The Journal of Experimental Biology 201, 1283–1294 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1167

NEURONAL MECHANISMS UNDERLYING THE FACILITATORY CONTROL OF UROPOD STEERING BEHAVIOUR DURING TREADMILL WALKING IN CRAYFISH

I. ANTAGONISTICALLY REGULATED BACKGROUND EXCITABILITY OF UROPOD MOTONEURONES

MAKI MURAYAMA* AND MASAKAZU TAKAHATA

Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

*e-mail: makim@bio.sci.hokudai.ac.jp

Accepted 5 February; published on WWW 20 April 1998

Summary

One of the postural reflexes of crayfish, the uropod steering response, is elicited by specific sensory inputs while the animal is walking. It is not elicited, however, by the same inputs when the animal is at rest. To clarify the neuronal mechanisms underlying this facilitatory control of body posture in the active animals, we used intracellular recordings to analyse the synaptic activities of uropod motor system neurones in an unanaesthetized whole-animal preparation. Several uropod motoneurones were found to receive sustained depolarizing inputs during walking, whereas the walking leg motoneurones sampled always showed rhythmic activity. The membrane conductance of the uropod motoneurones increased during the sustained synaptic activity. Premotor nonspiking interneurones showed depolarizing or hyperpolarizing membrane potential changes during walking that were also accompanied by increases in membrane conductance. Some of these interneurones enhanced uropod motoneurone activity, whereas others suppressed it during walking. These results suggest that the background excitability of uropod motoneurones is kept at an intermediate level during walking by the antagonistic inputs from premotor nonspiking interneurones so that the uropod motor system can be responsive to both further excitatory and inhibitory inputs resulting from postural changes.

Key words: crayfish, *Procambarus clarkii*, uropod, motoneurone, background excitability, walking, premotor nonspiking interneurone.

Introduction

Behavioural outputs depend not only on specific releasing stimuli and motor commands but also on the background excitation level of motor systems, which is extensively affected both by stimulus conditions (Pearson and Rowell, 1977) and by the behavioural context (Mori *et al.* 1991). Activation of one motor system leading to a specific behavioural act either suppresses systems controlling incompatible acts (Kovac and Davis, 1980; Krasne and Lee, 1988; Huang and Satterlie, 1990; Jing and Gillette, 1995; Shaw and Kristan, 1997) or facilitates those controlling related acts (Reichert and Rowell, 1989) by synaptically changing their excitation level. The physiological basis of behavioural hierarchy and motivation as ethological and psychological concepts (Tinbergen, 1951; Baerends, 1976; Dawkins, 1976) lies in this synaptic interaction. However, few neurophysiological studies have analyzed these synaptic processes directly, in particular the facilitatory process (Reichert and Rowell, 1985, 1989; Takakusaki *et al.* 1991), partly because it is often difficult to make intracellular recordings of the synaptic responses of nerve cells to controlled stimuli while the animal is behaving normally.

In crustaceans, the uropod steering response to a postural change is significantly facilitated when the animal is actively engaged in abdominal posture movements (Davis, 1968, 1971; Schöne *et al.* 1976; Yoshino *et al.* 1980; Newland, 1989). The synaptic mechanisms underlying this facilitatory interaction have been studied in detail in the crayfish *Procambarus clarkii* (Takahata and Hisada, 1986*a*,*b*; Takahata and Murayama, 1992). Functionally, however, the postural reflexes become most important when the animal is engaged in locomotion (Grillner, 1985; Orlovsky, 1991). It has been shown by quantitative behavioural observation and electrophysiological recording that the uropod steering response to tilting of the leg substratum is facilitated during free walking (Takahata *et al.* 1984) and during tethered standing (Newland, 1989). The synaptic mechanisms underlying this facilitatory interaction, however, have not been investigated intracellularly.

In the present series of studies, we take advantage of a whole-animal preparation of the crayfish that permits intracellular recording during tethered walking on a treadmill (Murayama and Takahata, 1996) to address two questions regarding the facilitation of steering behaviour during walking. First, how does the background excitation level of the motor system change during walking and how is it optimized synaptically? Second, how does the sensory input associated

with the leg substratum tilt interact with the background activity of the motor system and how is it mediated? In the first of two papers, we report here that the uropod motoneurones receive nonrhythmic, sustained excitatory inputs during walking while the leg motor system exhibits rhythmic activities. Previous studies revealed that the sustained excitation of motoneurones during the abdominal posture movement is mediated by premotor nonspiking interneurones (Takahata and Hisada, 1986*a*,*b*). In the present study, we have found additional nonspiking interneurones that receive sustained synaptic inputs to suppress motoneurone activity during walking. These results suggest that the background excitation level of motoneurones during walking is not simply raised in an unrestrained way but is deliberately kept at an intermediate level so that the motoneurones can be further excited or suppressed by sensory inputs to produce asymmetric configurations of the bilateral uropods. The synaptic events associated with this modulation of the background excitation are reported in the accompanying paper (Murayama and Takahata, 1998).

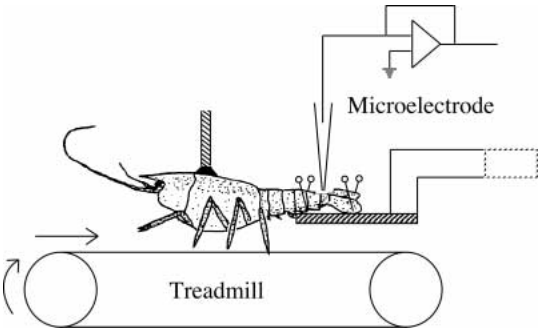

Fig. 1. Side view of the experimental procedure for obtaining intracellular recordings from a crayfish walking on a treadmill. The front of the animal was fixed to a holder using a nut glued onto the cephalothorax, and its abdomen was secured on its ventral surface to another holder. The relative positions of the cephalothorax and abdomen on the treadmill could be adjusted for individual preparations using the movable holders. Intracellular events were recorded using a microelectrode inserted into the terminal ganglion.

Materials and methods

Experimental animals and procedures

Experiments were performed on crayfish, *Procambarus clarkii* Girard, of both sexes ranging from 8 to 12 cm in body length. They were obtained commercially (Sankyo Lab, Tokyo), kept in laboratory tanks until used for experiments, and were fed weekly on a diet of raw potato and liver pieces.

Both chelipeds were cut away at least a week before experimentation. A steel nut was glued to the dorso-anterior region of the cephalothorax. An animal was first fixed dorsal side up in a chamber filled with crayfish saline (van Harreveld, 1936) cooled to approximately 5–10 °C. Abdominal tergites 2–6 were removed, and the dorsal abdominal artery and the intestine were ligated together so that their contents would not damage the nerve cells when the parts posterior to the ligature were removed. Both the superficial and deep abdominal extensor muscles were then removed to expose the fifth and the terminal (sixth) abdominal ganglia. The dorso-anterior portion of the telson cuticle was also removed. The dorsal and ventral rotator muscles and anterior and posterior telson flexor muscles were removed so that the motor bundles of the second and third roots of the terminal ganglion could be exposed.

The animal was then moved from the chamber to an experimental apparatus, where it was fixed, dorsal side up in the air, to the holder by the nut glued on the cephalothorax. The animal was suspended from the holder and provided with a treadmill as a leg substratum. The abdomen was mechanically immobilized with another holder (Fig. 1). The exposed abdominal cavity was flushed with cooled saline as frequently as possible during the experiment. The terminal ganglion was stabilized for microelectrode penetration by a small silver spoon positioned beneath the ganglion to lift it up slightly.

The treadmill was mechanically coupled to a d.c. motor (Mabuchi Motor, RE-280) by a gear-box. The velocity and direction of treadmill turning were controlled by an electrical signal applied to the motor. The velocity was adjusted to between 1.7 and 2.1 cm s^{-1} to be comparable with that of the freely walking animal. Recordings were made when the animal was walking forward in response to the treadmill turning in the opposite direction. Rotation of the treadmill shaft was monitored by a phototransistor coupled to a light-emitting diode. One cycle of oscillation in this monitor corresponds to one full rotation of a gear shaft connected with a reduction ratio of 8:1 to the treadmill shaft having a diameter of 25 mm, indicating that the linear velocity of the treadmill belt is 9.8 mm $cycle^{-1}$. Details of the treadmill are described in a previous paper (Murayama and Takahata, 1996). All experiments were conducted in air. The whole body of the animal was kept wet by occasionally spraying it with water.

Electromyogram and extracellular recordings

Electromyogram (EMG) recordings were obtained from the anterior basipodite depressor muscle of the third or fourth walking leg (Ayers and Davis, 1977) on either side. Recordings were made using pairs of Teflon-coated stainless-steel wires (127 μm in diameter) insulated except at their tips.

