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RESEARCH ARTICLE

Control of motor activity in crayfish by the steroid hormone 20-hydroxyecdysone *via* motoneuron excitability and sensory-motor integration

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SUMMARY

We studied the effects of the molting hormone 20-hydroxyecdysone (20E) on leg sensory-motor networks of the red swamp crayfish, *Procambarus clarkii*. The hormone was injected in isolated crayfish and network activity was analyzed 3 days after injection using electrophysiology on an *in vitro* preparation of the leg locomotor network. This 20E treatment deeply reduced motor activity, by affecting both intrinsic motoneuron (MN) properties and sensory-motor integration. Indeed, we noticed a general decrease in motor nerve tonic activities, principally in depressor and promotor nerves. Moreover, intracellular recordings of depressor MNs confirmed a decrease of MN excitability due to a drop in input resistance. In parallel, sensory inputs originating from a proprioceptor, which codes joint movements controlled by these MNs, were also reduced. The shape of excitatory post-synaptic potentials (PSPs) triggered in MNs by sensory activity of this proprioceptor showed a reduction of polysynaptic components, whereas inhibitory PSPs were suppressed, demonstrating that 20E acted also on interneurons relaying sensory to motor inputs. Consequently, 20E injection modified the whole sensory-motor loop, as demonstrated by the alteration of the resistance reflex amplitude. These locomotor network changes induced by 20E were consistent with the decrease of locomotion observed in a behavioral test. In summary, 20E controls locomotion during crayfish premolt by acting on both MN excitability and sensory-motor integration. Among these cooperative effects, the drop of input resistance of MNs seems to be mostly responsible for the reduction of motor activity.

Key words: Crustacea, stretch reflex, neuromodulation, behavior, posture, molt, ecdysteroids.

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INTRODUCTION

Arthropods are characterized by a rigid cuticle exoskeleton that is replaced regularly in order to allow growth or metamorphosis (Wigglesworth, 1972). 'Molt' (or 'molting') typically defines a very short and stereotyped sequence, i.e. the shedding of the old cuticle, also known as ecdysis. However, it also frequently designates the whole series of events that prepare and follow ecdysis, more precisely corresponding to 'premolt' and 'postmolt', respectively. Molting hormones, named ecdysteroids, trigger and control this crucial developmental period. Indeed, hormonal titers fluctuate and reach a large peak during premolt, declining before ecdysis. These fluctuations, during the rising as well as the falling phases of the peak, are able to orchestrate the whole cascade of events leading not only to the synthesis of a new cuticle (reviewed in Charles, 2010), but also to many other related changes, including changes in behavior. For example, in insects, it has been demonstrated that a precocious ecdysteroid rise is involved in the initiation of wandering behavior before metamorphosis (Riddiford, 1976), whereas the drop of ecdysteroid concentrations after the hormonal peak is generally associated with an arrest of locomotion and the onset of a stereotyped behavioral sequence leading rapidly to ecdysis (reviewed in Truman, 2005; Žitňan et al., 2007). Similarly, in crustaceans, animals become quiescent in late premolt (Lipcius and Herrnkind, 1982; Chang, 1995), and ecdysis depends on a drop in ecdysteroid concentrations (Maissiat and Graf, 1973). However, ecdysteroids may also have transient stimulating effects on behavior during early premolt, as observed for agonistic interactions in lobsters (Bolingbroke and Kass-Simon, 2001).

Numerous studies have emphasized that, in addition to the epidermis, the nervous system is one of the main targets of ecdysteroids, which are able to control neuron physiology at various levels, including neurogenesis and neurodegeneration, dendritic remodeling, synaptic connectivity or efficacy, as well as neuron intrinsic biophysical properties and ionic conductances (reviewed in Weeks and Levine, 1990; Levine et al., 1995; Consoulas et al., 2000; Truman, 2005). In insects, the effects of molting hormones are particularly intense during metamorphosis and have thus been mainly described during this crucial period. By contrast, in crustaceans, in which metamorphosis is absent or limited to the very first postembryonic molts, the effects of ecdysteroids on the nervous system appear more discreet (e.g. Cooper and Ruffner, 1998; Cromarty and Kass-Simon, 1998) and have not been extensively studied.

In our previous studies on our model species, the red swamp crayfish, we analyzed the electrophysiological properties of leg motoneurons (MNs) controlling posture and locomotion, using an *in vitro* preparation of the nervous chain (Cattaert et al., 1990; Cattaert et al., 2010). In the present study, we were interested in analyzing the effects of the main ecdysteroid, 20-hydroxyecdysone (20E), on this *in vitro* preparation. However, in order to combine an electrophysiological study with behavioral analysis, we injected the hormone into animals; some of them had their ventral nervous

chain dissected in order to analyze nerve activities and MN excitability, whereas the others had their behavior analyzed. The results presented below show that 20E, under our experimental conditions, leads to a decrease of MN excitability and premolt locomotion in the red swamp crayfish.

MATERIALS AND METHODS

Experimental animals

Experiments were performed on male form I adult red swamp crayfish [*Procambarus clarkii* (Girard 1832)] weighing 20–30 g. The animals were collected locally, in the Réserve Naturelle de Bruges, after prefectural authorization, and were maintained in isolation for at least 3 weeks before any experiment, in order to avoid interference from past social experience. They were kept at 18–20°C on a 12 h:12 h light:dark cycle, and fed once a week with shrimp pellets and carrots.

The natural occurrence of ecdysis was noted for each animal. Premolt, also known as D-stage in crustaceans (Drach, 1939), typically corresponds to the period extending from apolysis (i.e. separation of epidermis from cuticle) to ecdysis. Postmolt is the period of consolidation of the new cuticle, following ecdysis (A and B stages in crustaceans). In order to avoid disturbing their behavior, we did not manipulate the animals to examine the appendages or swimmerets (Aiken, 1973) to determine precisely the beginning of premolt and the further progression of the molt cycle. However, we considered that, in relation to the size of our animals, premolt extended at least over the 2 weeks before ecdysis and postmolt over the 3 weeks after ecdysis. The day of ecdysis and the day after were considered to be early postmolt. Intermolt was then considered to begin after postmolt (at least 3 weeks after a molt) and to end before premolt (a minimum of 2 weeks before the next molt).

Before any injection or dissection, animals were anesthetized by chilling in ice-cold water for 15–20 min.

Hormonal treatment

The steroid hormone 20E and saline components were purchased from Sigma-Aldrich (St Louis, MO, USA). 20E was dissolved at a concentration of 10 µg µl^{-1} in a saline solution composed of (in mmol l^{-1}) 195 NaCl, 5 KCl, 13 $CaCl_2$, 2 $MgCl_2$ and 3 HEPES, at pH 7.65. Treated animals received a single dose of 5 µg 20E per gram animal fresh mass, injected dorsally between two tergites into the abdominal muscles. Experiments made with lower doses were consistent with the observations of Krishnakumaran and Schneiderman (Krishnakumaran and Schneiderman, 1970), who noted that 20E concentrations higher than 3 µg g^{-1} fresh mass were necessary for premolt induction. For control conditions, animals were sham injected with 0.5 µl of saline solution per gram fresh mass. After injection, each animal was returned to isolation in its aquarium.

In vitro preparation

An *in vitro* preparation of the thoracic nervous system was made [following Sillar and Skorupski (Sillar and Skorupski, 1986) and El Manira et al. (El Manira et al., 1991)] from control animals or from 20E-injected animals, 3 days after injection. After anesthesia, each animal was decapitated. A careful dissection of the ventral nerve cord was made, containing the last three thoracic (T3–T5) and the two first abdominal (A1, A2) ganglia, and was removed with all the nerves of the two proximal segments of the left fifth leg (Fig. 1A). The preparation, pinned dorsal side up on a Sylgard-lined Petri dish, was continuously superfused with oxygenated saline solution (same composition as for injections, see above). The fourth and fifth thoracic ganglia, as well as the first abdominal ganglion, were desheathed to improve the perfusion of the central neurons and to allow for intracellular recordings (Fig. 1B).

