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**Chronic electromyographic analysis of
circadian locomotor activity in crayfish**

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Abstract

Animals generally exhibit circadian rhythms of locomotor activity. They initiate locomotor behavior not only reflexively in response to external stimuli but also spontaneously in the absence of any specific stimulus. The neuronal mechanisms underlying circadian locomotor activity can, therefore, be based on the rhythmic changes in either reflexive efficacy or endogenous activity. In crayfish *Procambarus clarkii*, it can be determined by analyzing electromyographic (EMG) patterns of walking legs whether the walking behavior is initiated reflexively or spontaneously. In this study, we examined quantitatively the leg muscle activity that underlies the locomotor behavior showing circadian rhythms in crayfish. We newly developed a chronic EMG recording system that allowed the animal to freely behave under a tethered condition for more than 10 days. In the LD condition in which the animals exhibited LD entrainment, the rhythmic burst activity of leg muscles for stepping behavior was preceded by non-rhythmic tonic activation that lasted for 1323 ± 488 msec when the animal initiated walking. In DD and LL free-running conditions, the pre-burst activation lasted for 1779 ± 31 and 1517 ± 39 msec respectively. In the mechanical stimulus-evoked walking, the pre-burst activation ended within 79 ± 6 msec. These data suggest that periodic changes in the crayfish locomotor activity under the condition of LD entrainment or free-running are based on activity changes in the spontaneous initiation mechanism of walking behavior rather than those in the sensori-motor pathway connecting mechanoreceptors with leg movements.

Key words: walking behavior; motor command; behavioral initiation; reflexiveness; spontaneity; invertebrate; crustacean.

Introduction

Animals show circadian rhythms in a variety of homeostatic functions and behavioral activities. These rhythms are thought to be endogenous [1-3], based on an internal clock or pacemaker that resides in a specific site in the central nervous system depending on animal species [4]. Among many kinds of behavioral rhythms, the most widely recognized in the animal kingdom is that observed in locomotor behavior including walking [5-7], swimming [8, 9], and flying [10, 11]. The neurophysiological mechanism underlying the circadian rhythms of locomotor behavior, however, remains largely unknown.

Crustaceans have been known to show circadian rhythms in hormonal secretion [12-15], neurosecretion [16], biogenic amine contents and actions in the central nervous system [17-19], mechanoreceptor sensitivity [20], photoreceptor sensitivity [20-36] and locomotor activity [37-43]. A previous study in the crayfish *Procambarus clarkii* showed that the circadian rhythm in leg movement disappeared when the circumesophageal commissures were surgically lesioned bilaterally, thus experimentally demonstrating the importance of descending signals from the brain to posterior ganglia [38]. Thus the rhythm of leg movements appears to be based on circadian changes in the motor commands generated endogenously in the brain.

It should be noted here that locomotor behavior could be evoked either endogenously or in response to external stimulation. In crayfish, walking behavior is initiated spontaneously in the complete absence of external stimuli that can be detected by human observers. Walking behavior of crayfish can be also initiated by slightly touching its body or putting some chemical substances in the water [44]. An electromyographic (EMG) study revealed that the temporal pattern of leg muscle

activation is statistically different between spontaneously evoked and stimulus-evoked walking [44]. It was reported that the spontaneous walking was characterized by sustained, non-rhythmic activation of leg muscles that preceded their rhythmic burst activities whereas the mechanical stimulus-evoked walking was initiated abruptly following the stimulus [44]. Further studies have revealed that descending neuronal signals indicative of readiness activities, which is defined as the spike activity that transiently precedes the voluntary initiation of behavior, in the crayfish brain [45,46]. The spike activities called readiness discharge precede the onset of leg muscle activation drive spontaneous walking in crayfish [45,46]. Those descending neurons (readiness discharge neurons) identified by intracellular techniques became active > 1 s before the behavioral initiation of walking and remained inactive at the onset of mechanical stimulus-evoked walking, suggesting that the central nervous mechanisms for initiating spontaneous and stimulus-evoked walking are quite different from each other [45,46].

In the present study, we addressed the following question: what physiological mechanism subserves the circadian rhythm of crayfish locomotor activity? One possible mechanism is periodic changes in the central nervous activities that are responsible for endogenous initiation of walking behavior. Alternatively, periodic changes in the functioning of sensori-motor pathways such as mechanoreceptor sensitivities and synaptic transmission efficacy might underlie the circadian rhythm observed in locomotor activity. We newly developed a chronic EMG recording system that allowed us to quantify the muscle and behavioral activities simultaneously over a long-term experimental period. Quantitative analysis of the EMG data suggests that the neurophysiological mechanisms underlying circadian locomotor activity in crayfish involve the neuronal pathways responsible for spontaneous initiation of walking.

2. Materials and Methods

2.1. Animals

Adult crayfish (*Procambarus clarkii* (Girard)) of both sexes (8.5 - 9.8 cm in body length from rostrum to telson) were used in this study. They were obtained commercially and maintained in laboratory tanks, fed weekly on pellet foods (Kyorin, Hyogo). Prior to the tethering of animals for electromyographic recording, they were individually kept in small aquarium and subjected to 12 h: 12 h light: dark (L:D) cycle for at least 2 weeks. The animals were then equipped with EMG harnesses and acclimatized to the experimental aquarium (Fig.1A) for 3 to 5 days under LD cycle conditions preceding the circadian experiments. They could freely obtain food that was placed at the feeding place with a sufficient dose for the experimental period (10 days ~). We tested 68 animals in this study. Especially in EMG analysis of circadian locomotor activity, only those 16 crayfish that were statistically judged to exhibit circadian rhythm as explained below were used as the experimental animal. We used 49 animals, including those for other experiments, in the final analysis.