The uropod steering movement is controlled by antagonistic motoneurones innervating the uropod opener and closer muscles. All the motoneurones are located in the terminal abdominal ganglion. The axons of motoneurones innervating closer muscles exit the ganglion through its second root, while those of opener motoneurones exit through the third root (Larimer and Kennedy, 1969) with a few exceptions (Higuchi, 1991). In the present paper, motoneurones exiting from the second root will be designated as closer motoneurones and those exiting from the third root as opener motoneurones. The activity of uropod motoneurones was monitored by extracellular recordings of spike activity in the motor bundles

of the second and third roots using suction electrodes. Electrical signals from the motor bundles and the muscles of walking legs were amplified (Nihon-Kohden MEG-1100) and led to a storage oscilloscope (Tektronix 5111A with 5A18N amplifiers).

Intracellular recording and staining

Glass microelectrodes filled with 3% Lucifer Yellow (Stewart, 1978) in 1 mol l^{-1} LiCl (d.c. resistance 30–50 MΩ in saline) were used for intracellular recording and staining. A silver wire coated with AgCl was used as the indifferent electrode. The microelectrode was connected to an amplifier with a high input impedance (Nihon-Kohden CEZ-3100) whose output was fed into the oscilloscope and to a magnetic-tape data recorder (TEAC XR-50H; frequency range d.c. to 2.5 kHz) together with the extracellular nerve and EMG signals. Current injection into interneurones to study their output to motoneurones was carried out using the bridge mode. Current injection experiments for examining the membrane conductance change during synaptic activities were made using the discontinuous current-clamp mode (Wilson and Goldner, 1975; Finkel and Redman, 1985). Constant-current pulses (1–5 nA in intensity, 160–200 ms in duration) were injected into the cell through the electrode during the injection mode (30% duty cycle). The current flowing through the electrode was monitored by the potential difference between the two ends of a current injection resistor (Purves, 1981). Since the preparation was not immersed in saline, stray capacitance in the recording system was low, thus enabling sampling frequencies of 1.0–3.0 kHz without any specific procedures. The optimal capacitance neutralization of the recording system was obtained by monitoring the voltage across the microelectrode with an additional oscilloscope. A home-made software program running on a personal computer (Apple Macintosh IIcx) was written to analyze the amplitude distribution in the membrane potential fluctuation. The membrane potential signal from the amplifier was fed to a digital oscilloscope (Tektronix TDS 340) and digitized at a sampling frequency of 1 kHz with eight-bit vertical resolution. In total, 1024 consecutive samples of the membrane potential were sent from the oscilloscope to the computer *via* a GPIB interface and analyzed to construct an amplitude histogram in which the ordinate indicates the number of samples that are in specific ranges of membrane potential, represented by the abscissa.

Identification of individual nerve cells, from which an intracellular recording was made, was based solely on the cell structure revealed by intracellular staining with Lucifer Yellow after the physiological experiment. For dye injection, a hyperpolarizing direct current of 10 nA was delivered through the electrode for 5–10 min. The ganglion was dissected out, dehydrated in alcohol, cleared in methyl salicylate and examined under a fluorescence microscope (Nikon Optiphoto II). The ganglion was optically sectioned at 1 μm intervals using a confocal laser scanning microscope system (Molecular Dynamics Sarastro 2000) consisting of an Argon-ion laser, a Nikon Optiphoto II epifluorescence microscope equipped with a ×10 planapo objective (numerical aperture 0.45) and a Silicon Graphics Iris Indigo XS24 workstation. The morphology of a whole cell was obtained by projecting all sections to the horizontal plane using the ImageSpace (version 3.0) software. In some

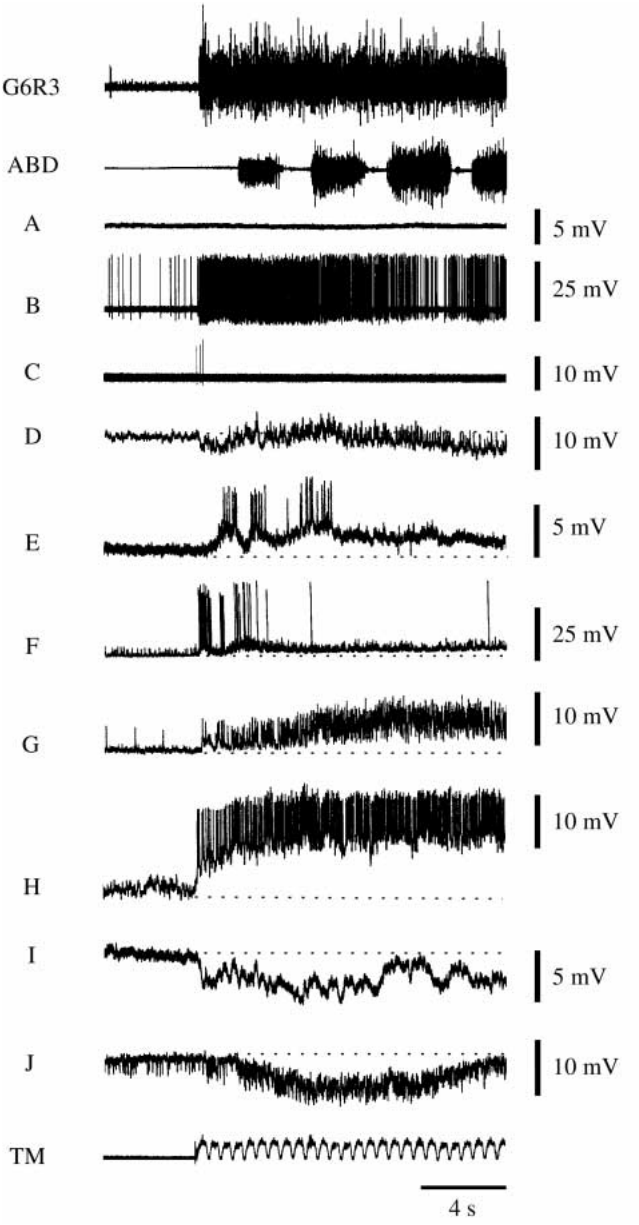

Fig. 2. Typical synaptic responses recorded in the terminal abdominal ganglion in association with walking, as represented by sustained excitation of uropod motoneurones (G6R3; spike activity of the third root on the left side) and rhythmic activation of leg muscles (ABD; electromyogram from the anterior basipodite depressor of the fourth leg). Some cells showed no specific response (A), but others showed a variety of responses, each characteristic of individual cells (B–J). See text for a detailed explanation. The oscillation in the bottom trace (TM) monitors the rotation of the treadmill axis. The dotted lines indicate the resting potential level.

preparations, the cell structure was obtained by *camera lucida* drawing.

Results

Intracellular recordings were obtained from 57 cells in the terminal abdominal ganglion of 96 animals. The results are summarized in Table 1. Response patterns associated with walking are illustrated in Figs 2 and 3. Many cells showed no activity change when the animal began, maintained or stopped walking (Fig. 2A). We stained seven of these cells to ascertain their structure: two were descending interneurones and five were motoneurones. This result was not meant to be exhaustive, however, since when we encountered such a nonresponsive cell we moved the electrode to find a responsive cell.

Four cells were found to show changes in spike activity: two of them increased (Fig. 2B) and one decreased their spike discharge rate when the animal began and maintained walking. The remaining cell transiently discharged spikes only when the animal began walking (Fig. 2C). No underlying synaptic activity was observed in these cells, and intracellular staining showed them to be descending interneurones.

All the other 46 cells showed changes in synaptic activity during walking. One nonspiking interneurone received both depolarizing and hyperpolarizing synaptic inputs concomitantly so that it showed no overall change in the mean membrane potential level when the animal began and maintained walking (Fig. 2D). Four cells (three motoneurones and one nonspiking interneurone) received transient subthreshold (Fig. 2E) or suprathreshold (Fig. 2F) depolarizing synaptic input when the animal began walking. Thirty-two cells received tonic subthreshold (Fig. 2G) or suprathreshold (Fig. 2H) depolarizing synaptic potentials (20 motoneurones and 12 nonspiking interneurones), while another four cells (one motoneurone and three nonspiking interneurones; Fig. 2I,J) received tonic hyperpolarizing input when the animal began and maintained walking. Each cell always showed the same pattern of synaptic activity during walking, and cells were never seen to show different activity patterns during different bouts of walking.