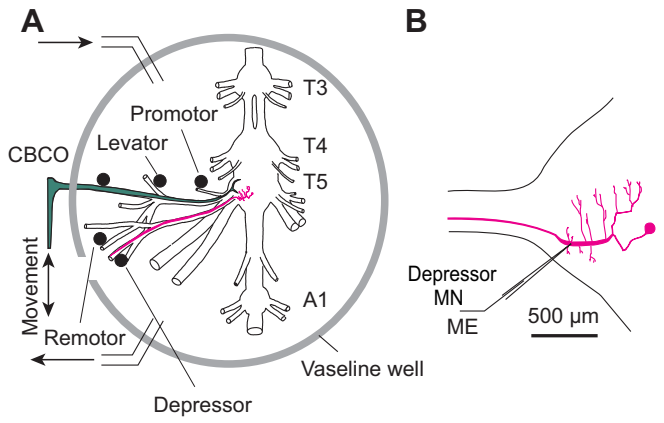

Fig. 1. *In vitro* preparation. (A) *In vitro* preparation of the crayfish thoracic locomotor system consisted of thoracic ganglia 3–5 (T3, T4 and T5) with motor nerves and proximal muscles of the left fifth leg (promotor, remotor, levator and depressor), as well as one or several abdominal ganglia (A1, first abdominal ganglion). The coxo-basipodite chordotonal organ (CBCO), a proprioceptor that encodes the vertical movements of the leg, was also dissected out. A mechanical puller was used to apply sine wave movements to the CBCO in order to mimic the vertical movements of the leg by stretching and releasing the CBCO strand. The central nervous system was isolated from the CBCO by a Vaseline well to perfuse the ganglia only. Activities of motor and sensory nerves were recorded with stainless steel electrodes (black circles). (B) Single or multiple intracellular recordings from motoneurons were performed within the neuropile using glass microelectrodes (ME) placed in the main neurite.

The coxo-basipodite chordotonal organ (CBCO), which monitors the movements of the second joint (coxo-basipodite; Fig. 1A), was also dissected out and kept intact. The distal end of its elastic strand was attached to an electromagnetic puller (VT101, Ling Dynamic Systems, Meudon-la-Forêt, France) controlled by a homemade function generator that allowed the application of sine wave movements to the CBCO strand (Fig. 1A) to mimic upward (during stretch) and downward (during release) movements of the leg.

More detailed information on the study of sensory-motor circuit has been already published (El Manira et al., 1991; Le Bon-Jego and Cattaert, 2002), and is described in the Appendix.

Behavioral tests and analyses

Crayfish were transferred individually in a dark container (10 cm diameter) to the center of an aquatic arena (50×25 cm; Fig. 2A), where they were maintained for 1 min. The container was removed after this period, and each animal was released into the aquatic arena where its exploratory behavior was recorded for 10 min with a video camera (Handycam, DCR-SR78, Sony, Tokyo, Japan). After recording, each animal was returned to its aquarium. The first test was performed before 20E or sham injection, the others at the same time (ca. 10:00 h) during each of the five following days.

We used EthoVision XT8 software (Noldus, Wageningen, The Netherlands) to analyze video records (dark line, Fig. 2B), more particularly by measuring total distance covered and instantaneous velocity during the last 8 min of recording; the first 2 min of recording, during which animals may have unusual reactions after their release from the dark container, were not taken into account.

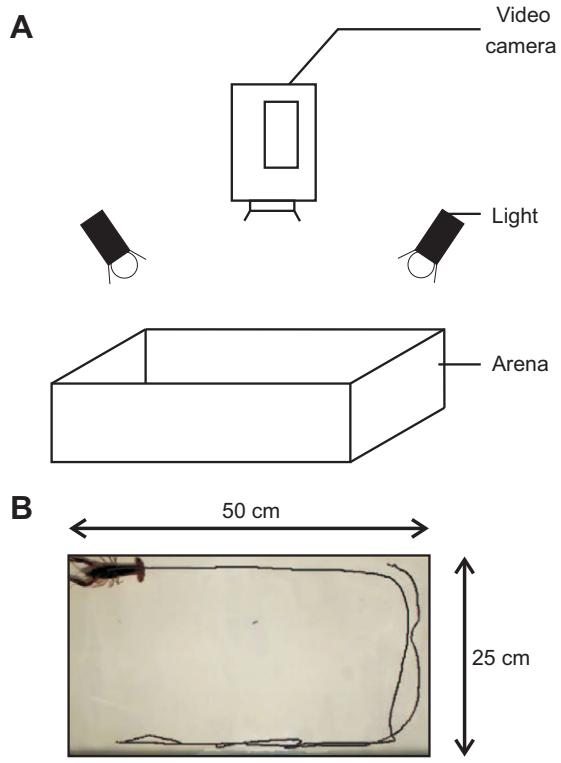

Fig. 2. Behavioral test. (A) Schematic diagram showing the aquatic arena (50×25 cm), in which locomotion of crayfish was recorded with a video camera for 10 min. Several spotlights uniformly illuminated the arena. (B) Crayfish displacements tracked by EthoVision software, which measured total distance covered and instantaneous velocity.

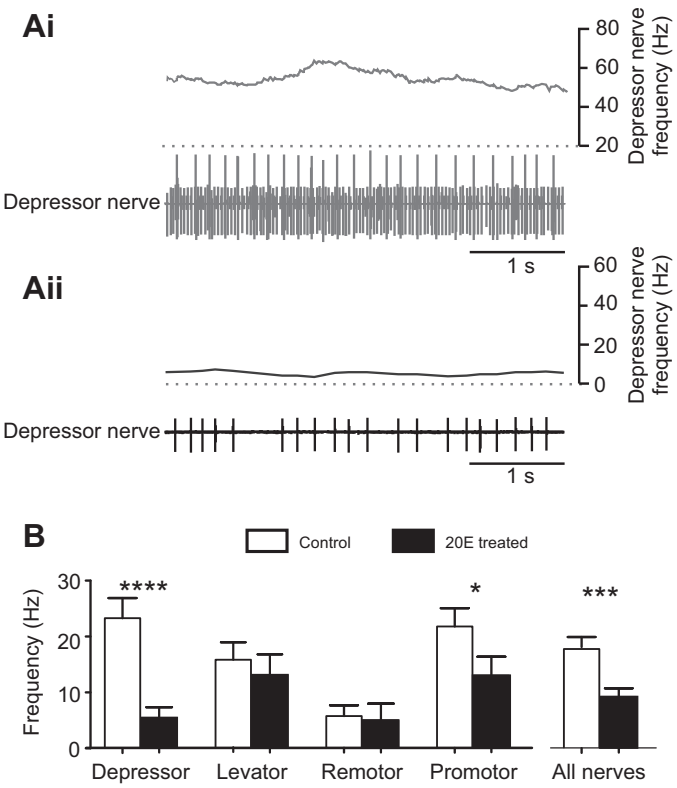

Fig. 3. Effect of 20E treatment on motor nerve tonic activities. (A) Depressor nerve recordings and corresponding average frequencies (calculated over 1 s) in a control (example in Ai) and a 20E-treated preparation (example in Aii). In both cases, depressor nerves displayed a tonic discharge (49.6 and 6.1 Hz, respectively). (B) Mean ± s.e.m. frequencies of nerve activities from controls (white bars, N=27) and treated preparations (black bars, N=22), showing depressor, levator, remotor and promotor nerve frequencies, separately and pooled (all nerves). Significant differences between control and treated preparations were observed for the mean depressor (****P<0.0001, Mann–Whitney) and promotor nerve frequencies (*P<0.05, Mann–Whitney), but not for levator (15.67±3.11 and 13.04±3.69 Hz, respectively, Mann–Whitney, P>0.05) or remotor nerve frequencies (5.69±1.91 and 4.92±2.98 Hz, respectively, Mann–Whitney, P<0.05). The pooled total activity of all nerves dropped significantly (***P<0.001, Mann–Whitney) from 17.86±1.80 Hz in control preparations to 9.19±1.56 Hz in treated ones.