2.2. Experimental apparatus

An experimental animal equipped with a harness, consisting of PVC insulated cables which was connected to Teflon insulated silver wires for EMG recording and their catches to a small plastic plate that was dorsally fixed to the carapace, was placed in an acrylic aquarium (400 mm long × 200 mm wide × 160 mm high, Fig.1A). The whole apparatus was placed in a wooden box (1100 mm long × 700 mm wide × 740 mm high)

that was completely shielded from the outside to avoid any visual and auditory disturbance. The box also functioned as a faraday-cage because its inward was covered a steel mesh connected to the ground. The aquarium was filled with water to the level of the animal's body height. The water temperature was kept at $20^{\circ}\text{C}\pm 1$. The L/D switching of a white LED was controlled by an electronic timer. The light period started at 6 o'clock in the morning and the dark period started at 6 o'clock in the evening. The illuminance of the light was maintained at 60 lx at the center part of the aquarium. The tether cable was connected with a slip ring (TSR4251, Tokyo communication equipment) mounted on the ceiling of the box to avoid disturbing animal's behavior by twisting of the tether cable. Animals could rest in a shelter space that was kept relatively dim compared to the surround (Fig.1A). On a platform place located above the water surface, their food was stored sufficiently for long-term experiment. We monitored locomotor activity of the crayfish using five pairs of infrared LEDs (Osram SFH 4350: peak emission at 850 nm) and phototransistors (Osram SFH 313 FA: maximum sensitivity at 870nm) that were mounted in the aquarium wall above the water surface. In every experiment, we started EMG recording at the onset of the dark period (6 o'clock in the evening). When the animal walked, the tether cable moved together and interrupted any one of the lines of infrared beam. The phototransistor signal was fed to a voltage comparator and a differentiator, and finally to a one-shot multi vibrator to generate a TTL pulse signal of 100msec duration. This was finally digitized every 15 msec by a 16-bit A/D converter (USB-6211, National Instruments) connected to PC1 that constantly recorded pulse trains. The data was recorded by a self-made software written in Objective-C using Cocoa framework on MacOS10.5.7.

2.3. Electromyographic recording

Crayfish were anesthetized with ice water for 10-15 min before implantation of chronic electrodes. We always used a pair of the fifth leg for EMG recording because the electrodes implanted here were less likely to tear loose during long-term chronic recording. A typical fifth leg is depicted in Fig. 1B. Extracellular recording was made by a pair of silver wires (250 μ m in diameter) coated with Teflon except at the cut tip. The cut tip was additionally cut in half lengthwise to make it easily inserted into the leg muscle. A pair of fine holes was drilled through the cuticle with a sharp micro pin, and the electrode tips were inserted into a relatively immobile region of the muscle. The holes were then sealed and the wires were fixed to the cuticle with quick-drying adhesive (Aron Alpha, Toagosei, Tokyo). All appendages, including the chelipeds, remained intact and mobile. After surgery, the animal was placed in the experimental aquarium (Fig. 1A) for 3 to 5 days for recovery and acclimation. Since, in the previous study [44], the mero-carpopodite flexor (MCF) muscle activity was found to be useful for discriminating between spontaneous and stimulus-evoked walking, electromyograms were recorded from the MCF in the meropodite throughout the current study. Since the musculature arrangement of the meropodite is complicated [47], we always had to be careful to make sure the recording was being done from the targeted one by checking its activity against the meropodite movement. EMG signals were differentially amplified (MEG- 2100, Nihon-Kohden), displayed on an oscilloscope (Tektronix 5115), and digitized at 2 kHz by using an A/D converter (Power Lab, AD Instruments) and the associated software (Chart version 5.5.6, and LabChart, AD Instruments). The light/dark condition in the experimental box was also monitored simultaneously by recording the current directed to the white LED. The EMG recording was maintained for 14 days at the longest together

with the behavioral recording.

Occurrence of walking behavior was inferred from the EMG by the following criteria: rhythmic bursting activities were clearly observed simultaneously in each leg at least three times, which corresponded to stepping leg movements during walking. Of the EMG activity was interrupted until starting state that lasted for more than 30 sec, the activity was regarded to be a separate bout distinguished from the preceding one.

2.4. Mechanical stimulation

Mechanical stimulation was used to exogenously induce walking [44]. The tail fan was slightly touched with a paintbrush operated manually. A pair of fine wire electrodes were attached parallel to the bristles of the paintbrush to generate electrical signals in the EMG to identify the exact timing of stimulation. The intensity of stimulus was manually adjusted so that the animal responded to the stimulation not by running or rushing but by walking behavior. The animal was tested under light (60 lx) or dark (dim red light) to determine its behavioral response to a brush touch stimulus. The timing of stimulus was determined by the stimulus artifacts in the EMG caused by the sudden change in the resistance between two electrodes when the brush contact was made. The animal was brushed approximately every 30 sec from the start of resting state with the brush duration of ~1 sec.

2.5. Analysis of locomotor activity data

The behavioral data was transformed to a readable form for the software ActogramJ (version v0.7 and v0.8, B. Schmid and T. Yoshii) [48] using the programming Macro (Microsoft, Excel). The transformed data was then analyzed with ActogramJ,

which is a free-software package based on ImageJ (version 1.44o, Wayne Rasband, NIH) for the analysis and visualization of chronobiological data. Time resolution of the produced actograms was set to 1 minute. Circadian rhythmicity was determined by using a chi-square periodogram. If there was a peak around 24 h in the periodogram above the 0.05 confidence level, then the activity was judged to be circadian.