Other types of activity were occasionally observed. One

Table 1. *Response patterns observed in the uropod motor system during walking*

Response pattern	Total	DIN	MN	NSI	Unidentified
No specific response	7	2	5	0	0
During walking					
Increase in spike discharge frequency*					
Tonic	3	3	0	0	0
Phasic	1	1	0	0	0
Increase in synaptic activity†					
Tonic, depolarizing	32	0	20	12	0
Tonic, hyperpolarizing	4	0	1	3	0
Tonic, no net potential change	1	0	0	1	0
Phasic, depolarizing	4	0	3	1	0
Rhythmic, depolarizing	1	0	0	1	0
Unclassified	2	1	1	0	0
Upon termination of walking					
Increase in synaptic activity†	2	0	0	1	1
Total	57	7	30	19	1

*No synaptic activity change observed.
†Includes both subthreshold and suprathreshold activities.
DIN, descending interneurones; MN, motoneurones; NSI, nonspiking interneurones.

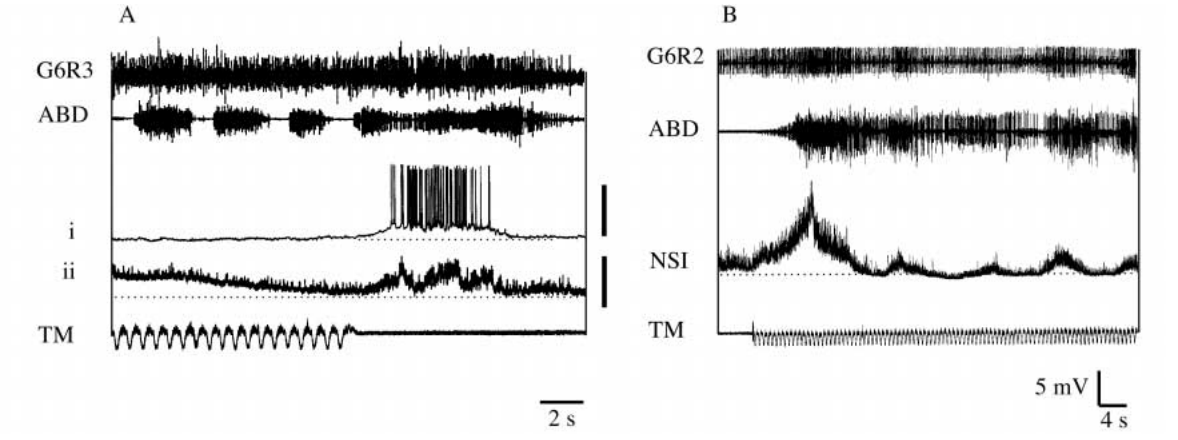

Fig. 3. Atypical synaptic responses associated with walking. (A) Transient excitatory responses of either (i) suprathreshold or (ii) subthreshold magnitude were observed only upon termination of walking, as characterized by sustained excitation of uropod motoneurones (G6R3) and rhythmic contraction of the leg muscle (ABD). The cell that showed a suprathreshold response could not be identified. The subthreshold response was recorded from a PL-type nonspiking interneurone having its cell body in the postero-lateral region of the ganglion. Neither cell showed any response upon initiation of, or during, walking. (B) Rhythmic synaptic activity was observed during walking in an AL-type nonspiking interneurone (NSI) having its cell body in the antero-lateral region of the ganglion. The dotted lines indicate the resting potential level. Calibration bars indicate 50 mV in Ai and 10 mV in Aii. TM, trace monitoring treadmill activity.

unidentified cell showed a burst of spike discharge accompanied by underlying synaptic inputs when walking terminated (Fig. 3Ai). One nonspiking interneurone received detectable synaptic inputs only when the animal stopped walking (Fig. 3Aii). The other nonspiking interneurone showed rhythmic membrane potential changes in association with walking behaviour (Fig. 3B). This was the only case in which rhythmic synaptic activities were observed during walking in the present study. We will describe in the following sections the characteristic synaptic activities observed during walking in projection interneurones descending from anterior ganglia, premotor nonspiking interneurones and uropod motoneurones.

Descending interneurones

We obtained intracellular recordings from seven descending interneurones. The synaptic activities of two interneurones that showed sustained activity changes during walking are illustrated in Fig. 4. One descending interneurone (Fig. 4Aiii) that suppressed opener motoneurone activities when depolarizing current was injected (Fig. 4Aii) discharged spikes at 2.7 spikes s^{-1} in the quiescent state. When the treadmill started to rotate, the animal began to walk. The EMG obtained from the anterior basipodite depressor (ABD) muscle of the fourth walking leg expressed a rhythmic pattern of alternate activation and inactivation, whereas the uropod opener motoneurones (G6R3) raised their activity in a sustained way (Fig. 4Ai). The discharge frequency of the descending interneurone dropped to 0.19 spikes s^{-1} during a bout of walking. This result indicates that the interneurone activity is suppressed to ensure the continuous excitation of motoneurones during

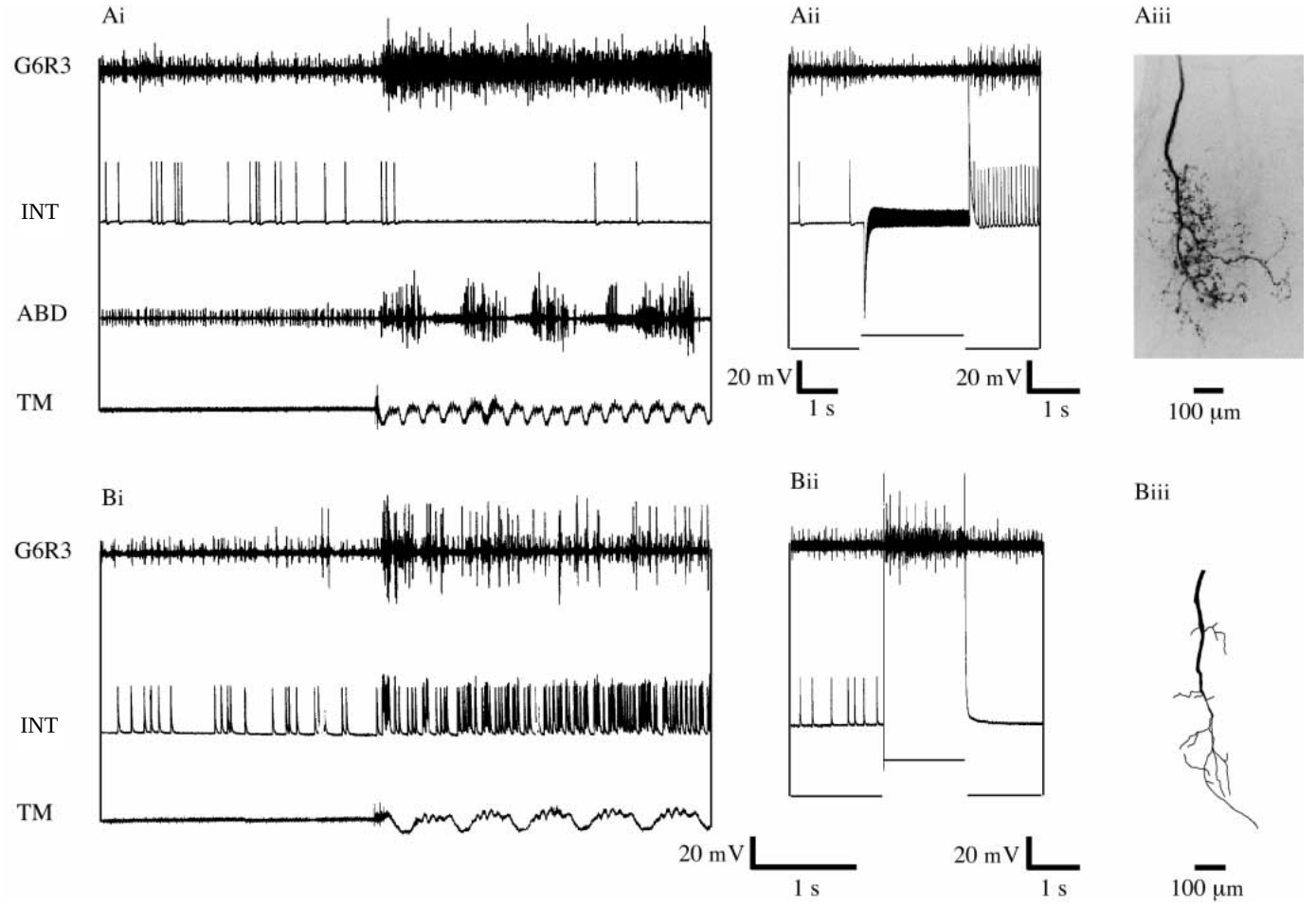

Fig. 4. Changes in spike activity in descending interneurones during walking. (A) Depression of spike activity during walking. The descending interneurone (INT) illustrated in Aiii continuously discharged spikes in the quiescent state and ceased to fire when the animal began walking, as characterized by sustained excitation of uropod motoneurones (G6R3) and rhythmic contraction of the leg muscle (ABD) (Ai). Activation of this interneurone by injected depolarizing current (5 nA) caused suppression of motoneurone spike activity (Aii). The structure of this cell was obtained by projecting on the horizontal plane optical sections made by a confocal laser scanning microscope (Aiii). (B) Elevation of spike activity during walking. The interneurone illustrated in Biii increased its spike discharge rate when the animal began walking (INT) (Bi). Activation of this interneurone by injected depolarizing current (8 nA) caused excitation of motoneurone spike activity (Bii). These interneurones respectively constitute inhibitory and excitatory pathways from the walking system to uropod motoneurones. The structure of this cell was obtained by *camera lucida* drawing (Biii). TM, trace monitoring treadmill activity.

walking, suggesting that this interneurone is not directly involved in the transmission of signals from the walking system to the uropod motor system.