Statistical analyses

Results are given as means ± s.e.m. Statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

20E decreases motor nerve tonic activities *in vitro*

Three days after the injection of 20E in treated animals or of saline in control animals, *in vitro* motor nerve activity was recorded from isolated preparations of the ventral nerve cord using extracellular electrodes placed close to the motor nerves that control movements of the leg. Fig. 3A shows examples of depressor nerve activity and corresponding mean frequency in a control (Fig. 3Ai) and in a treated preparation (Fig. 3Aii). Although the depressor nerve presented a tonic discharge in each condition, the mean frequency dropped after 20E treatment, both in terms of the number of active units and the unit discharge frequency. Such an analysis was repeated on the other motor nerves from 22 treated preparations and 27 control preparations (Fig. 3B). Very significant differences were confirmed for the tonic activity of the depressor nerve (23.30±3.59 Hz in controls *versus* 5.35±1.92 Hz in treated preparations; Mann–Whitney P<0.0001) and the promotor nerve (21.73±3.27 Hz in controls *versus* 12.79±3.39 Hz in treated preparations; Mann–Whitney P<0.05). By contrast, the levator and the remotor nerves did not display significant differences. However, their activities were not increased after 20E treatment (as might be expected in response to the decreases in their antagonist nerves), but rather a slight decrease occurred. Indeed, the pooled total activity of all motor nerves (depressor, levator, remotor and promotor) dropped significantly after 20E treatment (Fig. 3B, all nerves), from 17.86±1.80 Hz in control preparations to 9.19±1.56 Hz in treated preparations (Mann–Whitney, P<0.0001). Interestingly, 20E exerted maximal effects on depressor nerve activity, which is responsible for downward movements of the leg (i.e. rising posture) and for the power stroke phase of walking.

20E decreases the excitability of depressor motoneurons

Because the effect of 20E on the activity of the depressor nerve was particularly apparent, we analyzed the electrophysiological properties of depressor MNs with intracellular electrodes (Fig. 4). We measured their firing thresholds and the input resistances (R_{in}) in control and treated preparations (Fig. 4A) from their responses to different intensities of square current pulses. The current necessary to trigger a depressor MN discharge was lower in control preparations (example in gray trace, Fig. 4Ai) than in treated preparations (example in black trace). This discharge threshold was

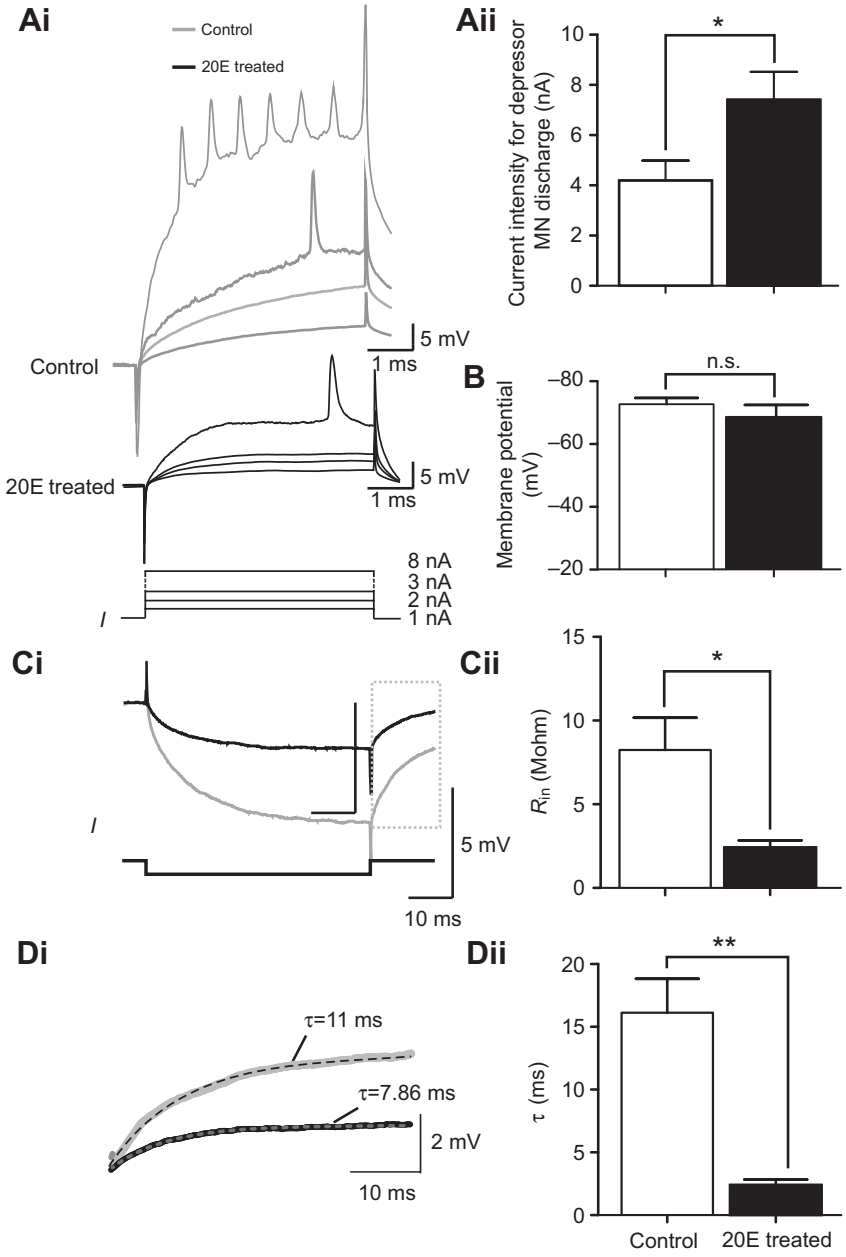

Fig. 4. Effect of 20E on depressor motoneuron (MN) excitability. (Ai) Intracellular activities of depressor MNs recorded from control (gray lines) and 20E-treated preparations (black lines). A series of increasing depolarizing current (*I*) from 1 to 8 nA was injected into depressor MNs. Depressor MN discharges were obtained at a higher current intensity in treated preparations than in controls (8 and 3 nA, respectively, in these examples). Resting membrane potentials of depressor MNs in control and treated preparations were ca. –78 mV. (Aii) Mean ± s.e.m. threshold current intensity values in control (white bars) and treated preparations (black bars). Depressor MN discharges required a lower current intensity in control than in treated preparations (**P*<0.05, Mann–Whitney). (B) Mean ± s.e.m. membrane potential measured in depressor MNs from control and treated preparation (n.s., *P*>0.05, Mann–Whitney). (Ci) Average traces of the membrane potential deflection induced by –1 nA current pulse injection in control and treated preparations; such injections produced a 5.4 mV hyperpolarization in the depressor MN of control preparations (gray trace) and a 2 mV hyperpolarization in the depressor MN of treated preparations (black trace). From these results, depressor MN R_{in} was estimated to be ca. 5 and 2 MΩ in control and treated preparations, respectively. (Cii) Mean ± s.e.m. input resistance (R_{in}) measured in depressor MNs from control and treated preparations (**P*<0.05, Mann–Whitney). (Di) Average traces during recovery after –1 nA current pulse injection into depressor MNs, fitted with exponential decay curves, indicated by the gray dotted rectangle in Ci. The time constant (τ) measured from these fitting curves decreased from 11 to 7.86 ms in control and treated preparations, respectively. Average traces were obtained on 10 current pulses. (Dii) Mean ± s.e.m. τ measured from control and treated preparations (***P*<0.01, Mann–Whitney).