2.6. Determination of the timing of muscle activity change

To confirm temporal relationship between the timing of leg movement and EMG burst, we tested it using a treadmill system equipped with a video camera. The animal was placed on a sphere-type treadmill in such a way that it could freely perform locomotor behavior in the air (Fig.1C). The M-C joint of the fifth leg was marked on both sides with white-spot made of manicure in order to easily judge the timing of the leg movement. A video camera (Ucam-dld200ba, Eelecom), filming at a rate of 30 frames per second, was used to record the movement of crayfish legs. EMG recording was carried out in the same manner as mentioned above. We also used LabChart Capture module for synchronizing video frames with EMG recordings. Details of the apparatus are described elsewhere [45].

We used the burst latency (the time difference between muscle activation and burst initiation) to determine whether the walking was initiated spontaneously or reflexively (Fig.2A-D). The minimum Akaike information criterion (AIC) procedure was applied to the EMG data in order to find out the time onset of muscle activation and bursting activity objectively (Fig.2E). Theoretical details and computational algorithms of the procedure are provided by [49]. Briefly, we chose one or more 1 sec block (time series of 2000 points under the sampling rate of 2 kHz) to apply the procedure. The block

containing the time of target onset could be divided into two blocks. To determine the dividing point, the block was provisionally divided into two blocks. Each block was fitted by an autoregressive model (highest autoregressive orders of 20) and the goodness of fit for each model was measured by the corresponding AIC. The dividing point was moved step by step (moved forward point by point), and if the sum of each AIC reached the minimum value, the dividing point was judged to provide the best model. The time of the dividing point served the time of activity onset. We performed the procedure using R programming software (2.15.1 version, R Development Core Team) and Timsac package (1.2.7 version, The Institute of Statistical Mathematics).

2.7. Statistical analysis

Except the analysis of circadian locomotor activity data, all statistical analyses were performed using the R programming software. For statistical analysis of differences in the burst latency between different conditions, one-way ANOVA was employed to determine the significance in the conditional effect. A Student's t-test was performed for analyzing the difference in the burst latency for pooled data between spontaneous and stimulus-evoked walking. For analysis of temporal correlation between the onsets of leg movement and burst activity, Pearson's product-moment correlation coefficient was calculated and tested for significance. For categorization of walking patterns in LD, DD and LL experiments, statistical analyses were performed by generalized linear mixed models (GLMMs) [50] using lme4 package (0.999999-0 version) in R software. For the burst latency data of spontaneous and stimulus-evoked walking, we constructed two models to explain the data: the spontaneous initiation model and the stimulus-evoked initiation model. We tested these models by a likelihood ratio test, asymptotically

applying the chi-square distribution with degrees of freedom equal to the difference in the number of identifiable parameters in the two models as described by [51]. In all statistical tests, the difference was considered to be significant when $P\text{-value} < 0.05$. With regard to the sample number, “N” indicates the number of individuals while “n” indicates the number of trials. If not otherwise specified, results are expressed as mean $\pm$ standard error (SE) of the indicated number of trials.

3. RESULTS

Chronic EMG recording was made from freely moving crayfish to test whether its locomotor activity that showed a circadian rhythm was initiated spontaneously by endogenous activity changes or reflexively by changes in the sensori-motor signal transmission efficacy. We first compared quantitatively the latency of stepping leg movements from the onset time of sustained, non-rhythmical EMG activity to that of rhythmic burst between locomotor activities initiated spontaneously and reflexively. We then analyzed the EMG pattern of locomotor activities under LD, DD and LL conditions to test whether the locomotor behavior was spontaneous or stimulus-evoked in its origin.

3.1. EMG activity analyses

The previous study [44] demonstrated that the spontaneous and stimulus-evoked walking could be reliably discriminated by the time difference between the onset of sustained muscle activation and that of leg movement in crayfish: spontaneous walking was characterized by longer sustained muscle activation than in stimulus-evoked walking. In the present experiment, we did not record any behavioral movie during the long-term EMG recording of locomotor activity. We instead focused on

the time difference between the onset of sustained muscle activation and that of rhythmical burst activity that was associated with leg movements. In the present study, we used this time difference, referred to as “burst latency”, as an indicator for quantitative discrimination of spontaneous (Fig.2A, B) and mechanical stimulus-evoked walking (Fig.2C, D).

We firstly compared the burst latency of spontaneous and stimulus-evoked walking to confirm that it could make reliable distinction between them. The walking behavior of crayfish was visually observed under both light and dark (low-intensity red light) conditions in the experimental chamber, and the leg muscle activity was recorded at the same time. Typical EMG activities around the onset of walking are shown in Fig.2A and Fig.2C for spontaneous initiation and stimulus-evoked initiation, respectively. In both conditions, sustained activation of MCF preceded the rhythmical bursting of the same muscle. We statistically compared the burst latency between spontaneous/stimulus-evoked, left/right legs, and dark/light conditions (Fig.3A). The burst latency was found to differ significantly between spontaneous and stimulus-evoked walking (ANOVA: $P = 1.51 \times 10^{-6} < 0.05$) but not between left and right legs (ANOVA: left/right; $P = 0.866 > 0.05$) and between light and dark conditions (ANOVA: $P = 0.119 > 0.05$). The data for the present analyses were obtained from 344 sessions of spontaneous walking (number of animals $N = 12$) and 290 sessions of mechanical stimulus-evoked walking ($N = 10$). The mean value of the burst latency was 1000 ± 51 msec and 79 ± 6 msec (mean $\pm$ standard error (SE)) for spontaneous and mechanical stimulus-evoked walking, respectively. The difference was statistically significant (Student's t-test; $P = 1.40 \times 10^{-16} < 0.05$, Fig.3B). These data were used for GLMMs analysis in following experiments. Since there was no significant time difference in the burst latency between

left and right legs, the data on each leg were pooled in the following analysis.