Another descending interneurone (Fig. 4Biii) had an excitatory effect on opener motoneurones upon depolarization by current injection (Fig. 4Bii). When the treadmill started to rotate, the discharge frequency of the interneurone increased from 7.3 to 27.9 spikes s^{-1}, while the opener motoneurones also increased their spike discharge rate (Fig. 4Bi). This result shows that this descending interneurone carries signals from the walking system to activate the uropod motor system continuously.

It should be noted here that the spike activity of these interneurones is as sustained as that of uropod motoneurones. A further three descending interneurones showed a transient change in their spike activity at the onset of walking (Fig. 2C), and two showed no activity change associated with walking. Only one interneurone, shown in Fig. 3B, was found to show a rhythmic activity change during walking. These findings suggest that the sustained excitation of uropod motoneurones during walking that is associated with rhythmic movements of thoracic legs is, for the most part, due to tonic descending inputs rather than to any conversion processes of rhythmic inputs to tonic signals in the terminal abdominal ganglion (see Discussion).

Nonspiking interneurones

Intracellular recordings were made from 19 nonspiking interneurones which received descending inputs while the animal was engaged in walking. These interneurones were less polarized at rest than the motoneurones and other spiking cells, as has also been observed in isolated nerve cord preparations (Takahashi and Takahata, 1995). The synaptic activities of one nonspiking interneurone are illustrated in Fig. 5 with its structure (Fig. 5D). When the treadmill started to rotate, the animal began to walk, as characterized by rhythmic activity in the EMG obtained from the anterior basipodite depressor muscle of the third walking leg. The spike discharge of opener motoneurones increased while the interneurone received sustained depolarizing synaptic input (Fig. 5A). This

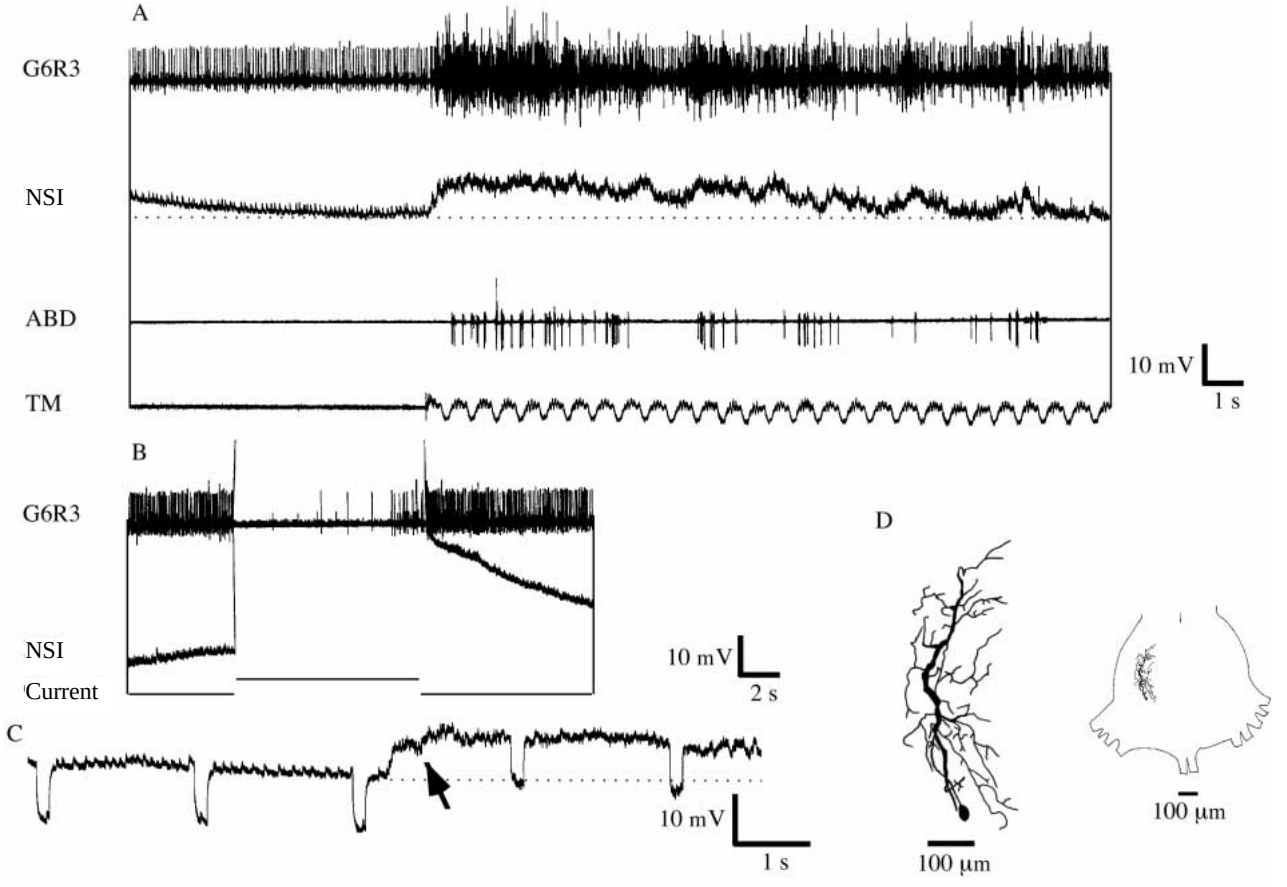

Fig. 5. Synaptic activity of a nonspiking interneurone that may be involved in suppression of motoneurones during walking. (A) The interneurone (NSI) showed sustained depolarization when the animal began walking, as characterized by sustained excitation of uropod motoneurones (G6R3) and rhythmic activation of leg muscles (ABD). (B) Artificial depolarization of this interneurone by current injection (3 nA) caused suppression of motoneurone spike activity. (C) Injection of hyperpolarizing current pulses (0.8 nA) into the interneurone revealed that the membrane conductance was increased during the sustained depolarization associated with walking, the onset of which is indicated by an arrow. (D) Projected image of the interneurone obtained by *camera lucida* drawing. The dotted lines in A and C indicate the resting potential level. TM, trace monitoring treadmill activity.

interneurone suppressed the opener motoneurone activity when experimentally depolarized by current injection (Fig. 5B). To examine the membrane conductance during the quiescent state and during walking, hyperpolarizing current pulses (0.8 nA, 120 ms at 2 Hz) were applied intracellularly to the nonspiking interneurone. The membrane conductance of the cell showed an increase of 8.5 % (P<0.05; two-sided Student's t-test) during the sustained depolarization (Fig. 5C), suggesting that this depolarization was based not on disinhibitory but on excitatory synaptic inputs. It is not known whether the membrane potential changes observed during walking (Fig. 5A,C) are of sufficient amplitude to affect the postsynaptic cells, as are those that occur during current injection (Fig. 5B). However, the fact that the depolarization of the interneurone (by more than 10 mV) during walking was the largest of the observed synaptic activities of the cell strongly suggests that it should be effective in changing the activity of postsynaptic motoneurones. It should be noted here that the synaptic activity of this nonspiking interneurone is not consistent with the actual activity of opener motoneurones since depolarization of this interneurone causes suppression of motoneurone activity (Fig. 5B). The observed increase in motoneurone activity (Fig. 5A) suggests that, although this interneurone has a suppressive effect on motoneurones during walking, it is overridden by other stronger excitatory inputs. Similar parallel antagonistic descending pathways have been reported in neuronal circuits involving both spiking (Kramer *et al.* 1981) and nonspiking (Takahata and Hisada, 1986*b*) nerve cells.