measured in 10 depressor MNs from 10 control preparations and in 12 depressor MNs from 10 treated preparations. The mean current threshold for spikes of depressor MNs from 20E-treated preparations was 7.42±1.10 nA, whereas it was only 4.2±0.79 nA in depressor MNs from control preparations (Mann–Whitney, $P<0.05$; Fig. 4Aii). This change in threshold current was not due to a difference in the resting potential of depressor MNs, which showed no significant difference between control and treated preparations (Fig. 4B, Mann–Whitney test, $P=0.29$)

A difference in MN R_{in} may help explain this difference in discharge threshold. Injection of a –1 nA square current induced a larger hyperpolarization in depressor MNs of control preparations than in MNs of treated preparations (see examples in Fig. 4Ci). Over all experiments, mean R_{in} dropped from 8.24±1.93 MΩ in control preparations ($N=14$) to 2.4±0.44 MΩ in 20E-treated preparations ($N=5$), which was a significant difference (Mann–Whitney, $P<0.05$; Fig. 4Cii). Similarly, depressor MN time constants (τ, measured by fitting, with one exponential decay time function, the recovery after –1 nA hyperpolarizing current pulses) were shorter in depressor MNs from treated preparations (black trace, Fig. 4Di) compared with control preparations (gray trace). Differences in depressor MN time constants measured in all experiments were consistent with differences in R_{in}. The mean time constant significantly decreased after 20E treatment (Mann–Whitney, $P<0.01$), from 16.13±2.69 ms in control preparations to 2.40±0.44 ms in treated preparations (Fig. 4Dii).

20E decreases the resistance reflex activity in depressor nerves *in vitro*

The activity of MNs also depends on the drives they receive from afferent inputs, including sensory inputs. Here we analyzed the effect of 20E on a sensory-motor loop that mediates a resistance reflex. This reflex consists of activation of the depressor nerve in response to an elevation of the leg, and it participates in coordination of locomotion (El Manira et al., 1991). We applied sine wave movements to the sensory receptor (CBCO) that controls vertical

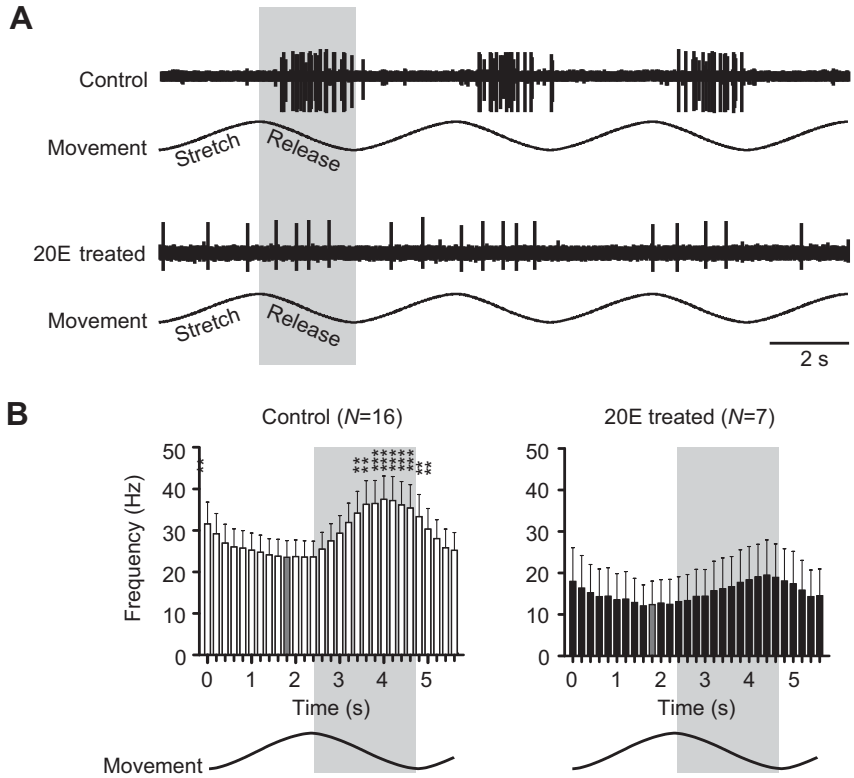

Fig. 5. Effect of 20E on the resistance reflex response recorded from the depressor nerve. (A) Examples of depressor nerve activities recorded from a control and a treated preparation (20E treated) in response to a sine wave movement continuously applied to the CBCO strand in order to mimic upward (during stretch) and downward (during release, light gray area) movements of the leg. The resistance reflex discharge recorded from the depressor nerve in the control preparation was strong and occurred during the release phase, whereas it was weaker and more dispersed in the treated preparation. (B) Mean ± s.e.m. depressor nerve frequencies from all control and treated preparations during 60 cycles of movement (300 s). The cyclic motor nerve activity was split into 29 bins.

position of the leg (Fig. 5A), and recorded the resistance reflex response from the depressor nerve. Extracellular spikes were observed during CBCO release that mimics an elevation of the leg, as previously described (El Manira et al., 1991). The reflex discharge was regular in control preparations (Fig. 5A, top), but weak and irregular in treated preparations (Fig. 5A, below).

In order to quantify these changes over all experiments, we divided cyclic motor activity into several bins (Fig. 5B). The discharge frequency of bins at the peak of the release was significantly higher (see asterisks above the nine bars centered around the discharge peak, between 3.2 and 4.8 s) than the minimum discharge frequency bin recorded during the stretch phase (see gray bar at 1.8 s in Fig. 5B, left). By contrast, in treated preparations, there was no significant difference between mean discharge frequency of stretch and release phases (Wilcoxon test, P=0.11, data not shown). The weakness of the reflex was confirmed by the lack of a significant difference between the peak response and the minimum frequency of the stretch phase (gray bar at 1.8 s, Fig. 5B, right). Similar results were found for the levator motor nerve resistance reflex discharge (data not shown).

20E decreases CBCO sensory activity

The smaller resistance reflex response observed in depressor nerves might be a consequence of a decrease in sensory inputs. Therefore, we analyzed the response of CBCO to sine wave movements in control and in treated preparations (Fig. 6) by examining the discharge frequency and the number of active CBCO units.

CBCO discharge frequency was much higher in control preparations (example in Fig. 6Ai) than in the treated preparations (example in Fig. 6Aii). The decrease of CBCO sensory activity after 20E treatment was consistently observed over 16 treated preparations. The mean CBCO discharge frequency, recorded during 300 s, significantly decreased (Mann–Whitney, P<0.05) from 94.22±7.70 Hz in control preparations to 67.61±10.47 Hz in treated preparations. However, the number of active CBCO units was not significantly different in control preparations (16.80±1.34 units) and in treated preparations (13.36±1.34 units, Fig. 6B, right). Therefore, the decrease of the resistance reflex was not due to silencing of CBCO sensory units.

20E decreases MN intracellular resistance reflex response

The resistance reflex can also be observed intracellularly in MNs kept hyperpolarized by injection of a continuous current in order to prevent spiking. Indeed, in a silent MN, application of sine wave movements to the CBCO strand elicits alternately depolarization and hyperpolarization of the membrane potential in response to synaptic inputs from CBCO units (El Manira et al., 1991; Le Ray and Cattaert, 1997). Two examples of intracellular responses of two depressor MNs in control (gray) and treated (black) preparations are presented in Fig. 7A. The amplitude of the depolarization during the release phase of CBCO movement is larger in the control preparation than in the treated preparation. This difference was confirmed (Mann–Whitney, P<0.01) by measuring reflex response amplitudes in intracellularly recorded depressor MNs from 18 control (1.11±0.15 mV) and eight treated (0.33±0.12 mV) preparations (Fig. 7B).

