard procedures [7,10]. The tissue was pinned out in a Slygard dish as to expose the muscle in a taut position. The muscles were viewed with a Nikon, Optiphot-2 upright fluorescent microscope using a 40× (0.55 NA) Nikon water immersion objective. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (205 mM NaCl; 5.3 mMKCl; 13.5 mM $CaCl_2$ $2H_2O$; 2.45 mM $MgCl_2$ $6H_2O$; 0.5 mM HEPES adjusted to pH 7.4) at 17 °C. The entire opener muscle is innervated by a single excitatory motor neuron [10], whereas the extensor muscle is innervated by a single tonic and a single phasic excitatory motor neuron [7].

2.4. Application of melatonin

To apply exogenous compounds to these preparations, the bathing medium was rapidly exchanged with saline containing melatonin (10 μM, Sigma). A fresh aliquot was used before each experiment from a frozen stock (1 mM).

2.5. Intracellular recorded evoked postsynaptic potentials (EPSPs)

Intracellular muscle recordings were made with a 3 M KCl-containing microelectrode. Short-term facilitation (STF) was induced by giving a 40-Hz train of 10 pulses at intervals of 10 s. To determine a facilitation index for the induced STF of the tonic EPSPs obtained from the opener muscle, the amplitude of the 10th EPSP within a train is compared with one of the preceding EPSPs amplitude within the response [16]. The subtraction of the numeral one ensures that if there is no facilitation occurring, the facilitation index (FI) will then be zero. Responses were recorded as previously described [15]. Stimulation of the opener motor neuron was carried out as described by Dudel and Kuffler [20].

2.6. Field recorded excitatory postsynaptic potentials (f EPSPs)

Synaptic potentials were measured with focal macropatch electrodes, allowing for a determination of the effect of melatonin on presynaptic vesicular events. The living terminals were visualized by exposure to vital fluorescent dye 4-Di-2-ASP (Molecular Probes [11,33]). The synaptic potentials were obtained using the loose patch technique by lightly placing a 10–20 μm fire polished glass electrode directly over a spatially isolated varicosity along the nerve terminal. The macropatch electrode is specific for recordings within the region of the electrode lumen. The seal resistance was in the range of 100 kΩ to 1 MΩ. The evoked field excitatory postsynaptic potentials (f EPSCs) and field miniature excitatory postsynaptic potentials (fmEPSPs) were recorded and analyzed to determine the mean quantal content (m) [12,13]. Mean quantal content was determined by the direct counting method for the low output tonic terminals. By direct counts of the evoked quantal events, the number of failures in evoked release was used as an index of altering synaptic function. If only one single event occurred after the spike, it was counted as one; when double events occurred, they were referred to as two, etc.

Quantal release over the time was also monitored by examining the area of the evoked potential. The time of the peak in evoked events varies due to latency jitter, so when multiple events occur, the measures of peak amplitude are not as reliable as the area measure [12]. To monitor quanta released over time, the area of the evoked potential was measured for each event. This approach was also used to quantify alterations in synaptic potentials for the tonic terminals. The tonic nerve was stimulated at a rate of 1 or 2 Hz in order not to facilitate the responses between trials. Since exposure to melatonin had a gradual effect on mean quantal content (m), samples for every 200 events were used [46,47]. Larger sample sizes of 800 to 1000 or more trials were used to estimate the quantal parameters for the effects of melatonin exposure on the number of releasing sites (n) and the probability of release at a site (p) [12,13,17].

2.7. Statistical analysis

Activity, glucose, or lactate levels were compared within each group of the early spring and late summer study with a one-way ANOVA with a Student–Newman–Keuls test. Mean activity, glucose, or lactate levels were compared between some groups of the above studies, and activity was compared between melatonin-treated and control animals in the early summer study, with a Student's t-test (SigmaStat software, SPSS, San Rafael, CA).

To quantitatively compare the change in the EPSP amplitudes and synaptic potentials from the f EPSPs, the measurements were normalized to a percent difference. The percent difference was calculated using the difference among the average during baseline recording prior to melatonin exposure and the average of the events at the maximum response during the exposure. The average was then divided by the baseline value as shown in the following equation: $[|\text{Baseline} - \text{maximum response}|/\text{Baseline}] \times 100 = \%\text{Difference}$. Numerical data were represented as the

mean in the percent difference from exposure to saline. When the basic assumption of parametric Student's *t*-test was valid, it was used; otherwise, the non-parametric Wilcoxon rank sum test was used.