Another nonspiking interneurone, illustrated in Fig. 6C, showed sustained hyperpolarization during walking when the opener motoneurones tonically increased their spike discharge

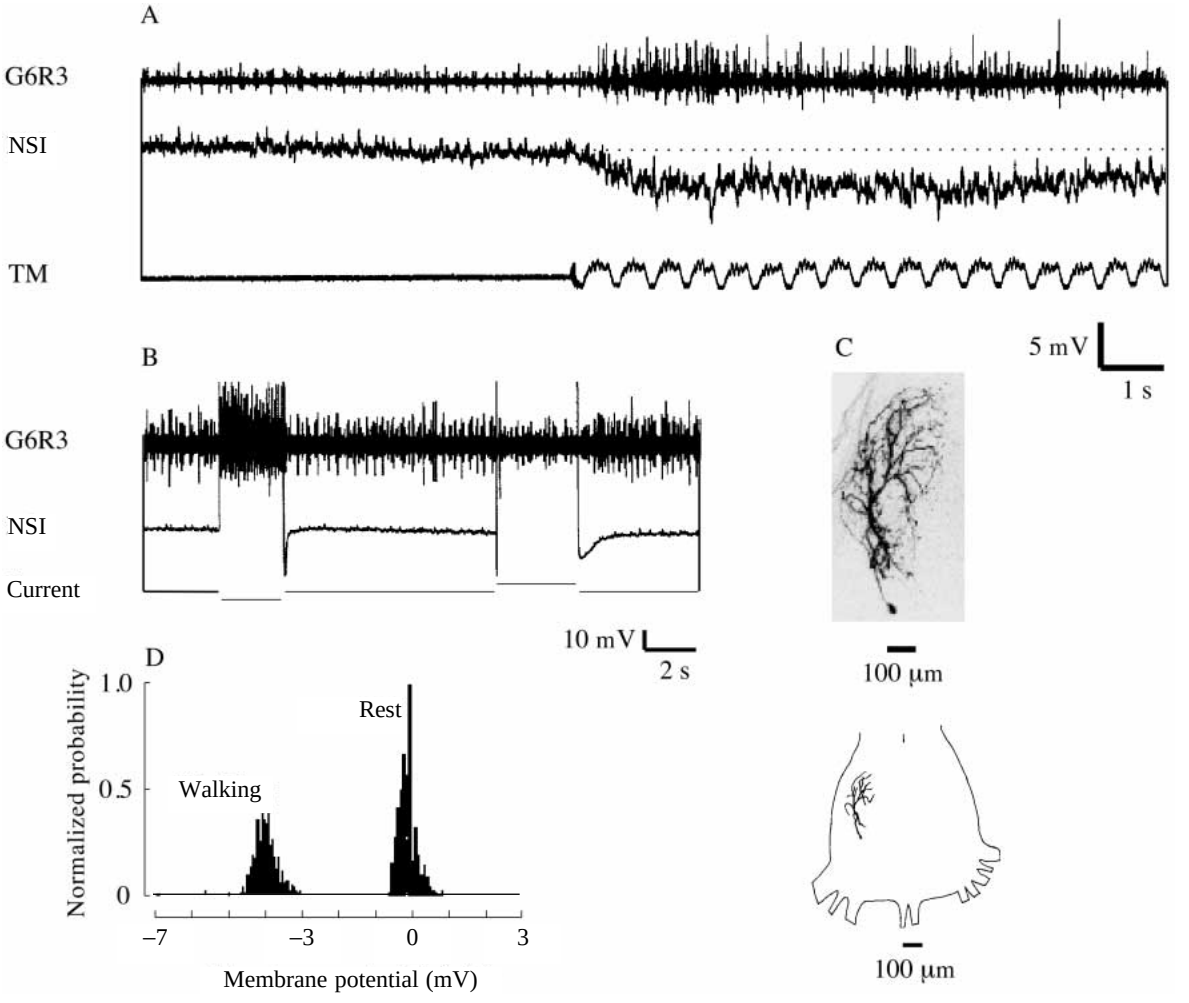

Fig. 6. Synaptic activity of a nonspiking interneurone involved in excitation of motoneurones during walking. (A) The interneurone (NSI) showed sustained hyperpolarization when the animal began walking. The dotted line indicates the resting potential level. (B) Artificial hyperpolarization of this interneurone by current injection (5 nA) caused an elevation of the spike activities of motoneurones, whereas depolarization suppressed activity. (C) Projected image of the interneurone obtained by confocal laser scanning microscopy. (D) Amplitude distribution in the membrane potential fluctuation when the animal was walking and at rest. Zero on the abscissa indicates the mean value of the membrane potential fluctuation in the quiescent state. The frequencies of each amplitude were normalized to the maximal value. G6R3, extracellular recording from the third motor root of the terminal ganglion. TM, trace monitoring treadmill activity.

frequency (Fig. 6A). This cell excited the opener motoneurones when it was hyperpolarized by current injection and suppressed them when depolarized (Fig. 6B). The fluctuation in membrane potential was greater when the animal began to walk than when it was at rest (Fig. 6D), suggesting that the sustained hyperpolarization during walking was produced by inhibitory, rather than disfacilitatory, inputs. The synaptic response of this nonspiking interneurone is consistent with its output effect on motoneurones. It is thus suggested that the interneurone receives hyperpolarizing synaptic input during walking and thereby excites the motoneurones to increase their spike activity.

The premotor nonspiking interneurones in the terminal abdominal ganglion are classified into two major morphological types, PL- and AL-types, according to the location of their cell bodies in the ganglion (Hisada *et al.* 1984). Thirteen of nineteen nonspiking interneurones that received descending inputs during walking belonged to the PL-type. The other six were of the AL-type. Nine of the 13 PL-type and five of the six AL-type interneurones received depolarizing inputs during walking, whereas one AL-type and two PL-type cells received hyperpolarizing inputs. One of the PL-type interneurones received both depolarizing and hyperpolarizing inputs during walking, so that no net change was observed in its membrane potential (Fig. 2D).

Regarding their output effect, eight PL-type interneurones were found to suppress the opener motoneurone activity upon depolarization (as in Fig. 5B) and to increase it upon hyperpolarization by current injection. Four of these continuously received depolarizing synaptic inputs (as in Fig. 5A), whereas two cells continuously received hyperpolarizing inputs during walking. One cell received transiently depolarizing input and one cell received rhythmic input. The other 11 interneurones (five PL-type and six AL-type cells) had no output effect on the opener motoneurone activity. Of these, three AL-type cells suppressed closer motoneurone activity when they were depolarized by current injection, whereas one PL-type cell increased closer motoneurone activity. The remaining seven cells had no output effect on either opener or closer motoneurone activities. Thus, the morphological types of nonspiking interneurones could not be related definitively to any functional differences. This uneven functional distribution of AL- and PL-type cells was probably due to our sampling bias during electrode probing.

Motoneurones

We made intracellular recordings from 30 uropod motoneurones that received synaptic inputs while the animal was engaged in walking. Sixteen motoneurones received sporadic synaptic inputs and discharged spikes spontaneously (0.07–2.80 spikes s^{-1}) in the quiescent state without any specific stimulus being applied. These motoneurones are described as tonic in the following discussion. The other 14 cells, termed phasic, were synaptically silent when the animal was at rest. Tonic motoneurones received sustained depolarizing synaptic input that was either subthreshold or suprathreshold for eliciting spikes, whereas phasic motoneurones received a train of small, discrete synaptic potentials when the animal was engaged in walking (Fig. 2G).

The synaptic activities of tonic motoneurones associated with walking are illustrated in Fig. 7 with their morphology (Fig. 7Aii, Bii). When the treadmill started rotating, the animal began to walk and exhibited a sustained increase in both the opener and closer motoneurone activities (Fig. 7Ai) and rhythmic EMG activities in the fourth walking leg (Fig. 7Bi). Intracellular recordings from motoneurones revealed that they received a sustained depolarizing synaptic input with spikes superimposed. Periodic injection of constant hyperpolarizing current pulses into the motoneurone showed that the sustained depolarization was associated with an increase of 17.7 % (P<0.05) in the membrane conductance (Fig. 7Bi). This result indicates that the sustained depolarization of motoneurones during walking is caused not by disinhibitory but by excitatory synaptic inputs.

Eight of the phasic motoneurones received subthreshold synaptic inputs during walking while the other six received no synaptic inputs. Eleven of sixteen tonic motoneurones received sustained depolarizing synaptic input and increased their spike discharge frequency during walking. Three other cells received only subthreshold inputs occasionally superimposed by spikes, and the remaining cell received a train of discrete depolarizing synaptic inputs without any sustained depolarization. No uropod motoneurone receiving rhythmic synaptic inputs during walking was encountered in this study.