In the previous study [44], the spontaneous and stimulus-evoked walking could be discriminated by the latent period from the onset of muscle activation in EMG to that of actual leg movement as revealed by video recording. This initial leg movement, although it eventually leads to rhythmical stepping movements, was slight in its magnitude and non-rhythmical in its temporal profile corresponding to sustained activation of the muscle. In the present study, on the other hand, we did not use video recording to monitor actual leg movement in order to ensure complete darkness during the dark condition in the LD and DD cycle experiment. Instead, we intended to measure the behavioral latency, i.e., the time difference between the onset of EMG activity and that of the burst activity. Before proceeding to systematic observation, we therefore had to examine quantitatively the temporal relationship between the burst and behavioral latencies in order to confirm a consistency with the previous study [44], demonstrating that the non-rhythmical muscle activation reliably preceded the initiation of burst activities in EMG that corresponded to rhythmical stepping movements [44]. In this groundwork study, we made video and EMG recordings from MCF of the fifth legs at the time of initiation of spontaneous and stimulus-evoked walking in the light condition. Data were obtained from 6 animals moving on the treadmill (Fig. 4A and B). The mean time difference between the onset of muscle activation and that of leg movement was 595 ± 35 msec and was 128 ± 12 msec in spontaneous and stimulus-evoked walking, respectively. The mean burst latency was 1069 ± 56 msec and 75 ± 8 msec in spontaneous and stimulus-evoked walking, respectively. Regardless of how they initiated walking, there was statistical correlation between the burst and behavioral latencies ($r = 0.637$ and $P = 1.741 \times 10^{-9} < 0.05$ in spontaneous walking, $r = 0.820$ and $P = 1.110 \times 10^{-11} < 0.05$ in stimulus-evoked walking,

Fig.4A and B). Leg movement constantly preceded bursting activity in both conditions: the time difference between the onset of leg movement and that of bursting activity was 474 ± 43 msec and 53 ± 7 msec. The temporal correlation between these two latencies ensured consistency with the previous study to enable discrimination of spontaneous and stimulus-evoked walking on the basis of the burst latency in this study.

3.2. Circadian locomotor activity and its EMG pattern under LD condition

We examined circadian locomotor activity of the crayfish and simultaneously recorded the EMG from MCF of fifth legs on both sides in 12:12 LD cycle. We then analyzed the burst latency of walking behavior under the LD condition to judge whether the walking was initiated spontaneously or reflexively in response to external stimuli. Under the LD condition that lasted for 10 days after the acclimation periods (see Materials and Methods), crayfish exhibited typical locomotor activity rhythms entrained to the LD cycle (Fig.5A). Averaged locomotor activity over a period of 10 days is shown in Fig. 5B, representing the general activity pattern in 24 h period. The 12:12 LD entrainment was confirmed by chi-square periodogram analysis (Fig.5C). The mean time of the first peak in the periodogram was 23.99 ± 0.06 h (mean $\pm$ standard deviation (SD)) for the total population ($N = 5$). We further counted the walking bouts using EMG data for the same period of the behavioral record of locomotor activity. The walking bout histogram (Fig.6, $N = 5$, $n = 1746$) showed the same general pattern with the averaged locomotor activity (Fig.5B), demonstrating noticeable consistency between walking bout count recording by the behavioral monitoring system and locomotor activity recorded by EMG. The animal was more active in the dark than in the light condition, and generally exhibited two clear peaks of locomotor activity after the switching of light condition (Fig.

6). It increased abruptly at the time of lights-off and slowly declined overnight (box-plot at 6:00 in Fig. 6). There was an increase in the activity approximately 2 hours before lights-on estimated by visual judgment from Fig.6, and a further increase occurred transiently at the time of lights-on (box-plot at 18:00 in Fig. 6). In contrast to the LD condition, there was no clear peak in averaged walking counts in the DD (Fig.8C) and LL condition (Fig.9C).

The burst latency of each walking bout is shown with the mean and SE for each hour along the 24 h period starting from 18:00 in Fig.7. Although the latencies varied throughout the record, the mean latency in each hour was maintained above the mean latency of stimulus-evoked walking. In general, the EMG activity pattern of MCF muscle in each walking bout in the LD cycle was observed to be similar to that of spontaneous walking: a sustained, non-rhythmic activity preceded the bursting activity. The mean burst latency in the LD condition was 1323 ± 488 msec, which was remarkably longer than that of stimulus-evoked walking (79 ± 6 msec). By statistical modeling analysis using the generalized linear mixed model [50, 51], the spontaneous initiation model was accepted for this data (likelihood ratio test: $P = 1.20 \times 10^{-16} < 0.05$). The results indicate that the locomotor activity entrained to the LD cycle is spontaneously.

3.3. Circadian locomotor activity and its EMG pattern under constant dark/light conditions

We next analyzed the circadian locomotor activity and the burst latency under the DD condition in order to test whether the locomotor activity in the free-running situation was initiated spontaneously or reflexively. Crayfish were exposed to the DD condition for

10 days after entrainment to LD cycles. The actogram obtained from two animals showed that the activity peak ran freely under the DD condition, with a period shorter than 24 h (Fig.8A). This tendency was confirmed by chi-square periodogram analysis (Fig.8B). The mean time of the first peak in periodgram was 23.12 ± 1.24 h (mean $\pm$ SD) for the total population (N = 6). Under the LL condition, it was longer than 24 h with the mean of 26.62 ± 1.41 h (mean $\pm$ SD; N = 5; Fig. 9A). This tendency was also confirmed by chi-square periodogram analysis (Fig. 9B).