20E modified post-synaptic potentials triggered by CBCO units in depressor MNs

Intracellular recordings allowed us access to synaptic inputs. Depressor MNs exhibit post-synaptic potentials (PSPs), both excitatory (EPSPs) and inhibitory (IPSPs). Synaptic inputs triggered by CBCO units during imposed sine wave movements were analyzed in control and treated preparations (see Materials and methods). Examples of superimposed unitary EPSPs are presented in Fig. 8Ai and 8Aii (left), respectively, for control and treated

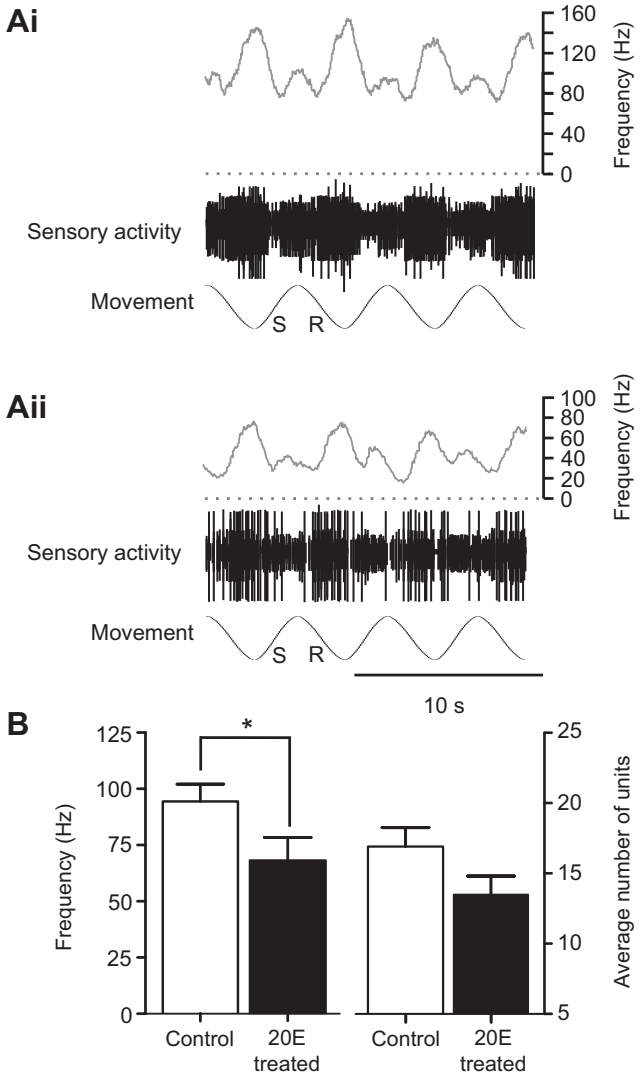

Fig. 6. Effect of 20E treatment of CBCO sensory activity. (A) Examples of CBCO sensory activity recorded during sine wave movements continuously applied to the CBCO strand [stretch (S) and release (R) phases] from a control and a treated preparation (20E treated). Sensory activities were estimated as average frequencies (calculated in a 1 s floating window). In the control preparation, the mean discharge frequency of the CBCO reached 120.0±1.4 Hz during release and 91.8±0.3 Hz during stretch (Ai). In the treated preparation, the mean discharge frequency of CBCO during release and stretch decreased to 48.4±0.7 and 41.2±1.6 Hz, respectively (Aii). (B) Mean ± s.e.m. CBCO discharge frequency (left) and number of CBCO units (right) measured from control (white bars, N=15) and treated (black bars, N=16) preparations (*P<0.05, Mann–Whitney).

preparations, together with the corresponding averages from all EPSPs (Fig. 8Ai,ii, right). Note that after the spike in CBCO units, the peak of a monosynaptic EPSP is rapidly reached (black arrows). In addition, various polysynaptic components (gray arrows) combine during the decay phase of monosynaptic EPSPs. However, the shapes of these EPSPs were different in control and treated preparations. Indeed, the very slow decaying phase of control EPSPs (Fig. 8Ai, right) is due to the presence of polysynaptic components (Fig. 8Ai, left). By contrast, in depressor MNs of treated preparations, EPSPs rarely present polysynaptic components (Fig. 8Aii, left) and the average trace of EPSPs presents a faster decay phase (Fig. 8Aii, right).

The mean peak amplitude of these EPSPs was similar in control and treated preparations (Fig. 8B). However, EPSP decay measured 15 ms after peak (see gray arrows in Fig. 8A), which represents the polysynaptic components, significantly decreased (Mann–Whitney, P<0.05) from 0.14±0.02 mV (in 17 control preparations) to 0.07±0.01 mV over all unitary EPSPs (in eight treated preparations) (Fig. 8B). This 50% decrease has important functional consequences by limiting the temporal summation of CBCO EPSPs, and thereby the amplitude of the resistance reflex. Note that the mean number of CBCO units contacting a single depressor MN was not significantly different between control (5.12±0.73) and treated preparations (3.5±0.76).

IPSPs recorded on depressor MNs were also analyzed (Fig. 8C). No IPSPs were found in depressor MNs recorded from the eight treated preparations (Table 1). In contrast, eight IPSPs were recorded from depressor MNs in 17 control preparations. The mean peak amplitude of these IPSPs was −0.17±0.03 mV and the amplitude measured 15 ms after IPSP peak was −0.05±0.03 mV.

These results demonstrate that 20E reduces sensory motor integration. However, the effects on sensory inputs are less important than those observed on polysynaptic components *via* interneuron silencing. Nevertheless, these effects were also weaker than those observed above on intrinsic properties of MNs. All these effects lead to MN decreased activity, which might lead to decrease of locomotion. Therefore, we analyzed the functional consequences of 20E treatment on crayfish locomotion.

20E decreases crayfish walking activity *in vivo*

20E-induced premolt

After sham or 20E injection, the total distance walked and the mean instantaneous velocity were measured every day for 5 days. Values were expressed as a relative change from day 0 value (i.e. before treatment). The relative distance walked of sham controls remained unchanged during all test days (Kruskal–Wallis test, n.s., N=9; Fig. 9A, left), indicating that crayfish did not become accustomed to the arena, and that the injection procedure itself had no effect on crayfish behavior. In contrast, 20E treatment significantly changed the total distance walked (two-way ANOVA, N=9, P<0.05; Fig. 9A, left). At the third day after 20E treatment, 94% of crayfish decreased their global activity (Tukey's *post hoc* test, P<0.05). This difference was even more significant (P<0.01) on the fifth day. Similarly, 20E treatment significantly changed the relative mean instantaneous velocity (two-way ANOVA, P<0.01; Fig. 9A, right).

In order to further study the effect of 20E on walking velocity, we compared the distribution of instantaneous velocities before and 5 days after 20E treatment. These two distributions were significantly different (Chi-square test for trend, P<0.0001). The median of the distribution shifted from 1.55 cm s^{-1} before treatment (Fig. 9B, left) to 0.87 cm s^{-1} 5 days after 20E injection (Fig. 9B, right). The shape of the distribution clearly shows that before treatment crayfish walked similarly at a variety of speeds between 0.5 and 2.5 cm s^{-1}, whereas after treatment the histogram presents a peak of walking speed at 0.5 cm s^{-1}, and animals very rarely walked faster than 1.5 cm s^{-1}.