3. Results

3.1. Activity and metabolites

3.1.1. Early Spring

Control crayfish were significantly more active ($P<0.001$) during mid- to late-photophase than during scotophase (Fig. 1a); glucose and lactate levels were both significantly elevated ($P<0.001$ for both) during the active phase (Fig. 1b). Melatonin injected at the onset of scotophase caused a significant nocturnal rise ($P=0.011$) followed by a diurnal decrease in activity (Fig. 1a). Glucose was higher in scoto- and lower in photophase than control levels, though overall glucose levels were still lower in scoto- than photophase (Fig. 1c); i.e., a reversal of pattern did not occur. Lactate levels in melatonin-treated crayfish were in phase with activity levels (Fig. 1d), with a significant ($P=0.008$) scotophase increase.

3.1.2. Late Summer

Control crayfish were significantly more active ($P=0.013$) in scotophase (Fig. 2a), and glucose and lactate levels were also elevated ($P=0.001$ and 0.018, respectively) in scotophase (Fig. 2b). Melatonin injected at the onset of scotophase resulted in a decreased amplitude of the scotophase activity peak, with no significant change over

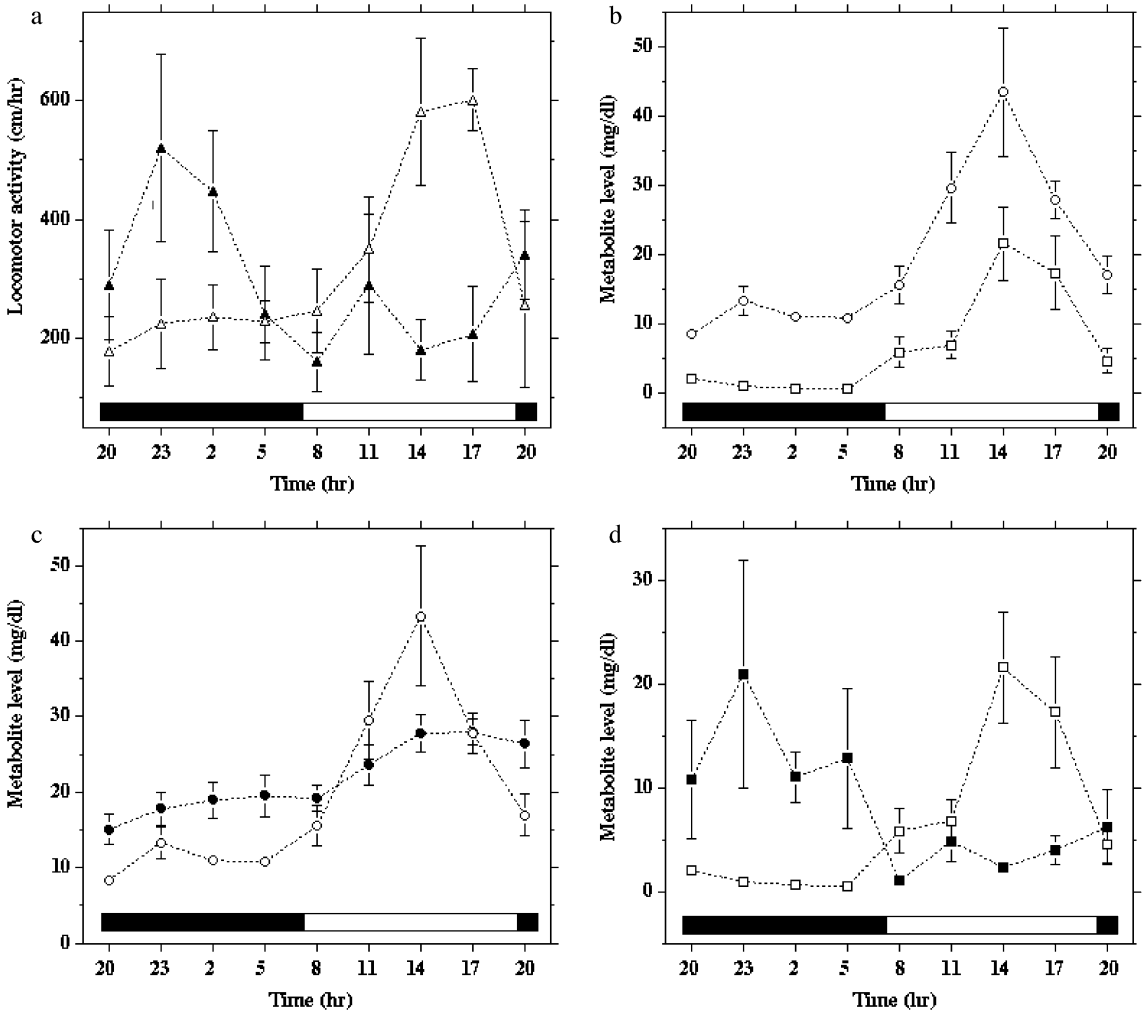

Fig. 1. (a) Activity levels (distance moved in 1 h) in *P. clarkii* in early spring in response to saline (△) or melatonin (▲) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 10 crayfish monitored for 1 h. Dark and white bars represent scotophase and photophase. (b) Hemolymph glucose (○) and lactate (□) levels in *P. clarkii* in early spring in response to saline injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase. (c) Hemolymph glucose levels in *P. clarkii* in early spring in response to saline (○) or melatonin (●) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase. (d) Hemolymph lactate levels in *P. clarkii* in early spring in response to saline (□) or melatonin (■) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase.