The EMG activity pattern in the DD or LL cycle was observed to be similar to that of spontaneous walking: a sustained, non-rhythmic activity preceded the bursting activity (Fig. 10, N = 6, n = 1146 for DD; N = 5, n = 905 for LL). The mean values were 1779 ± 31 msec (Fig. 10A) and 1517 ± 39 msec (Fig. 10B) for the DD and LL condition, respectively. The burst latency under constant dark or light conditions was remarkably longer than that of stimulus-evoked walking, and was more close to that of spontaneous walking. It was statistically confirmed by making the generalized linear mixed model in either the DD or LL condition (likelihood ratio test: $P < 0.05$). The result suggests that the free-running activity under the constant dark/light conditions is spontaneously generated. Taken together, our data indicate that the locomotor activity showing a circadian rhythm is controlled by yet unidentified spontaneous factors exhibiting an underlying circadian rhythm.

3.4. EMG pattern of locomotor activity in active and inactive phases

The locomotor activity showed a circadian rhythm in the DD/LL free-run condition or a transient increase in response to light-on/off in the LD condition. It should

be noted here that the activity was observed during not only the active phase but also the inactive phase of these conditions although the occurrence frequency was lower in the latter phase (Fig. 5A, 8A, and 9A). We compared the burst latency of walking behavior in the active and inactive phases under LD, DD, and LL conditions to test whether there was any difference in the EMG activity pattern between the two phases. The active phase was visually defined as a major peak in the circadian rhythm, where the activity dominantly occurred on an actogram. The inactive phase was defined as the period not included in the active phase during the 24 hours cycle (LD condition) or during the circadian rhythm cycle (DD/LL condition). We randomly sampled the EMG data from the recording illustrated in Fig.7 (LD) and Fig.10 (DD and LL). The burst latency was measured on both sides in 10 walking bouts for each animal (N = 5, n = 100 for LD; N = 6, n = 120 for DD; N = 5, n = 100 for LL). No significant difference in the latency was observed between the active and inactive phase under any condition (Fig.11, t-test: $P = 0.179 > 0.05$ in LD; $P = 0.288 > 0.05$ in DD; $P = 0.199 > 0.05$ in LL). The mean values were 1409 ± 99 msec and 1626 ± 126 msec for active and inactive phases in the LD condition, 1505 ± 81 msec and 1644 ± 103 msec for active and inactive phases in the DD condition, and 1586 ± 148 msec and 1364 ± 87 msec for active and inactive phases in the LL condition, respectively.

3.5. Diurnal variation in the EMG pattern of stimulus-evoked walking

The mean burst latency of stimulus-evoked walking for comparison with the spontaneously initiated walking was obtained from the experiment that was conducted mostly in daytime, but not assigned to any specific time in the 24 hour cycle. We examined diurnal variations in the burst latency of stimulus-evoked walking in order to

confirm that the latency was not dependent on the time a day. We used five animals under the LD condition and carried out stimulation experiment in four time zones: 6:00-7:00, 12:00-13:00, 18:00-19:00 and 24:00-1:00. The burst latency was measured on both sides in 4 walking bouts for each animal ($n = 40$ for each time zone). No significant difference was observed in the burst latency among time zones (Fig.12, ANOVA: $P = 0.20 > 0.05$). The mean values were 57 ± 8 msec, 47 ± 5 msec, 42 ± 5 msec, and 56 ± 4 msec for 6:00-7:00, 12:00-13:00, 18:00-19:00 and 24:00-1:00, respectively. The burst latency of these stimulus-evoked walking pattern was remarkably shorter than that of spontaneous walking, and was more close to that of stimulus-evoked walking in the control experiment (Fig.3B and Fig.12). It was statistically confirmed by making the generalized linear mixed model (likelihood ratio test: $P < 0.05$).

4. Discussion

Circadian rhythms have been reported in a variety of physiological functions of crayfish ranging from behavioral [37-39,41,43] to cellular [21-36] and molecular levels [12-19]. Many of these are thought to be under the control of endogenous circadian oscillators. The circadian rhythms of locomotor activity and sensory input, in particular, have been intensively studied to clarify the neuronal mechanisms of the rhythm by using surgical interference, ablation, biochemical and molecular biological techniques [3, 38, 43]. Although these researches provided evidences that the circadian locomotor activity was attributed by endogenous factors functioning in the brain [3, 38,43], it remained unknown how the endogenous factors affect the locomotor control system in the central nervous system. The locomotor behavior can be initiated in general either spontaneously or reflexively [44-46]. In the crayfish *Procambarus clarkii*, a previous study

demonstrated that the spontaneous and stimulus-evoked initiation of walking behavior could be distinguished from each other on the basis of the activity of leg muscles: a sustained, non-rhythmical activation of leg muscles preceding their rhythmical burst activities was found to be critical for distinction of spontaneous and stimulus-evoked walking [44]. In this study, we newly developed a chronic EMG recording system that could be maintained for more than 14 days to analyze muscle activities over a long period of LD and LL/DD conditions. With the exception of [52] that developed an analog recording system for monitoring locomotor circadian rhythm of a scorpion *Androctonus australis*, this is the first case of EMG analysis conducted on the circadian rhythm of locomotor behavior in invertebrate species.

4.1. Circadian locomotor activity and EMG burst latency

Our results on the circadian locomotor activity under three light conditions (e.g. LD, DD, and LL) conformed to typical rhythmic patterns in adult crayfish. In the LD condition, the entrainment pattern of locomotor activity showed two peaks at the time of lights-off and lights-on as well as a gradual increase before light-off (Fig. 6). The pattern was consistent with the previous reports [37-39, 43]. In the free running conditions, the mean periodicity was shorter than 24 h in DD (Fig.8), but longer than 24 h in LL (Fig.9). These results on periodicity in constant conditions were also consistent with a previous study [39], where the crayfish *Procambarus clarkii* exhibited free-running rhythm with a longer periodicity under LL than that under DD. This tendency conformed to the empirical Aschoff's rule, according to which, typical nocturnal species would be expected to have shorter periodicity under DD and the opposite to be observed under LL condition [53].