Natural molts

We also analyzed the evolution of the total distance walked during natural molts (Fig. 10A). Animals were regularly observed (at least twice a week, over ca. 3 months) during the course of their natural molting cycle. Among the 11 animals studied, nine ecdysed and were recorded during the whole intermolt–molt sequence. Observations were averaged after being synchronized by the day

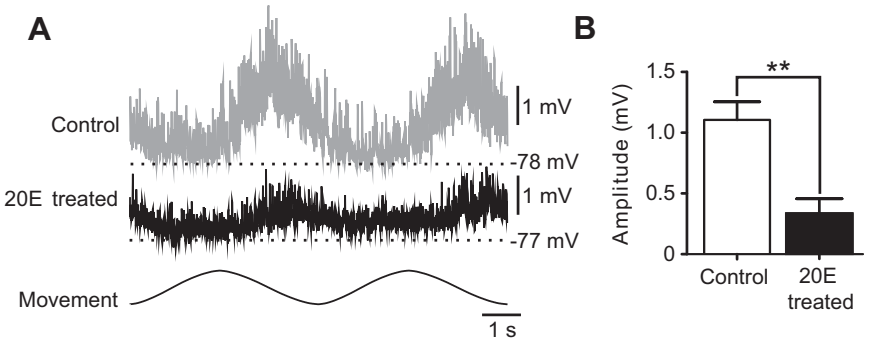

Fig. 7. Effect of 20E treatment on the resistance reflex response amplitude measured from depressor MNs. (A) Examples of depressor MN membrane potential recorded from a control preparation (gray line) and a treated preparation (20E treated, black line) during sine wave movement applied to the CBCO strand. The amplitude of the depolarization during the release phase of CBCO movement is larger (1.8±0.2 mV) in the control preparation than in the treated preparation (0.9±0.2 mV). (B) Mean ± s.e.m. resistance reflex response amplitude measured from all control (white bar) and treated preparations (black bar).

of ecdysis; premolt was considered to correspond to the 2 weeks before ecdysis (gathering observations made from 6 to 1 days before ecdysis for the first week, and from 12 to 7 days before ecdysis for the second week). During premolt and postmolt phases, the total distance walked decreased (Fig. 10B) in all but one animal (Fig. 10C). As a result, the mean total distance walked recorded during premolt phases was significantly different (Wilcoxon matched-pairs signed rank test, $P<0.05$), switching from 341.6±65.9 to 199.4±69.0 cm during molt (42% decrease) (Fig. 10C). All these data show that premolt, either natural or induced by 20E, is characterized by a decrease in locomotion, which is consistent with the decrease of MN excitability observed *in vitro*.

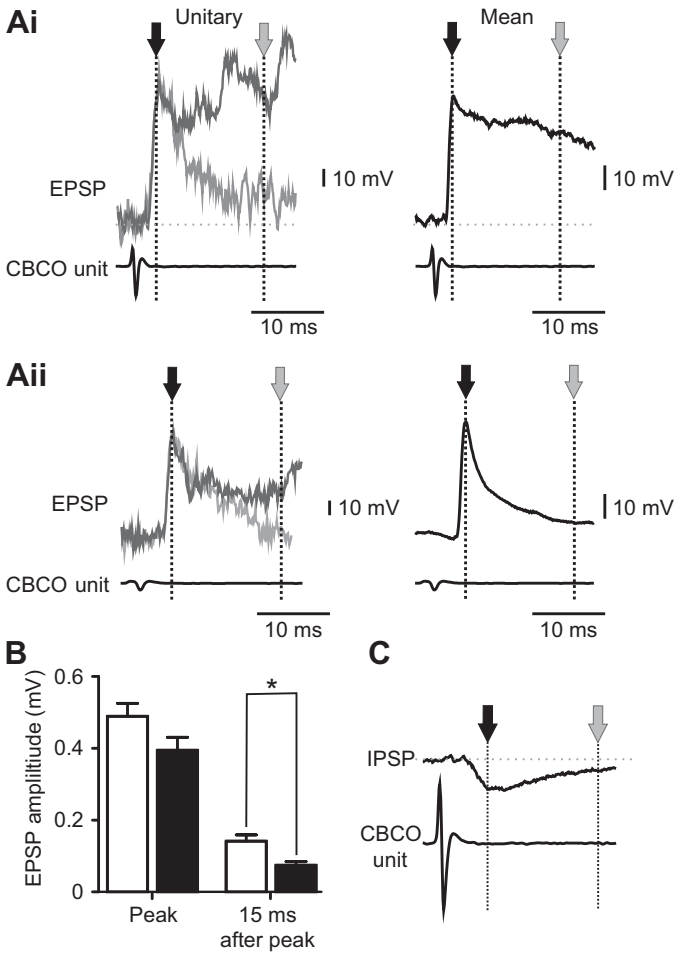

Fig. 8. Effect of 20E treatment on CBCO-unit-triggered post-synaptic potentials (PSPs) in depressor MNs. (A) Unitary excitatory PSPs (EPSPs) recorded from a depressor MN of a control (Ai) and a treated preparation (20E treated, Aii). For each condition, superimposed examples (raw data) of unitary EPSPs triggered by a single CBCO unit are presented on the left side. Corresponding averaged EPSP traces are presented on the right side. Black arrows represent EPSP amplitude at peak and gray arrows 15 ms after peak. (B) Mean ± s.e.m. EPSP amplitudes measured at peak and 15 ms after peak in depressor MNs from all control (white bar) and treated (black bar) preparations (*$P<0.05$, Mann–Whitney). (C) Example of IPSP recorded from a depressor MN in a control preparation. Arrows are as in A.

DISCUSSION

Hormonal effects on MNs excitability and sensory motor activity.

Injection of a sufficient amount of 20E into crayfish is known to induce a rapid premolt, but this accelerated development is frequently fatal (Krishnakumaran and Schneiderman, 1970). Indeed, our treated animals generally failed to ecdyse and died ca. 7 days after treatment under our experimental conditions. However, neurons studied in our isolated preparations 3 days after injection (or even 5 days after, data not shown) had no observable characteristics of dying cells; they maintained a constant membrane potential for at least 3 h *in vitro* at a voltage similar to that of control preparations. Moreover, dying neurons, if any, would be characterized by an increase of spike frequency due to uncontrolled depolarization, whereas MNs of our treated preparations were almost silent due to a maintained hyperpolarization; thus, the changes described in this study after 20E injection were clearly not due to neuronal death.

More precisely, we observed a modification of neuronal plasticity in crayfish locomotor networks following 20E treatment, mainly affecting motor outputs and, to a lesser extent, sensory inputs to MNs. Indeed, hormone injections induced changes in depressor MN excitability because of the alteration of cell membrane properties, more particularly the decrease of input resistance. It is important to note that this decrease in input resistance was not due to a change in membrane resting potential, which remained constant before and after 20E treatment under our experimental conditions. Previous studies in insects have already shown that input resistance may strongly differ after metamorphosis in leg MNs that persist from larvae to adults, suggesting a possible role for developmental

Table 1. Inhibitory post-synaptic potential (IPSP) amplitudes recorded from control and 20E-treated preparations

	Control	20E treated
Amplitude (mV)	–0.17±0.03	–
Late amplitude (mV)	–0.05±0.03	–
Number of IPSPs	8	0

Data are means ± s.e.m.
Late amplitude was recorded 15 ms after peak.
–, no IPSPs in treated preparations.

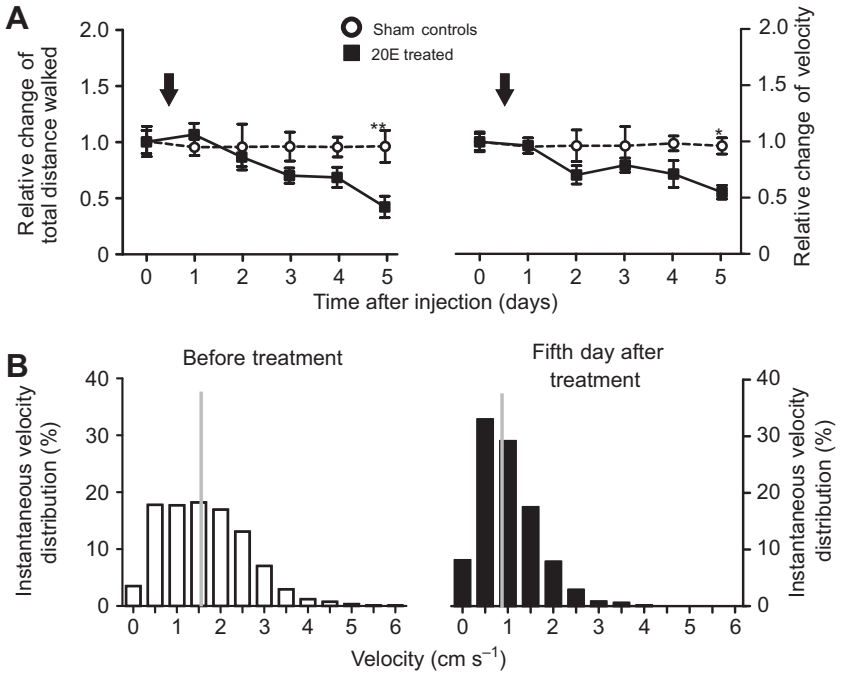

Fig. 9. Effect of 20E on locomotion behavior. (A) Variation in relative changes in total distance walked and instantaneous velocity for sham controls (white circles) and 20E-injected (black squares) crayfish. Abscissa represents time in days after injection (0 is immediately before injection). Injection is represented by black arrows. (B) Distribution (%) of instantaneous velocity for 20E-injected crayfish before treatment (white bars, left) and 5 days after treatment (dark bars, right). Gray lines represent distribution medians.