Discussion

The mechanism underlying the facilitatory control of postural reflexes during locomotory behaviour in the crayfish is the synaptic summation of subthreshold sensory input with the excitatory input from the locomotor system (Takahata, 1990). This summation occurs in motoneurones themselves as well as in proprioabdominal interneurones descending from anterior ganglia to the uropod motor system (Takahata and Murayama, 1992). However, the way in which the rhythmic motor outputs of the locomotor system interact with the sustained outputs of the postural reflex systems is not known. Furthermore, it is crucial for this summatory interaction to keep the excitatory input from the locomotor system at an appropriate level because uncontrolled strong inputs would shift the operating point of the cell where the interaction occurs close to saturation level (Burrows and Siegler, 1978; Wilson and Phillips, 1982), thus invalidating further inputs from the sensory source.

In the present study, we addressed these interactions and have found that the sustained excitability of uropod motoneurones during walking is kept at an intermediate level by the antagonistic inputs from premotor nonspiking interneurones so that the uropod motor system can be responsive to both further excitatory and inhibitory inputs resulting from postural changes.

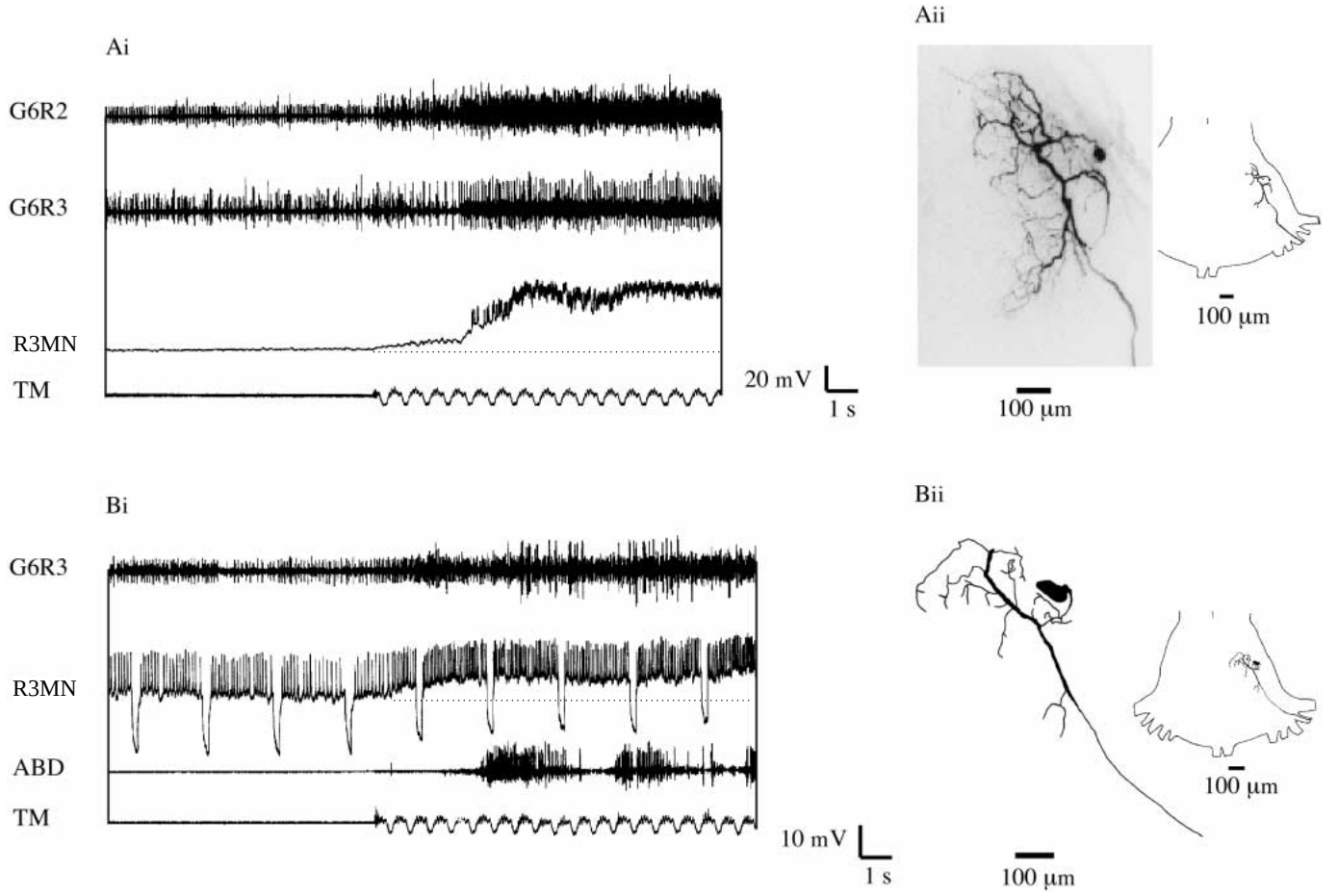

Fig. 7. Synaptic activity of uropod motoneurones during walking. The opener motoneurone illustrated in Aii showed sustained depolarization that was initially subthreshold and became suprathreshold (R3MN) during walking, as characterized by sustained excitation of both closer (G6R2) and opener (G6R3) motoneurones (Ai). The cell structure obtained by confocal laser scanning microscopy (Aii). In the closer motoneurone illustrated in Bii, intracellular injection of hyperpolarizing current pulses (0.4 nA) revealed that the membrane conductance increased during the sustained depolarization (R2MN; Bi). In this experiment, the opener motoneurone activity (G6R3) was recorded together with the fourth leg muscle activity (ABD). Rotation of the treadmill is monitored by oscillation in the bottom trace (TM). The dotted lines in Ai and Bi indicate the resting potential level. The cell structure was obtained by *camera lucida* drawing (Bii).

Sustained facilitatory inputs to the uropod motor system during walking

Uropod motoneurones were found to receive sustained synaptic inputs when the animal was engaged in walking and while the leg motor system showed rhythmic outputs (Figs 3–5). There are two possible explanations for the synaptic mechanism of sustained inputs to the uropod motor system during rhythmic walking. One possibility is that the descending interneurones, including the proprioabdominal interneurones (Takahata and Murayama, 1992), carry a tonic train of action potentials from the walking system to the uropod motor system. It is well known that a tonic driving signal is required to activate oscillatory circuits such as the locomotor system (Grillner, 1975; Roberts *et al.* 1981). Thus, tonic corollary signals derived from the command for stepping pattern generation would cause nonspiking interneurones and motoneurones to produce a sustained membrane potential change. Alternatively, rhythmic inputs from the walking system in the thoracic ganglia to the terminal abdominal ganglion could be integrated there to produce sustained potential changes in the uropod motor system. In this case, there must be a circuit-level integrator in the terminal abdominal ganglion since, although premotor nonspiking interneurones have relatively long membrane time constants (30–50 ms; Takahashi and Takahata, 1995), they are unlikely by themselves to integrate signals in phase with the walking rhythm, which has a period of more than 1 s (Figs 3–5).

In this study, we found several descending interneurones that showed tonic activity changes during walking (Fig. 4). No descending interneurone was encountered that showed a rhythmic activity change in phase with the leg movements. The results suggest that the sustained signal is formed in the anterior ganglia during walking and is transmitted to the terminal abdominal ganglion, where the uropod motor system is located. Quantitative behavioural analyses have revealed that walking behaviour is preceded and accompanied by postural

movements of the abdomen (Takahata *et al.* 1984). Uropod motoneurones and premotor nonspiking interneurones show sustained membrane potential changes during fictive abdominal posture movements (Takahata and Hisada, 1986*a*,*b*). It seems, therefore, that the tonic excitation of uropod motoneurones during walking is provided by the abdominal posture system, which is also activated tonically during walking on the treadmill (Murayama and Takahata, 1996). It remains to be determined, however, whether the activation of the abdominal system is required for, or just independently accompanies, walking behaviour in crayfish. It is interesting to note here that an increase in postural tonus is associated with walking in cats (Mori *et al.* 1991) and that an experimental change in the postural tonus can elicit and suppress stepping leg movements (Takakusaki *et al.* 1991).

Our finding that one of the 19 nonspiking interneurones examined showed a rhythmic membrane potential change which was apparently associated with rhythmic movements of legs also suggests the existence of some descending interneurones carrying rhythmic activity from the walking system to the uropod motor system. Such interneurones were not detected in the present study. In the lobster *Homarus gammarus*, rhythmic swimmeret beating was reported to be well coordinated with the walking rhythm (Cattaert and Clarac, 1983). This observation strongly suggests that rhythmic signals associated with leg movements during walking are transmitted from thoracic to abdominal ganglia. However, the functional role of rhythmic activities in the uropod motor system is not known. Behavioural observations and EMG analyses showed no reliable rhythmic movements in uropods during free walking (Takahata *et al.* 1984). Further studies are needed to examine whether such oscillatory membrane potential changes of nonspiking interneurones are eventually transformed to the sustained excitation of the uropod motoneurones or whether they are further directed to some other systems.