Our results on the EMG analysis of the leg muscle activity pattern demonstrate that the locomotor activity showing the circadian rhythm is initiated spontaneously regardless of the light/dark conditions. The locomotor activity of crayfish was classified to be initiated either spontaneously or reflexively by mechanical stimulation according to the burst latency that was defined in this study as the time difference between the onset time of sustained muscle activation and that of bursting activity. The burst latency was found to be significantly longer in the spontaneous walking than in mechanosensory stimulus-evoked walking in the experimental chamber (Fig. 3). The latent period from the onset of muscle activation to the initiation of leg movements was temporally correlated with the burst latency on the current treadmill system (Fig. 4). Although the experimental situation of the spherical treadmill in the air was different from the underwater flat

chamber in the previous study, the behavioral and burst latencies measured on the treadmill system were comparable to those measured in the underwater flat chamber used in the previous study [44] (Fig.3 and 4).

In the LD condition, the burst latency was generally maintained longer than that of stimulus-evoked walking ($P < 0.05$). Although we observed a certain range variation of the burst latency depending on the time of observation (Fig. 7), it would be safe to assume that a common motor control system is employed to initiate and control the locomotor activity throughout 24 hours entrained to the LD condition. It remains unknown, however, how these variations are generated. It might be due to circadian changes in some unidentified internal factors affecting the locomotor output, but further study is needed to test this possibility.

4.2. Constancy of responsiveness to mechanosensory stimulus

Tactile stimulation to the tail part of crayfish (see materials & methods) has been shown to elicit walking behavior with the burst latency shorter than that observed in the spontaneously initiated walking (Fig.3 and 12). We showed that the burst latency in the mechanical stimulus-driven walking was constant throughout the 24-hour cycle period (Fig.12). It was significantly shorter than that observed in the spontaneously initiated walking throughout the period (Fig.11 and 12).

It has been reported in the green crab *Carcinus maenas* that reflex responses of optomotor neurons to tactile stimulation applied to its carapace exhibited diurnal changes [20]. In this study, we could not confirm such circadian variations of reflexiveness to mechanoreceptive stimulation. Our results, however, do not completely exclude the possibility for the following reasons. First, since we stimulated the animal with a fine

brush manually, the stimulus intensity could not be controlled strictly. This might have caused a change in the sensitivity of mechanoreceptors responsible for initiating stimulus-evoked walking. Second, since we only studied the animal behavior by EMG recording, any change in the mechanoreceptor sensitivity could not be observed if the synaptic transmission in the reflex pathway had also changed to compensate for the changes in mechanoreceptor sensitivity. Finally, since we repeated the stimulation, a habituation process might have also obscured the changes in mechanoreceptor sensitivity. Further study is needed to finally determine whether the mechanoreceptors on the crayfish tail show any rhythmic change in their sensitivity.

4.3. Effects of mechanosensory stimuli of different intensity on initiation of locomotor behavior

The most likely candidate of mechanical stimulation, if any, that would elicit the locomotor behavior of crayfish was minute vibration transmitted from the ground. This vibration would stimulate a specific type of mechanoreceptors on the tail as a water-borne stimulus [54]. It would also stimulate other mechanoreceptors on or in the walking legs as a substrate-borne stimulus. But since sufficient information is not available for the latter mechanosensory system, we examined only the former system in this study.

Since mechanosensory stimulation of the animal was carried out manually by touching the tail fan slightly with a fine paintbrush, we could not precisely control the stimulus intensity. In the course of finding out the adequate intensity of mechanical stimulation so as not to elicit escape or rushing behavior (Materials and Methods), we confirmed that the stimulus at the intensity lower than a certain level elicited no reliable behavioral response of the animal whereas the stimulus at the intensity higher than

another level elicited running or escape behavior. Stimuli of different intensity within this range elicited similar behavioral response although no quantitative analysis was carried out.

Even the spontaneously initiated walking could have been caused by different factors including not only purely endogenous activity changes in the neuronal system controlling walking but also some motivational systems that are upstream to the locomotor center in the brain [45, 46]. The minute vibration from the ground could initiate locomotor behavior by activating these motivational systems as in chemical stimulus-driven walking [44]. In this case, however, we are no longer able to distinguish between spontaneous and stimulus-evoked initiation of locomotor behavior.

4.4. Effects of retinal and extraretinal input on the circadian rhythm of locomotor behavior

Crayfish have three photoreceptive systems: retinal photoreceptors on compound eyes, extraretinal brain photoreceptors, and caudal photoreceptors in its 6th abdominal ganglion. Previous studies using *Procambarus bouvieri* showed that there was a correlation between the rhythms of ERG and locomotor activity in intact and brainless crayfish [27], and that its caudal photoreceptors functioned as a circadian photoreceptor [55]. Recent research using *Procambarus clarkii* and *Cherax quadricarinatus* suggests that the caudal photoreceptor contributes to the entrainment of circadian locomotor activity [56, 57]. Meanwhile, other studies using *P. clarkii* demonstrated that the brain photoreceptors are necessary for the entrainment of locomotor activity rhythms to light stimuli [38], and that they can function in the absence of the compound eyes and caudal photoreceptors [37, 43]. Whatever photoreceptors are the predominant factor for LD

entrainment, phonic input sensitivity of any photoreceptors must be strongly coupled to circadian locomotor behavior.