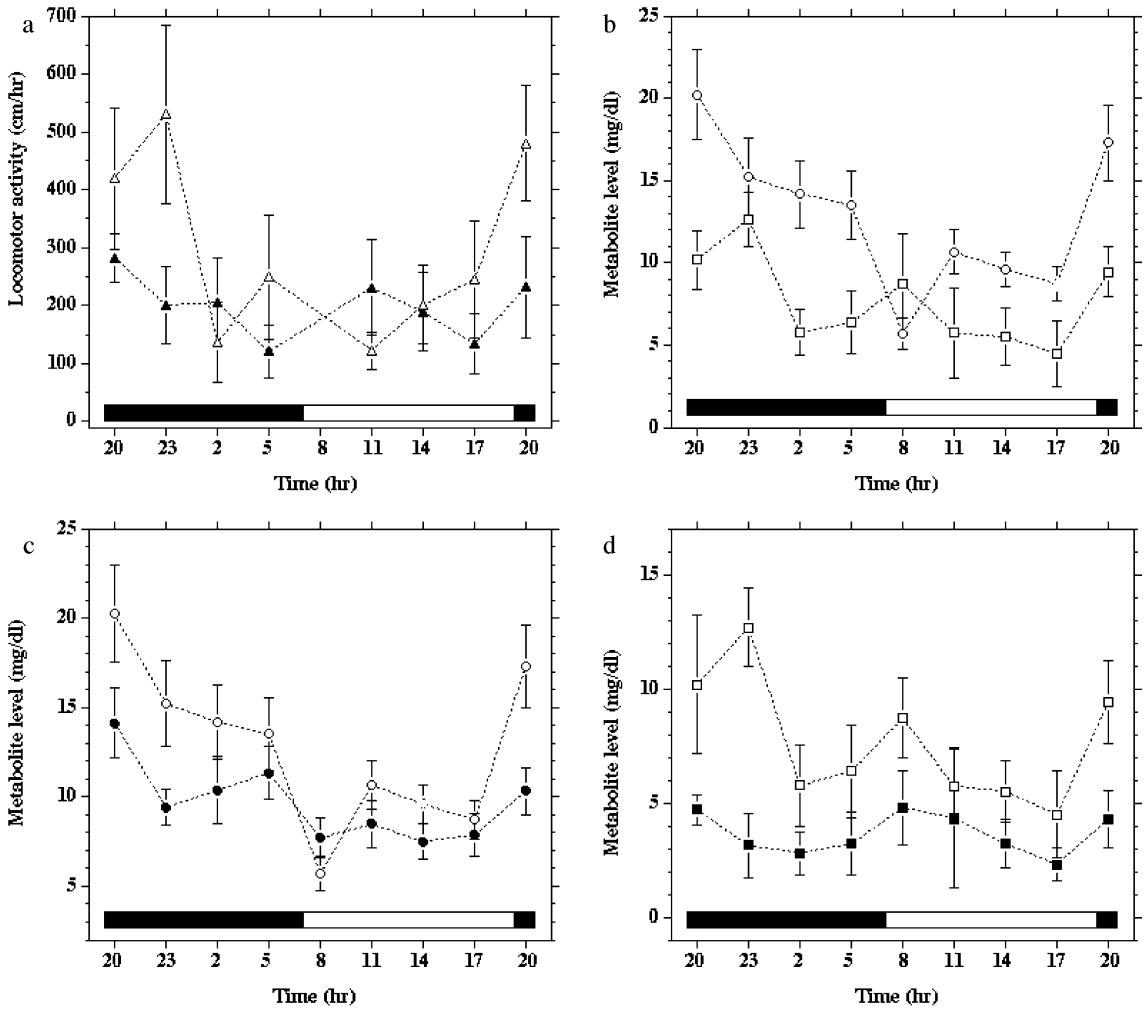

Fig. 2. (a) Activity levels (distance moved in 1 h) in *P. clarkii* in late summer in response to saline (△) or melatonin (▲) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 10 crayfish monitored for 1 h. Dark and white bars represent scotophase and photophase. (b) Hemolymph glucose (○) and lactate (□) levels in *P. clarkii* in late summer in response to saline injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase. (c) Hemolymph glucose levels in *P. clarkii* in late summer in response to saline (○) or melatonin (●) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase. (d) Hemolymph lactate levels in *P. clarkii* in late summer in response to saline (□) or melatonin (■) injection at the first scotophase onset. Each point represents the mean ± S.E.M. of 12 crayfish. Dark and white bars represent scotophase and photophase.