Antagonistic input pathways to motoneurones mediated by nonspiking interneurones

Premotor nonspiking interneurones, like the motoneurones that are controlled by them, showed a sustained membrane potential shift in either a depolarizing or a hyperpolarizing direction during walking (Figs 5, 6). It is generally believed that nonspiking interneurones having a less negative resting potential than spiking cells, release neurotransmitters continuously at rest, and that both depolarization and hyperpolarization are effective in changing their output by increasing or decreasing the amount of transmitter released (Burrows and Siegler, 1978). We found in this study not only nonspiking interneurones receiving hyperpolarizing inputs that would disinhibit motoneurones (Fig. 6) but also others receiving depolarizing inputs that would tend to inhibit them while the animal was walking on a treadmill (Fig. 5). The former interneurones are of the same type as those reported previously (Takahata and Hisada, 1986*b*; Takahata and Murayama, 1992) and may be involved in gating the descending motor pathway. The latter interneurones, in contrast, apparently antagonize the former by partially nullifying their disinhibitory effect. Since the total activity of both opener and closer motoneurones increased while the animal was engaged in walking, the inhibitory action of these nonspiking interneurones was not consistent with the actual activity of the motoneurones. Similar opposing parallel connections have been observed in many other systems (Kramer *et al.* 1981; Dumont and Robertson, 1986).

Antagonistic connections that are apparently nonfunctional can be also seen in the synaptic connection from the abdominal posture system to the uropod motor system. Namba *et al.* (1994), using the isolated nerve cord preparation, reported that, when the abdominal posture system was activated by electrical stimulation of one extension-evoking command fibre located in area 81 (Wiersma and Hughes, 1961) of the abdominal connective, the opener motoneurones were excited while one closer motoneurone was inhibited. However, the closer muscles are in fact activated during abdominal extension under natural conditions (Takahata *et al.* 1984), and the closer motoneurones are excited during fictive abdominal extension in the air without a leg substratum (Takahata and Murayama, 1992) and on the treadmill (Fig. 7). It has been shown by Evoy and Kennedy (1967) that there is a diverse set of abdominal posture command fibres in the ventral nerve cord, each of which is capable of eliciting a different pattern of abdominal movement. In a study of the rhythmic movements of swimmerets that are also controlled by a set of command fibres, Davis and Kennedy (1972) concluded that a full behavioural act should be elicited and controlled by the collective activities of all of the command fibres. The findings so far reported thus suggest that inhibitory inputs to uropod motoneurones, such as that mediated by the nonspiking interneurone shown in Fig. 5, are overridden by major excitatory inputs during walking and abdominal postural movements under natural conditions. The synaptic mechanism of this interaction between the excitatory and inhibitory inputs remains to be studied.

Feedforward system for setting an appropriate level of excitation of motoneurones

The functional significance of antagonistic inputs has been unclear. Why are cells inhibited while simultaneously receiving excitatory input? Referring to the experimental results obtained in the insect flight system (Robertson and Pearson, 1983, 1985) and the crayfish escape circuitry (Dumont and Wine, 1987), Dumont and Robertson (1986) pointed out the possibility that not all neuronal connections are functional and that the contradictory circuits may be vestigial, reflecting their evolutionary constraints. Kramer *et al.* (1981) suggested that the apparently contradictory input found in the crayfish escape circuit may have a function in some unknown aspects of behavioural control. Similar potential functionality has been assumed for the parallel input pathways mediated by nonspiking interneurones (Namba *et al.* 1994). However, no experimental support has been provided for this hypothesis. In the present study, we have shown that uropod motoneurones also receive antagonizing inputs, including inhibition (Fig. 5)

and disinhibition (Fig. 6), during walking. This inhibition, which can be overridden by disinhibitory and excitatory inputs, is more likely to be functional than vestigial: we assume that the hidden inhibitory inputs would function by counteracting the excitatory and disinhibitory inputs so as to set the membrane potential of motoneurones to an appropriate level, thus preventing them from saturating in excitability.

The steering behaviour of uropods is elicited by body tilt stimuli provided that the uropod motoneurones and other relevant elements are sufficiently depolarized (Takahata and Murayama, 1992). This is because the excitatory input from the statocyst to the uropod motoneurones is subthreshold, so that it can generate spikes in motoneurones only after synaptic summation with depolarizing inputs from other sources (Takahata, 1990). This depolarizing input is provided by the walking and abdominal posture systems (see above). It should be noted here, however, that steering behaviour consists of opening one uropod and closing the opposite one (Yoshino *et al.* 1980). This bilaterally asymmetric behaviour is performed by either an increase or a decrease in motoneurone activities from a certain excited level (Takahata *et al.* 1984). These observations indicate that the uropod motoneurones should be excited not to the maximal extent but to a moderate level for steering behaviour to be elicited by external stimuli. The antagonism between synaptic inputs to motoneurones during walking observed in the present study thus led to the hypothesis that the inhibitory input, which appears to be futile, will function in limiting the excitation of motoneurones so that they can be responsive to further inputs, both excitatory and inhibitory; nonspiking interneurones mediating inhibition of uropod motoneurones during walking would thus constitute a sort of feedforward system setting an appropriate level of excitation of motoneurones.

The importance of an appropriate level of background excitability for initiating behavioural acts has been appreciated in a number of motor systems (Arshavsky *et al.* 1985; Roberts *et al.* 1985; Takakusaki *et al.* 1991). To accomplish complex behaviour by ensuring a maximal degree of freedom of movement, the motoneurones (as the final common pathway) should be responsive to both excitatory and inhibitory inputs. A unique feature of the uropod motor system of crayfish is that this is achieved by parallel circuits of nonspiking interneurones having antagonistic effects on the same motoneurones. The functional significance of nonspiking interneurones in the gating control of steering behaviour has been discussed elsewhere (Takahata and Murayama, 1992). A major driving circuit paralleled by a feedforward limiting circuit could be a general scheme in motor control systems.

This work was supported in part by Grants-in-Aid (07554071, 09440274) from MESSC Japan.

References

ARSHAVSKY, Y. I., MORI, S., ORLOVSKY, G. N., PAVLOVA, G.A. AND POPOVA, L. B. (1985). Effects of stimulation of midline inhibitory area within the pontine tegmentum on scratch reflex. *Brain Res.* **343**, 360–362.

AYERS, J. L. AND DAVIS, W. J. (1977). Neuronal control of locomotion in the lobster *Homarus americanus*. I. Motor programs for forward and backward walking. *J. comp. Physiol.* **115**, 1–27.

BAERENDS, G. P. (1976). The functional organization of behaviour. *Anim. Behav.* **24**, 726–738.

BURROWS, M. AND SIEGLER, M. V. S. (1978). Graded synaptic transmission between local interneurones and motoneurones in the metathoracic ganglion of the locust. *J. Physiol., Lond.* **285**, 231–255.

CATTAERT, D. AND CLARAC, F. (1983). Influences of walking on swimmeret beating in the lobster *Homarus gammarus*. *J. Neurobiol.* **14**, 421–439.

DAVIS, W. J. (1968). Lobster righting responses and their neural control. *Proc. R. Soc. Lond. B* **170**, 435–456.

DAVIS, W. J. (1971). The integrative action of the nervous system in crustacean equilibrium reactions. In *Gravity and the Organism* (ed. S. A. Gordon and M. J. Cohen), pp. 237–250. Chicago, London: Chicago University Press.

DAVIS, W. J. AND KENNEDY, D. (1972). Command interneurons controlling swimmeret movements in the lobster. I. Types of effects on motoneurons. *J. Neurophysiol.* **35**, 1–12.

DAWKINS, R. (1976). Hierarchical organization and postural facilitation: Rules for grooming in flies. *Anim. Behav.* **24**, 739–755.

DUMONT, J. P. C. AND ROBERTSON, R. M. (1986). Neuronal circuits: an evolutionary perspective. *Science* **233**, 849–853.

DUMONT, J. P. C. AND WINE, J. J. (1987). The telson flexor neuromuscular system of the crayfish. III. The role of feedforward inhibition in shaping a stereotyped behaviour pattern. *J. exp. Biol.* **127**, 295–311.

EVOY, W. H. AND KENNEDY, D. (1967). The central nervous organization underlying control of antagonistic muscles in the crayfish. I. Types of command fibers. *J. exp. Zool.* **165**, 223–238.