hormones (Duch and Levine, 2000; Rose and Levine, 2000). To our knowledge, however, our observations on crayfish represent the first report showing that 20E is able to control this parameter in the absence of any structural change of the locomotor apparatus. Yet it must be noted that 20E may also control cell excitability of some other neurons, in particular insect neurosecretory cells, without changes in input resistance (Hewes and Truman, 1994).

Together with the decrease of MN excitability, 20E treatment also modified sensory inputs, as observed with the strong alteration of MN synaptic potentials in our model. Indeed, the depolarisation of depressor MNs induced by sensory inputs from the CBCO was also decreased after 20E treatment. A hormonally mediated weakening of synaptic connections between the sensory and motor neurons has already been observed during insect metamorphosis, involving MN dendrites, breaking contacts with sensory neurons (Weeks et al., 1997). However, our results indicate that monosynaptic sensory–motor connections were not modified in our model after 20E treatment. Indeed, we observed that the decrease of reflex response after 20E treatment was mainly related to a change in the decay phase of EPSPs (Fig. 9), indicating a lower activity of excitatory interneurons. Moreover, our data show that the decrease of reflex response was not due to an increase of the activity of inhibitory interneurons, because they became silent. In our model, sensory-motor activity involves at least three types of interneurons (Le Ray et al., 1997; Le Bon-Jego and Cattaert, 2002; Le Bon-Jego et al., 2004). Our results show that the recruitment of two of them, i.e. the excitatory and inhibitory interneurons of resistance reflex, was decreased or suppressed by 20E treatment (our experimental

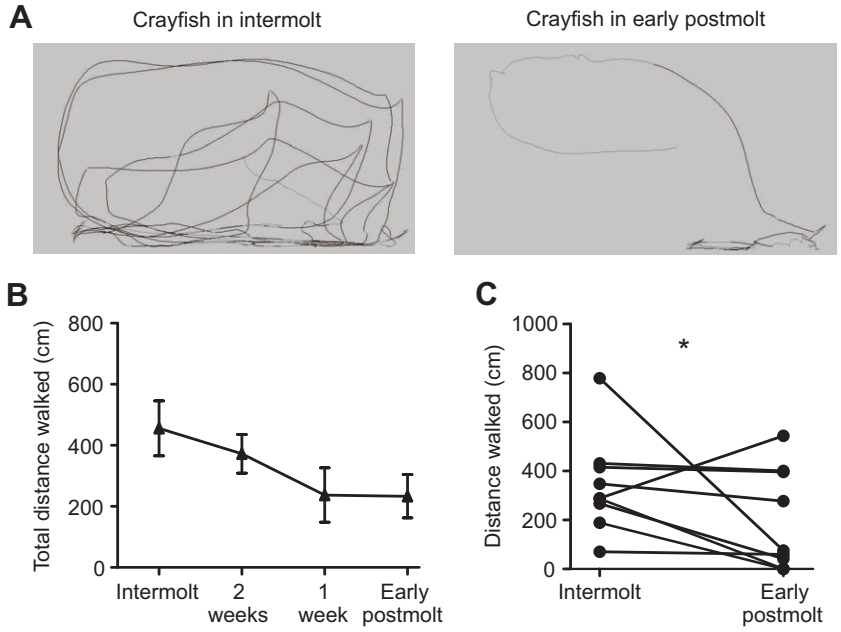

Fig. 10. Effect of natural molt on locomotion behavior. (A) Examples of crayfish tracking (black lines) during intermolt and early postmolt (less than 24 h after molt). The intermolt crayfish walked 778.8 cm, whereas the postmolt crayfish walked 75.0 cm during the same experimental time. (B) Mean ± s.e.m. variations of total distance walked at different molt periods (N=11 animals); premolt animals were assayed ca. 2 weeks and 1 week before molt. (C) Variations of individual distances walked at intermolt and early postmolt (*P<0.05, Wilcoxon matched-pairs signed rank test, N=9 animals).

conditions did not allow us to analyze the effects of 20E on the third type, the assistance reflex interneurons). The question remains open whether 20E effects involves specific targets in both excitatory and inhibitory interneurons or are rather the consequence of a similar decrease in interneuron excitability as observed in MNs.

In summary, 20E controls both MN excitability and sensory-motor integration in crayfish leg networks. However, between these two types of cooperative effects, the drop in input resistance of MNs (70%) seems to be mostly responsible for the reduction of motor activity.

Mode of action of 20E on neurons

Neuron excitability is generally thought to involve endogenous conductance, as a result of ion channels, i.e. membrane proteins. Numerous ion channels have been identified or described in vertebrates and invertebrates (reviewed in Armstrong and Hille, 1998). Though we have not yet identified the types of conductance involved in the membrane properties analyzed in our experiments in crayfish, it seems likely that ecdysteroids may act, either directly or indirectly, on such membrane protein(s). Preliminarily, however, it remains to establish whether the effects of 20E were direct on leg MNs. Of course, ecdysteroids are able to produce direct changes in neurons, as shown by cell culture approaches in insects (Levine and Weeks, 1996). In crayfish, direct and fast effects of 20E have already been described on neuromuscular junctions by local applications of 20E (Cooper and Ruffner, 1998). However, our results were obtained only after 20E injections into animals and our attempts to reproduce them by applying 20E directly on our *in vitro* preparation did not yield any noticeable change, neither in network activity nor in MN properties, even after 1 day of application (data not shown). This suggests that the 20E effects observed in this study were possibly indirect, either involving another organ or, more probably, some relay in superior nervous centers absent from our *in vitro* preparation, likely the brain. Indeed, such a role for the brain as the primary site of 20E action has already been documented in the control of insect wandering behavior (Dominick and Truman, 1986; Miller and Levine, 2006).

Another possible explanation could be that the effects of 20E observed in our study might nevertheless be direct on MNs, but after a longer cascade, extending for more than a day. Indeed, though steroid hormones, including ecdysteroids, may act through short-term pathways, interfering directly with membrane receptors that they may share with neurotransmitters [e.g. GABA (Okada et al., 1998); dopamine (Srivastava et al., 2005)], they more frequently have long-term effects, involving nuclear receptors that control gene expression (reviewed in Spindler, 1997; Spindler et al., 2009). The 20E effects observed in the present study (recorded 3 days after hormone injection) were obviously long-term effects, suggesting some possible control(s) on gene expression. Ecdysteroids are indeed able to trigger a cascade of events, involving a complex interplay of nuclear receptors, that allow coordinated and time-delayed expression of numerous proteins, as illustrated by the control of dopa-decarboxylase gene expression (reviewed in Hiruma and Riddiford, 2009), cuticular protein secretion (Charles, 2010) or neuroendocrine cascade regulating ecdysis (reviewed in Žitňan et al., 2007). Importantly, similar long-term effects of ecdysteroids on identified ion channels have been described in insects (see Grünewald and Levine, 1998; Börner et al., 2006; Garrison and Witten, 2010).