24 h; $P=0.341$ (Fig. 2a). Glucose and lactate were also reduced compared with controls, with no significant changes over 24 h for lactate ($P=0.426$) (Fig. 2c and d). A recording failure prevented the collection of data at 0800 h in this study.

3.1.3. Early Summer

Control crayfish in June had higher activity levels at 1200 and 2100 h than at 1500 and 1800 h; and melatonin had different effects on locomotor activity when injected at different times during the day (Fig. 3). Melatonin significantly increased activity during mid photophase, at 1200 h ($t=2.9$; $P=0.0095$) and 1500 h ($t=2.89$; $P=0.0097$); had no effect ($t=0.956$; $P=0.35$) at late photophase (1800 h), and significantly decreased activity ($t=2.94$; $P=0.0087$) in early scotophase (2100 h).

3.2. Amplitude of tonic EPSPs

The opener muscle in the first pair of walking legs in crayfish has been used to investigate the effects of neuromodulators and synaptic plasticity for a number of years [19,46]. Therefore, we also used this same preparation for comparative purposes to earlier works on the effects of other neuromodulators, such as 5-HT, octopamine [18] and ecdysone [9]. Since the opener muscle displays a relative regional variation in the amplitude of the induced EPSPs, we chose to consistently use the 3rd to the 5th muscle fiber bundles from the most distal end of the opener. This allowed us to avoid measuring responses from the relatively high output motor terminals which innervate the proximal fibers and the very low output terminals that innervate the large central-to-proximal muscle fibers [11,13,29,34]. A represen-

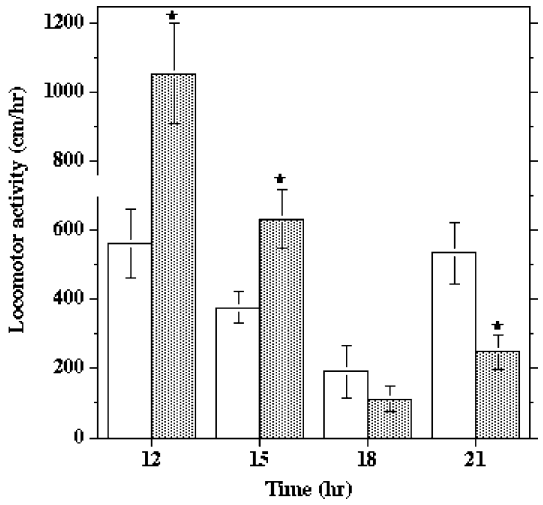

Fig. 3. Activity levels (distance moved in 1 h) in *P. clarkii* in early summer in response to saline (white bars) or melatonin (shaded bars) injected just prior to monitoring at 1200, 1500, 1800, and 2100 h; each bar represents the mean ± S.E.M. of 10 crayfish.

tative response to a 40-Hz train of 10 pulses is shown in Fig. 4. Upon addition of melatonin (10 uM) each of the EPSPs within the train shows an increase in amplitude. The response depicted for the effects of melatonin was measured after the preparation had been exposed for 10 min.

The effect of melatonin in enhancing the amplitude of the EPSPs was gradual, and in all five preparations the enhancement reached a plateau after approximately 10 to 15 min. The amplitude during the plateau response was used to determine a percent increase in the EPSP amplitude for both the 5th and 10th responses within the train. A representative response to exposure to melatonin is shown in Fig. 5A. Since there is variation in the initial amplitudes of the EPSP among preparations when only exposed to saline, the absolute changes observed during exposure to melatonin are not practical for comparisons. Thus, a percent change in the amplitude of the EPSPs was determined within each preparation relative to basal conditions during the initial saline exposure. As shown in Fig. 5B there is a significant increase ($n=5$, $P<0.05$ Wilcoxon rank sum) in the amplitude of the 10th EPSP to exposure to melatonin.

The facilitation index revealed that melatonin does not alter the extent of STF under the paradigm used to induce STF ($n=5$). A typical measure of facilitation before and during exposure to melatonin is shown in Fig. 5C. The measures shown were determined from the amplitudes of the 5th and 10th EPSPs depicted in Fig. 5A.