Crayfish have been reported to exhibit negatively phototactic locomotion when its eyes were illuminated [58-60]. In addition, the previous studies demonstrated that the caudal photoreceptor of crayfish could trigger walking by their response to light stimulation [60-62]. In our experiments under the LD condition (Fig. 7), the burst latency of the first walking bout at the onset of the light period (included in the box-plot at 6:00 in Fig.7) was longer than that of tactile-stimulus evoked walking (t-test; $p < 0.05$) and statistically comparable with that of spontaneous walking ($p > 0.05$), suggesting that the light stimulus might have activated the motor system that was also activated in spontaneous walking. However, the relatively long burst latency was possibly due to a low effective intensity of the light stimuli. A corollary to this hypothesis is that the burst latency decreases as the effective light intensity increases. This possibility is suggested by the presence of a neural pathway for light-evoked walking, and this pathway involves retinal and extraretinal (brain and 6th abdominal ganglion) photoreceptor neurons that can be activated when an effective light stimulus is presented. But if such a pathway for light-evoked walking dominantly functioned on the circadian locomotor activity, then the burst latency reduction should be generally present as a trail. Since we found no positive evidence for this hypothesis in the current study, we conclude that the light stimulus makes no significant contribution to the circadian rhythm of locomotor behavior even if there exists the possible reflexive pathway for light-evoked walking.

5. Conclusions

In the current study, we confirmed that the crayfish showed a circadian rhythm in

their locomotor activity either entrained to the LD cycle or free-running in the constant dark/light condition. We demonstrated by chronic EMG recording that the locomotor behavior was initiated spontaneously, not reflexively by any external stimulus. It has been proposed that the locomotory circadian rhythmicity of *P. clarkii* is generated by clock proteins and regulated by hormonal and neuromodulator secretion in the brain [3,41,43] and also is regulated by photoreceptors in the brain [37,38,43] or in the 6th abdominal ganglion [56, 57]. Although the details of interaction cascades among those factors remain unknown, the brain should play an important role for the circadian locomotor activity [3, 38, 43]. This contribution of the brain function is consistent with our results, suggesting that the motivational system in the brain for spontaneous initiation of walking is linked with the endogenous factor for locomotory circadian rhythm. Thus, our results provide an intersection of the studies on neural mechanisms underlying initiation of spontaneous behavior and underlying circadian locomotor activity.

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Figure captions

Fig.1

Experimental setup for chronic EMG recording. (A) Experimental apparatus. The animal was tethered to a slip-ring on the ceiling by PVC insulated cable for EMG recording. Locomotor activity was monitored by five pairs of infrared LED/ phototransistor combination, each separated by an interval of 70 mm at 80 mm height from the aquarium bottom. The phototransistor signal was fed to a personal computer (CPU1) through a series of electronic devices. The EMG signal was fed into another personal computer (CPU2) for continuous recording throughout the experiment. (B) Posterior-lateral view of the fifth left leg and the target muscle for the recording. EMG recording was made from the mero-carpopodite flexor (MCF) muscle of the fifth leg on both sides using a pair of silver wires. M-C joint, mero-carpopodite joint; MCE, mero-carpopodite extensor; MCF, mero-carpopodite flexor. (C) Treadmill system. The animal was fixed to a rod that was mounted to a lever system and positioned on the top of a water-supported white Styrofoam sphere so that the animal could walk on it while fixed at the cephalothorax. The sphere (diameter 15 cm; mass 60 g) was placed in a Petri dish (150 mm; 15 mm) partly filled with water and stabilized in a constant position by four brass bars that could freely rotate along the long axis. The amount of water in the dish was adjusted so that the sphere was just in contact with those bars in order to rotate smoothly. The lever could move vertically around a pivot so that the animal held at one end of the lever could freely change its standing posture. A counterbalance was attached at the other end of the lever so that the animal was virtually free from any load in the fixed experimental condition. Since the sphere weight was larger than the animal body weight, the possibility that the moving sphere might have a momentum causing unusual feedback modification of the walking

motor system could not be completely excluded. The animal was provided with water drips guided by a strip of gauze onto the dorsal part of the cephalothorax to prevent dehydration. A video camera was used to record the movement of crayfish legs. EMG recording was carried out in the same manner as mentioned above.

Fig.2

MCF muscle activities at the initiation of spontaneous and tactile stimulus-induced walking. (A) Electromyograms recorded at the onset of spontaneous walking from the mero-carpopodite flexor (MCF) of the right (R) and left (L) fifth legs. (B) Temporal expansion of the record indicated by the gray rectangle in A. A sustained, non-rhythmic increase in the leg muscle activity (black arrowhead) preceded the bursting activity (gray arrowhead). The difference between these activity onsets was defined as the burst latency. (C) Electromyograms recorded at the onset of mechanical stimulus-evoked walking from the mero-carpopodite flexor (MCF) of the right and left fifth legs. (D) Temporal expansion of the record indicated by the gray rectangle in C. The vertical line (black arrow) indicates the time of stimulation. The burst latency was shorter than that in spontaneous walking. In B and D, the black arrowhead indicates the time when the muscle showed a sustained, non- rhythmic activation preceding its rhythmic burst activities. The gray arrowhead indicates the time when the bursting activity was initiated. (E) Statistical estimation to find the time onset of muscle activation and burst activity from EMG. An example EMG from MCF of the right fifth leg at the onset of spontaneous walking is shown in the upper trace. In order to determine objectively the activation time of leg muscles and the beginning of burst activity, the minimum AIC procedure was applied to the EMG record: (a) light gray rectangle for muscle activation and (b) dark gray rectangle for burst activity. In (a) shown at the lower left, the black arrowhead indicates the time onset of a sustained, non- rhythmic muscle activation. In (b) shown at the lower right, the black arrowhead indicates the time onset of bursting activity.