Finally, it is also very probable that the response of crayfish neural networks to 20E might be a complex summation of rapid and direct effects, such as those described on neuromuscular junctions (Cooper and Ruffner, 1998), together with long-term and possibly indirect effects, as suggested by our results.

Behavioral consequences

Our behavioral observations on the effect of natural or induced premolt in crayfish are consistent with previous studies on insects (reviewed in Truman, 2005) and crustaceans (Lipcius and Herrnkind, 1982; Chang, 1995) reporting a reduction of locomotor behavior during premolt, particularly at the approach of ecdysis. This decreased motor activity is generally related to the weakness of the new exoskeleton on which muscle attachments are progressively transferred in late premolt. However, such a decrease of locomotion is more surprising when considering that, at the onset of premolt, animals have to find an appropriate place to molt quietly. This is particularly true in our crayfish model, which is a very aggressive and omnivorous species; if animals are maintained in high-density groups in the laboratory, every individual undergoing a molt is rapidly devoured by its congeners (authors' unpublished observations). It is thus obvious that, in natural conditions, animals should leave the plants where they generally feed, sometimes in high density, to find an isolated place. If so, this behavior would be comparable to the wandering behavior described before insect metamorphosis. Insect wandering behavior is in part controlled by a small rise in ecdysteroid titers (Riddiford, 1976), whereas the arrest of locomotion accompanying ecdysis behavior is dependent on the drop in molting hormone (Truman, 2005). Thus, one should consider the possibility that our injections of high concentrations of 20E into crayfish only highlighted the inhibition of locomotion by high and subsequently declining 20E concentrations, but not a possible excitation by a discrete hormonal rise. However, our attempts to verify this possibility using injections of lower quantities of 20E only gave intermediate inhibitory results, but no excitatory response in our test conditions (data not shown). It remains possible, however, that our behavioral test (isolated animals in an open field) was not adequate to reveal a behavior that may depend on more complex environmental interactions (e.g. grouped animals in presence of shelters), or that single 20E injections did not sufficiently mimic the subtle fluctuations of 20E occurring during the normal induction of premolt. An alternate possibility is that locomotion might be inhibited by any concentration of ecdysteroids in crayfish leg networks, but is activated at the level of tail-flip networks, as this kind of escape swimming varies in premolt animals (Tamm and Cobb, 1978; Cromarty and Kass-Simon, 1998; authors' unpublished observations). Indeed, ecdysteroids may also have opposite effects in different MNs (Weeks and Truman, 1985; Cromarty and Kass-Simon, 1998).

In conclusion, our experiments demonstrate that 20E is able to durably reduce the excitability of crayfish leg MNs and, more generally, the activity of the whole postural and locomotor network. From the electrophysiological MN properties after 20E treatment, it appears likely that this network would be less capable to respond to descending commands during the molting period than during intermolt.

APPENDIX

Sensory-motor circuit

The depressor MNs receive sensory input from the CBCO. This organ consists of an elastic strand of connective tissue that is attached proximally to the dorsal edge of the coxopodite and distally to an apodeme at the proximal-dorsal edge of the basipodite. Embedded within this strand are approximately 40 sensory neurons that project to the ipsilateral neuropile (the region in which neurons form their

synaptic contacts) and make monosynaptic and polysynaptic connections with the depressor MNs (El Manira et al., 1991; Le Bon-Jego and Cattaert, 2002). Half of the CBCO sensory neurons are activated when the CBCO strand is stretched, and the other half are activated when the band is released (El Manira et al., 1991). Thus, this proprioceptive organ monitors movements of the limb in the vertical plane. In the *in vitro* preparation, a pin holds the proximal end of the CBCO while a mechanical puller imposes movements to its distal end (Fig. 1A).

To ensure that the CBCO was not damaged during the dissection, we only used preparations with robust sensory neuron activity in response to movements imposed on the CBCO strand. In order not to damage the CBCO during the experiment, stretch movements were applied starting from the most released position of the CBCO strand, and the total amplitude of the movement was one-third of the released CBCO strand length (1–1.8 mm). The movement control voltage traces were visualized on an oscilloscope and stored on a computer.

Recordings

Extracellular recordings from the motor nerves innervating the four leg muscles and from the sensory nerve of the CBCO were made using stainless steel pin electrodes contacting the nerves and insulated with Vaseline (VWR BDH Prolabo, Strasbourg, France). The differential extracellular signals were amplified (×5000–10,000) and filtered (high-pass 30 Hz, low-pass 30 kHz, 50 Hz notch filter) using Grass Technologies AC preamplifiers (Warwick, RI, USA). The bath solution was grounded using a small silver plate that was previously chlorided using chlorine bleach. Stimulation of nerves was carried out with a programmable pulse generator (Master-8, A.M.P.I., Jerusalem, Israel) and a stimulus isolation unit (Isoflex, A.M.P.I.). Intracellular recordings from depressor MNs (Fig. 1B) were made using glass micropipettes (Clark Electromedical Instruments, Reading, UK) filled with 3 mol l^{-1} KCl (resistance 10–30 MΩ). The intracellular electrodes were connected to an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) used in current-clamp mode. In Crustacea, the somata of MNs lie outside the neuropile (Fig. 1B) and are linked to the arbor of the neuron *via* a thin process, and so do not participate in the electrical activity of the neuron. For these reasons, intracellular recordings were made from the main neurite, where EPSPs could be recorded (Fig. 1B).

Depressor MNs were identified following the procedure used in Hill and Cattaert (Hill and Cattaert, 2008). The resting membrane potential of MNs was usually in the range of –80 to –65 mV. Stability of resting membrane potential over a long period of time (>30 min) was used as a criterion for evaluation of cell health during recordings. In crustacean MNs, soma and neurites do not actively convey spikes. Therefore, spike amplitude was generally small (<20 mV) at the recording site. Data were digitized and stored on a computer hard disk through an appropriate interface (Power1401) and software (Spike2) from Cambridge Electronic Design (Cambridge, UK).

Electrophysiological data analysis

Data were analyzed using Spike2 analysis software. Spikes recorded from the CBCO nerve were identified according to their waveform based on a template matching protocol (wavemark). Templates were built automatically with a duration corresponding to the mean duration of sensory spikes (ca. 1.5 ms in duration). The sampling rate for CBCO nerve recording was set to 20 kHz, which resulted in templates containing ca. 30 points. The procedure used two criteria to identify a spike: (1) more than 90% of the points should be in the confidence limits of the template; and (2) the maximum amplitude change for a match was less than 5%. This procedure was applied off-line. After the completion of this protocol, each identified CBCO unit (spike shape) was assigned an arbitrary number. Subsequently, a spike-triggered average was performed for each CBCO unit, allowing us to observe in a given MN the occurrence of any postsynaptic events related to this unit.

LIST OF SYMBOLS AND ABBREVIATIONS

20E	20-hydroxyecdysone
CBCO	coxo-basipodite chordotonal organ
EPSP	excitatory post-synaptic potential
I	depolarizing current
IPSP	inhibitory post-synaptic potential
MN	motoneuron
PSP	post-synaptic potential
R_{in}	input resistance
τ	time constant

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AUTHOR CONTRIBUTIONS

This study is a part of the PhD thesis of J.B.-C., who performed most of the experiments and data analyses reported here. D.C. supervised electrophysiological investigations and analyses. J.P.D. supervised the behavioral and hormonal studies. P.F. added some of its electrophysiological data and participated, with J.B.-C., D.C. and J.P.D., in the interpretation of data. F.B. gave efficient technical assistance concerning animal care, hormonal injections and behavior experiments. J.B.-C., P.F., D.C. and J.P.D. collaborated on the writing of the manuscript.

COMPETING INTERESTS

No competing interests declared.

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