3.3. Quantal release measured by f EPSPs

Since the enhancement in the intracellular recorded EPSPs can be due to a composite of pre- and post-synaptic actions by melatonin, we set out to determine the presynaptic contribution of melatonin's actions by direct quantal counts of release [17]. This measure allows one to determine if more or fewer synaptic vesicles are being recruited for transmission by an evoked response during exposure to melatonin.

A single evoked action potential-induced depolarization of the nerve terminal will result in the recruitment of some synapses for the release of transmitter. Monitoring a given

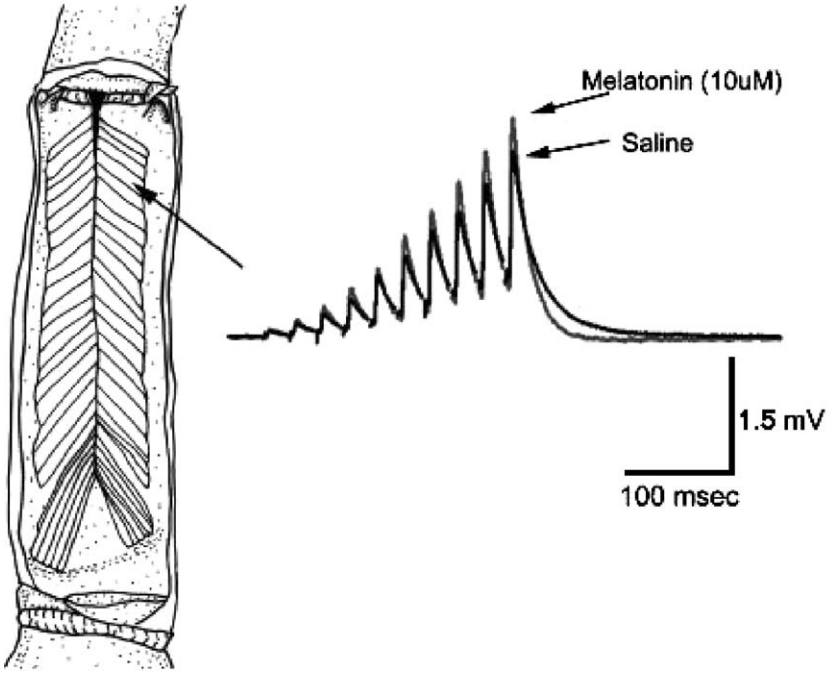

Fig. 4. Schematic view of crayfish opener muscle with ventral orientation. A typical response is shown when the excitatory motor nerve is stimulated by a 40-Hz train containing 10 pulses in saline and during exposure to melatonin (10 μM). The 5th and 10th pulse amplitudes were measured for effects of melatonin.