FINKEL, A. S. AND REDMAN, S. J. (1985). Optimal voltage clamping with single microelectrode. In *Voltage and Patch Clamping with Microelectrodes* (ed. T. G. Smith Jr, H. Lecar, S. J. Redman and P. W. Gage), pp. 95–120. Bethesda, MD: American Physiological Society.

GRILLNER, S. (1975). Locomotion in vertebrates – central mechanisms and reflex interaction. *Physiol. Rev.* **55**, 247–304.

GRILLNER, S. (1985). Neurobiological bases of rhythmic motor acts in vertebrates. *Science* **228**, 143–149.

HIGUCHI, T. (1991). Innervation pattern of some tonic muscles in the uropod of the crayfish, *Procambarus clarkii*. *Zool. Sci.* **8**, 193–196.

HISADA, M., TAKAHATA, M. AND NAGAYAMA, T. (1984). Structure and output connection of local non-spiking interneurons in crayfish. *Zool. Sci.* **1**, 41–49.

HUANG, Z. AND SATTERLIE, R. A. (1990). Neuronal mechanisms underlying behavioral switching in a pteropod mollusc. *J. comp. Physiol.* **166**, 875–887.

JING, J. AND GILLETTE, R. (1995). Neuronal elements that mediate escape swimming and suppress feeding behavior in the predatory sea slug *Pleurobranchaea*. *J. Neurophysiol.* **74**, 1900–1910.

KOVAC, M. P. AND DAVIS, W. J. (1980). Neural mechanisms underlying behavioral choice in *Pleurobranchaea*. *J. Neurophysiol.* **43**, 469–487.

KRAMER, A. P., KRASNE, F. B. AND WINE, J. J. (1981). Interneurons between giant axons and motoneurons in the crayfish escape circuitry. *J. Neurophysiol.* **45**, 550–573.

KRASNE, F. B. AND LEE, S. C. (1988). Response-dedicated trigger neurons as control points for behavioral actions: selective inhibition of lateral giant command neurons during feeding in crayfish. *J. Neurosci*. **8**, 3708–3712.

LARIMER, J. L. AND KENNEDY, D. (1969). Innervation patterns of fast and slow muscles in the uropods of crayfish. *J. exp. Biol*. **51**, 119–133.

MORI, S., SAKAMOTO, T. AND TAKAKUSAKI, K. (1991). Interaction of posture and locomotion in cats: Its automatic and volitional control aspects. In *Neurobiological Basis of Human Locomotion* (ed. M. Shimamura, S. Grillner and V. R. Edgerton), pp. 21–32. Tokyo: Japan Scientific Societies Press.

MURAYAMA, M. AND TAKAHATA, M. (1996). Sensory control mechanisms of the uropod equilibrium reflex during walking in the crayfish *Procambarus clarkii*. *J. exp. Biol*. **199**, 521–528.

MURAYAMA, M. AND TAKAHATA, M. (1998). Neuronal mechanisms underlying the facilitatory control of uropod steering behaviour during treadmill walking in crayfish. II. Modulation of uropod motoneurone excitation by leg proprioception. *J. exp. Biol.* **201**, 1295–1305.

NAMBA, H., NAGAYAMA, T. AND HISADA, M. (1994). Descending control of nonspiking local interneurons in the terminal abdominal ganglion of the crayfish. *J. Neurophysiol*. **72**, 235–248.

NEWLAND, P. L. (1989). The uropod righting reaction of the crayfish *Procambarus clarkii* (Girard): an equilibrium response driven by two largely independent reflex pathways. *J. comp. Physiol*. **164**, 685–696.

ORLOVSKY, G. N. (1991). Cerebellum and locomotion. In *Neurobiological Basis of Human Locomotion* (ed. M. Shimamura, S. Grillner and V. R. Edgerton), pp. 187–199. Tokyo: Japan Scientific Societies Press.

PEARSON, K. G. AND ROWELL, C. H. F. (1977). Functions of tonic sensory input in insects. *Ann. N.Y. Acad. Sci.* **290**, 114–123.

PURVES, R. D. (1981). *Microelectrode Methods for Intracellular Recording and Ionophoresis*. London: Academic Press.

REICHERT, H. AND ROWELL, C. H. F. (1985). Integration of nonphase-locked exteroceptive information in the control of rhythmic flight in the locust. *J. Neurophysiol*. **53**, 1201–1218.

REICHERT, H. AND ROWELL, C. H. F. (1989). Invariance of oscillator interneurone activity during variable motor output by locusts. *J. exp. Biol.* **141**, 231–239.

ROBERTS, A., DALE, N., EVOY, W. H. AND SOFFE, S. R. (1985). Synaptic potentials in motoneurons during fictive swimming in spinal *Xenopus* embryos. *J. Neurophysiol*. **54**, 1–10.

ROBERTS, A., KAHN, J. A., SOFFE, S. R. AND CLARKE, J. D. W. (1981). Neural control of swimming in a vertebrate. *Science* **213**, 1032–1034.

ROBERTSON, R. M. AND PEARSON, K. G. (1983). Interneurons in the flight system of the locust: distribution, connections and resetting properties. *J. comp. Neurol*. **215**, 33–50.

ROBERTSON, R. M. AND PEARSON, K. G. (1985). Neural circuits in the flight system of the locust. *J. Neurophysiol*. **53**, 110–128.

SCHÖNE, H., NEIL, D., STEIN, A. AND CARLSTEAD, M. (1976). Reactions of *Palinurus vulgaris* to substrate tilt (I.). *J. comp. Physiol.* **107**, 113–128.

SHAW, B. K. AND KRISTAN, W. B., JR (1997). The neuronal basis of the behavioral choice between swimming and shortening in the leach: Control is not selectively exercised at higher circuit levels. *J. Neurosci.* **17**, 786–795.

STEWART, W. W. (1978). Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell* **14**, 741–759.

TAKAHASHI, M. AND TAKAHATA, M. (1995). Dendritic properties of uropod motoneurons and premotor nonspiking interneurons in the crayfish *Procambarus clarkii* Girard. *J. comp. Physiol*. **176**, 503–512.

TAKAHATA, M. (1990). The crayfish posture control system as a model for studying mechanisms underlying behavioral variability. In *Frontiers in Crustacean Neurobiology* (ed. K. Wiese, W. D. Krenz, J. Tautz, H. Reichert and B. Mulloney), pp. 301–308. Basel: Birkhäuser Verlag.

TAKAHATA, M. AND HISADA, M. (1986*a*). Sustained membrane potential change of uropod motor neurons during the fictive abdominal posture movement in crayfish. *J. Neurophysiol*. **56**, 702–717.

TAKAHATA, M. AND HISADA, M. (1986*b*). Local nonspiking interneurons involved in gating of the descending motor pathway in crayfish. *J. Neurophysiol*. **56**, 718–731.

TAKAHATA, M., KOMATSU, H. AND HISADA, M. (1984). Positional orientation determined by the behavioural context in *Procambarus clarkii* Girard (Decapoda: Macrura). *Behaviour* **88**, 240–265.

TAKAHATA, M. AND MURAYAMA, M. (1992). Multiple gate control of the descending statocyst–motor pathway in the crayfish *Procambarus clarkii* Girard. *J. comp. Physiol*. **170**, 463–477.

TAKAKUSAKI, K., OHTA, Y. AND MORI, S. (1991). Membrane potential oscillations of hindlimb alpha-motoneurons associated with alternating hindlimb loading in reflexively standing cats. In *Neurobiological Basis of Human Locomotion* (ed. M. Shimamura, S. Grillner and V. R. Edgerton), pp. 159–166. Tokyo: Japan Scientific Societies Press.

TINBERGEN, N. (1951). *The Study of Instinct.* London: Oxford University Press.

VAN HARREVELD, A. (1936). A physiological solution for fresh water crustaceans. *Proc. Soc. exp. Biol. Med*. **34**, 428–432.

WIERSMA, C. A. G. AND HUGHES, G. M. (1961). On the functional anatomy of neuronal units in the abdominal cord of the crayfish, *Procambarus clarkii* (Girard). *J. comp. Neurol*. **116**, 209–228.

WILSON, J. A. AND PHILLIPS, C. E. (1982). Locust local nonspiking interneurons which tonically drive antagonistic motor neurons: Physiology, morphology and ultrastructure. *J. comp. Neurol.* **204**, 21–31.

WILSON, W. A. AND GOLDNER, M. M. (1975). Voltage clamping with a single microelectrode. *J. Neurobiol*. **6**, 411–422.

YOSHINO, M., TAKAHATA, M. AND HISADA, M. (1980). Statocyst control of the uropod movement in response to body rolling in crayfish. *J. comp. Physiol*. **139**, 243–250.