Fig.3

Comparison of the burst latency between spontaneous and stimulus-evoked walking. The ordinate shows time differences between MCF activation and initiation of burst. Both types of walking conditions include data on each leg (L/R) and light/dark conditions. The mean value for spontaneous walking ($N = 12$, $n = 344$) was significantly greater than that for mechanical stimulus-evoked walking ($N = 10$, $n = 290$). No significant difference was observed within spontaneous walking and within stimulus-evoked walking. Box plots show the median, first and third quartiles of the data distribution. Whiskers denote the minimum–max range of the data within 1.5 times the length of box. Outliers are shown with open circles.

Fig.4

Temporal relationship between leg movement and bursting EMG activity. (A) Correlation between the behavioral latency of leg movement and the EMG latency of bursting activity in spontaneous walking ($N = 6$, $n = 72$). The correlation coefficient was 0.637, and was statistically significant ($P < 0.05$). (B) Correlation between leg movement latency and the burst latency in stimulus-evoked walking ($N = 6$, $n = 60$). The correlation coefficient was 0.820, and was statistically significant ($P < 0.05$). The onset of sustained, non-rhythmical muscle activation was used as the base time for these latencies.

Fig.5

Locomotor activity under the LD condition. (A) A typical activity record from two crayfish (a) and (b) under LD condition for 10 days. The actogram shows a nocturnal pattern of locomotor activity. (Note that black bars at top of figure indicate the dark condition (D); white bars the light condition (L); the data are double-plotted). (B) Averaged activity of the data shown in Fig.5A. The activity was persistently high from the start of D period to the middle. At the start and the end of L period, there was a transient increase in the activity. 1 bin corresponds to 1 min. (C) Chi-square periodogram analysis. The analysis produced calculated period of 24.03 h and 24.02 h for these crayfish in LD cycle, indicating entrainment to the cycle.

Fig.6

Walking counts measured from the electromyogram. The histogram shows the average frequency of walking bout per time bin of 14.4 min counted under the LD condition for the 24 h cycle ($N = 5$, $n = 1746$). The distribution of walking bouts reflected the averaged locomotor activity shown in Fig.5B. Two asterisks indicate the peaks after lights-off and lights-on events.

Fig.7

Burst latency under the LD condition. The ordinate shows the burst latency of each walking bout per hour in the 24 h period represented by the abscissa. The black box on the left side and the white box on the right side represent the dark and light condition, respectively. The solid and dashed horizontal lines represent the averaged burst latency of spontaneous and stimulus-evoked walking, respectively, shown in Fig.3. The burst latency in the LD cycle was close to that of spontaneous walking ($P < 0.05$).

Fig.8

Locomotor activity under the DD condition. (A) A typical activity record from two crayfish (c) and (d) under the LD condition for 4 days and DD condition for 10 days. Black arrowhead indicates the start day of the DD condition. The double-plotted actogram shows a free-running rhythm of locomotor activity in the constant dark period. (B) Chi-square periodogram analysis. The analysis produced the calculated period of 23.19 h and 22.08 h for these crayfish in the DD condition. Under this condition, crayfish was able to follow a free-running rhythm with a periodicity of 23.12 ± 1.24 h (mean $\pm$ SD) in average (N = 6). (C) Walking counts measured from the electromyogram. The histogram shows the average frequency of walking bout per time bin of 14.4 min counted under the DD condition for the 24 h cycle (N = 6, n = 1146).

Fig.9

Locomotor activity under the LL condition. (A) A typical activity record from two crayfish (e) and (f) under the LD condition for 4 days and LL condition for 10 days. Black arrowhead indicates the start day of the LL condition. The double-plotted actogram shows a free-running rhythm of locomotor activity in the constant light period. (B) Chi-square periodogram analysis. The analysis produced the calculated period of 26.90 h and 27.63 h for these crayfish in LL condition. Under this condition, crayfish was able to follow a free-running rhythm with a periodicity of 26.62 ± 1.41 h (mean $\pm$ SD) in average (N = 5). (C) Walking counts measured from the electromyogram. The histogram shows the average frequency of walking bout per time bin of 14.4 min counted under the LL condition for the 24 h cycle (N=5, n = 905).

Fig.10

Burst latency under the DD and LL conditions. The ordinate shows the burst latency of each walking bout under the DD (left box, N = 6, n = 1146) and LL (right box, N = 5, n = 905) conditions. The mean value was 1.779 ± 0.031 sec and 1.517 ± 0.039 sec for the DD and LL conditions, respectively. The solid and dashed horizontal lines represent the averaged burst latency of spontaneous and stimulus-evoked walking, respectively, shown in Fig.3. The burst latency both in the DD and LL cycles was close to that of spontaneous walking ($P < 0.05$).

Fig.11

Burst latency of walking bouts in active and inactive phases. Active phase was visually judged as a major peak following the circadian rhythm, where the activity dominantly occurred in frequency on an actogram. Inactive phase was the period other than the active phase in the actogram. The locomotor activity occurred less frequently compared to the active phase ($P < 0.05$). The ordinate shows the EMG burst latency. These data were randomly sampled from the same recording shown in Fig.7 (LD) and Fig.10 (DD and LL). The sampled data consists of the burst latencies on both sides from 10 walking bouts per individual ($N = 5$, $n = 100$ for LD; $N = 6$, $n = 120$ for DD; $N = 5$, $n = 100$ for LL). No significant difference was observed between the active and inactive phases in any condition (t-test, $p = 0.05$).

Fig. 12

Burst latency of stimulus-evoked walking at four time zones. The ordinate shows the EMG burst latency of stimulus-evoked walking. Stimulation experiments were conducted at four time zones: 6:00-7:00, 12:00-13:00, 18:00-19:00 and 24:00-1:00 (N = 5, n = 40 for each time zone). No significant difference was observed among those time zones (ANOVA: $P = 0.20 > 0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12