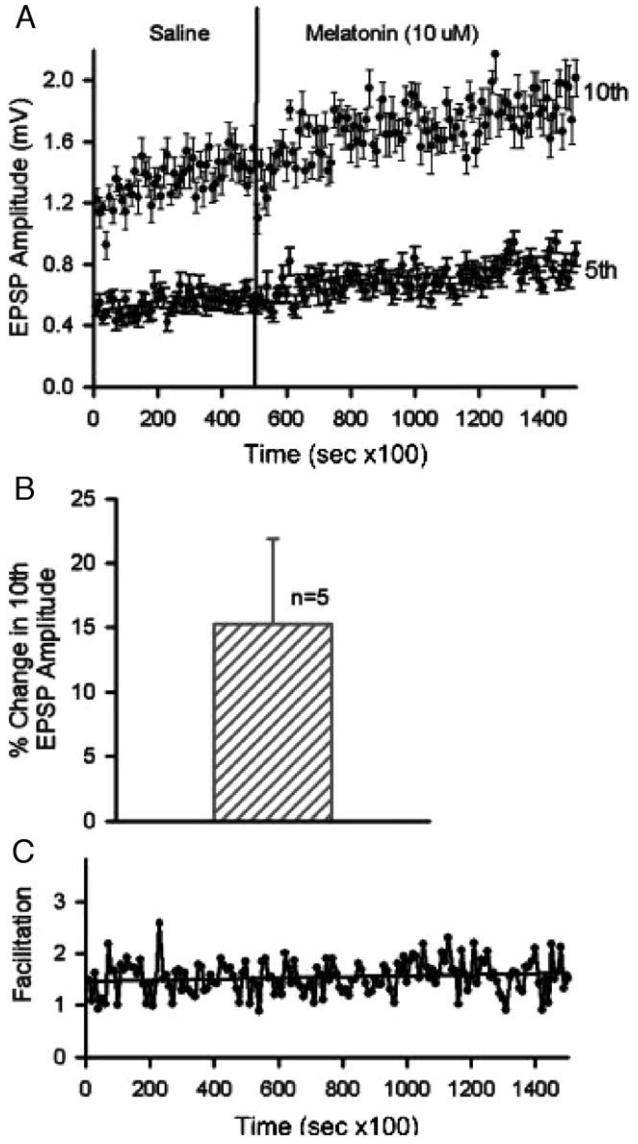

Fig. 5. Mean amplitudes of the intracellular recorded EPSPs for every 100 s plotted versus time (A). The bars show the standard error. The addition of melatonin (10 μM) causes a slight increase in the mean EPSP amplitude for both the 5th and 10th EPSPs. A percent change in the amplitude of the EPSPs for the 10th pulse was determined for the five preparations (B). The average responses measured between 200 and 500 and between 1200 and 1500 periods were used to calculate the percent change as shown in part A. Exposure to melatonin did not result in any change in facilitation (C).

length of the nerve terminal (~ 10 μm) with a macropatch electrode allows a subset of the nerve terminal to be assessed and discrete quantal events to be detected for a measure of synaptic efficacy. The vesicular glutamate released binds to ionotropic ligand-gated receptors of a quisqualate type [21,43], resulting in a rapid inward current. This current is detected by the focal macropatch electrode [48], and since we use the axoclamp as a differential amplifier (i.e., bridge mode), a field electrical potential is measured from inside the electrode lumen to outside in the surrounding bath.

Each single trace from an evoked stimulation of the excitatory motor axon may result in a failure of response, an evoked synaptic response with one quantal event, or responses with multiple releases in the defined region of the nerve terminal being monitored (Fig. 6A). With direct counting of each quantal event for each trace, the gradual changes in the observed quantal occurrences can be compiled (Table 1). These responses indicate an increased number of single and multiple evoked events upon exposure to melatonin (10 μM).

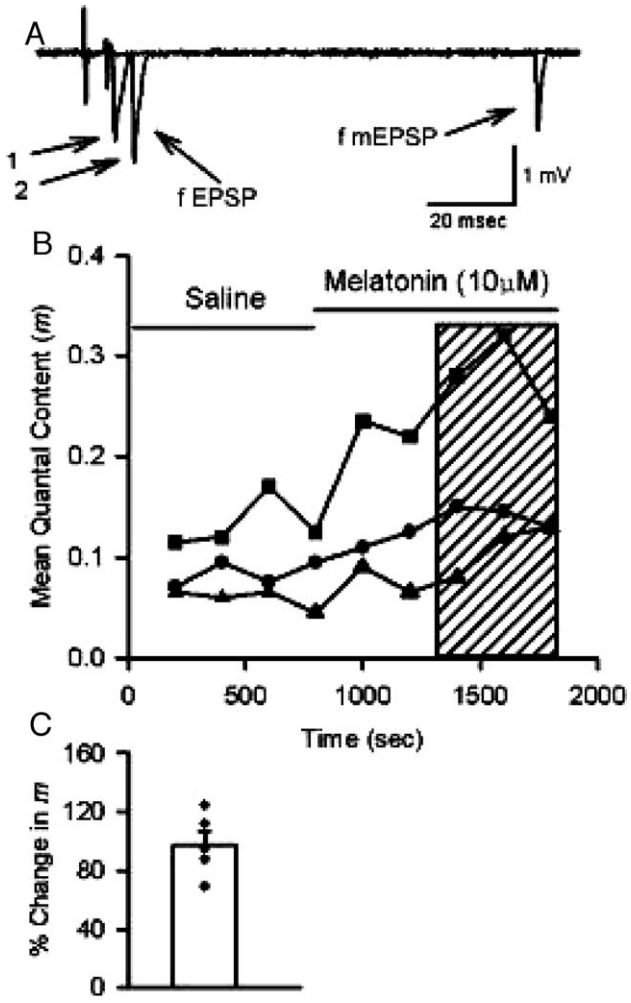

Fig. 6. Influence of melatonin on synaptic currents as recorded with a focal macropatch electrode from a spatially isolated varicosity. Representative single traces are shown with one containing two evoked events and a superimposed trace with a miniature excitatory postsynaptic potential (f mEPSP). Note in the evoked field excitatory postsynaptic potentials (f EPSPs) individual quanta can be counted (arrows) (A). A series of evoked events induced at 1 HZ stimulation to the motor nerve before and after application of melatonin. A typical time series for three preparations is shown (B). The time bins are divided into 200 trials for assessment of mean quantal content (*m*). The hatched area indicates the period taken, the last three values, to obtain an average value to determine a percent change due to melatonin's action. A mean percent change in the mean quantal content for five preparations indicates an excitatory presynaptic action of melatonin (C). The individual values for each of the five preparations are superimposed on the bar chart as closed circles.

Table 1
Quantal parameters: number of releasing sites (n), the probability of release at a site (p), and mean quantal content (m)

Prep	Events	Saline	Melatonin
1	0	755	905
	1	43	93
	2	2	2
	n	Poisson	2
	p		0.049
	m	0.059	0.097
2	0	696	750
	1	102	241
	2	2	9
	n	2	2
	p	0.066	0.13
	m	0.133	0.259
3	0	734	869
	1	65	130
	2	1	1
	n	2	2
	p	0.042	0.066
	m	0.084	0.132
4	0	2883	2768
	1	113	230
	2	4	1
	3		1
	n	1	Poisson
	p	0.038	
	m	0.040	0.078
5	0	2885	2809
	1	113	191
	2	2	
	n	1	Poisson
	p	0.038	
	m	0.039	0.064

In a Poisson distribution, the n is high and the p is small. Accuracy in estimating n and p is low for Poisson distribution and so they are not shown. Mean quantal content is determined by direct counts. The n and p for binomial distributions are determined by best fits.

The parameters: mean quantal content (m), the number of release sites (n), and the probability of release at the sites (p) suggest melatonin enhances the nerve terminal to release more vesicles. Estimates were made by a maximum likelihood estimation. Number of the discrete events indicated as: 0, failures; 1, ones; 2, twos; etc., are given.

3.4. Quantal parameters

An advantage of directly counting evoked quanta is that one may make estimates regarding the quantal parameters of n (number of release sites), and p (the average probability of release at the sites) based on the distribution of events, and further, on the effects that neuromodulators have on these parameters. Gradual or abrupt changes can also be detected by analyzing groups of 200 events as well as an entire set of 1000 events (Fig. 6B). The individual bins of 200 events for the most part mimics the larger grouped data set. It is apparent that the m increases in the presence of melatonin due to fewer failures of release which in turn mean an increase in the overall probability of release. Determination of n is a standard approach by assessing if the distribution is best fit by a binomial or a Poisson distribution. If the fit is a binomial one, then an average p for n sites is estimated for the closest fit to the observed distribution. Since a Poisson distribution indicates many release sites (i.e., high n) with a low probability, it is difficult to estimate with accuracy n and p and therefore estimates are not reported. We present the estimates of n and p for the binomial distribution since the field is familiar with this approach and may use it for comparative purposes. However, one should note that n is likely to be underestimated since not all single quantal events appear to arise from the same site given there is a wide variation in the areas and clusters of large and small areas measured from f EPSPs [58].

The index of m over time indicates a temporal effect of melatonin on mean quantal content (Fig. 6B). A percent change in m was determined by using an average value while exposed to saline to the average of the last three of the 200 binned periods (see hatched region in Fig. 6B) obtained while exposed to melatonin. Since an increase in the efficacy of synaptic transmission occurred in all cases ($n=5$), the effect of melatonin on the presynaptic transmitter release is significant (Fig. 6C, $n=5$, $P<0.05$; Wilcoxon rank sum).

To further examine the presynaptic actions of melatonin on the quantal release in addition to using the golden standard of direct counts, the influence on the area measures for f EPSPs were compared before and after application of melatonin. Melatonin increased the overall average of the f EPSPs since more occurred, and the area in some of the f EPSPs was increased since more multiple quantal events occurred. The quantal events deemed as singles did not show an increase in overall potential area. Likewise in the few runs in which fmEPSPs could be detected there was no significant difference in the average response during exposure to melatonin. A representative distribution shows the

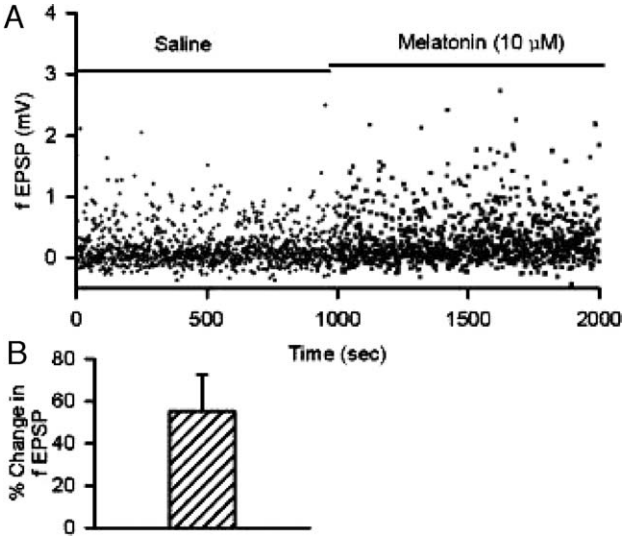

Fig. 7. A representative scatter plot showing the influence of melatonin on f EPSP area before and during exposure to melatonin (A). A mean in the percent increase caused by melatonin exposure indicates a significant increase in overall synaptic efficacy by melatonin (B). Five out five preparations showed this enhancement.

increase in the occurrences of the f EPSPs, as measured by the EPSP area, over time due to melatonin exposure. Such measures as the area or amplitude of the f EPSPs can be undertaken when one is not confident in the direct quantal counting method due to the lack of discerning multiple releases within a single f EPSP. So by using both types of analysis a clearer understanding of the mechanisms by which melatonin influences synaptic transmission can be ascertained. Since in a few preparations we were not confident in the direct quantal counts, we utilized the index for area of the f EPSP to assess a change in transmission instead of the counting method. An average in the percent increase in the area of the f EPSPs from before to during melatonin exposure revealed approximately a 60% increase in all five preparations ($n=5$, $P<0.05$ Wilcoxon rank sum) (Fig. 7).

4. Discussion

Melatonin affects activity and metabolite levels, and has an excitatory effect at the NMJ; however, the effects appear to vary with the activity phase of the animals and the timing of melatonin application. Crayfish are reportedly nocturnally active [2,23,24,38], with scotophase peaks of CHH and glucose [30,31]; yet we observed diurnal activity in controls in early spring. Two possible explanations for the diurnal activity are: First, the crayfish may have entrained to a daytime feeding regimen. Though we randomized feeding and did not feed animals for 5 days preceding an experiment, the crayfish were purchased from a farm-raised and -fed population. Fernández de Miguel and Aréchiga [23,24] showed that day-fed crayfish develop a photophase activity pattern that can be instated with a single feeding and persists for up to 2 weeks without food re-entrainment. Second, *P. clarkii* is active in the daytime during early spring rainstorms or cloudy days but not sunny days [38]. The activity stimulus is not known but may involve levels of light intensity. The light level in our lab (420 lx) is substantially lower than that of bright sunlight (>20,000 lx) and may have had a 'cloudy day' effect in the spring study.

Melatonin caused an increase in scotophase and a decrease in photophase activity, glucose, and lactate in the diurnally active early spring crayfish. Though we have data for only a single 24-h cycle, melatonin may be able to phase-shift an induced diurnal activity pattern to the more typical nocturnal one. In separate studies, both a photophase [1] and a scotophase [6] melatonin peak have been shown in *P. clarkii*; these conflicting results could be related to seasonal, acclimation, feeding schedule, sex, molt stage, reproductive cycle, population, or other differences. However, these studies do demonstrate that the timing of the melatonin peak is variable. In our late summer study, melatonin decreased the amplitude of the scotophase peaks of activity, glucose, and lactate but did not affect the timing of these events. In early summer, there were both photophase and scotophase activity increases, with melatonin enhancing activity in photophase but inhibiting activity in scotophase. Likewise, in fiddler crabs melatonin increased activity at some times of day but decreased it at others [53].

Our contradictory early spring, early summer, and late summer results may be due to seasonal or acclimational changes in both the timing of melatonin production and the timing of the animals' sensitivity to melatonin. Similarly, melatonin had opposing effects in two studies of visual activity in *P. clarkii*: Melatonin affected both the period and amplitude of the electroretinogram (ERG): the period was shortened and the amplitude was increased, indicating dark-adapting effects that were consistent with the finding of a scotophase melatonin increase [6]. Yet in another study, melatonin induced migration of retinal shielding pigments and changes in photoreceptor potential that resembled light-adapting responses [45], consistent with the finding of a photophase melatonin peak [1].

Melatonin receptors in invertebrates have not yet been studied, and the precise target tissues are not known. In previous studies [52,53], we have demonstrated that melatonin affects locomotor activity, glucose, and lactate in both intact and eyestalk-ablated crabs, indicating that that these effects probably are not mediated by eyestalk CHH and NDH. Melatonin has direct receptor-mediated effects on mammalian hepatocytes [40] and pancreatic islet cells [39], and may likewise affect the crustacean hepatopancreas. In addition to the hepatopancreas, muscle may be a source of hemolymph glucose [42], and we have shown here that melatonin has an excitatory effect on muscle cells. Melatonin may be a component of the complex system driving rhythmic processes in invertebrates as it is in vertebrates. The behavioral and physiological effects of melatonin are likely due to central modulation of neural activity rather than solely due to localized neuromodulation at the NMJ. However, the crayfish NMJ provides an ideal model system for the study of general nervous system neuromodulation and provides information on possible central and peripheral mechanisms of action.

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