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Intersegmental Coordination of Walking Movements in Stick Insects

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Ludwar, Björn Ch., Marie L. Göritz, and Joachim Schmidt. Intersegmental coordination of walking movements in stick insects. *J Neurophysiol* 93: 1255–1265, 2005. First published November 3, 2004; doi:10.1152/jn.00727.2004. Locomotion requires the coordination of movements across body segments, which in walking animals is expressed as gaits. We studied the underlying neural mechanisms of this coordination in a semi-intact walking preparation of the stick insect *Carausius morosus*. During walking of a single front leg on a treadmill, leg motoneuron (MN) activity tonically increased and became rhythmically modulated in the ipsilateral deafferented and deefferented mesothoracic (middle leg) ganglion. The pattern of modulation was correlated with the front leg cycle and specific for a given MN pool, although it was not consistent with functional leg movements for all MN pools. In an isolated preparation of a pair of ganglia, where one ganglion was made rhythmically active by application of pilocarpine, we found no evidence for coupling between segmental central pattern generators (CPGs) that could account for the modulation of MN activity observed in the semi-intact walking preparation. However, a third preparation provided evidence that signals from the front leg's femoral chordotonal organ (fCO) influenced activity of ipsilateral MNs in the adjacent mesothoracic ganglion. These intersegmental signals could be partially responsible for the observed MN activity modulation during front leg walking. While afferent signals from a single walking front leg modulate the activity of MNs in the adjacent segment, additional afferent signals, local or from contralateral or posterior legs, might be necessary to produce the functional motor pattern observed in freely walking animals.

INTRODUCTION

Locomotion requires coordinated body movements that provide propulsion and maintain body stability. In walking animals, these needs are fulfilled by alternating subsets of legs, one of which is on the ground (stance phase) and hence provides stability and propulsion, whereas the other subset is lifted and relocated (swing phase). As locomotion speed changes, the makeup of these subsets is modified to assure the stability of the animal and, in particular, can involve changes in the number of legs simultaneously on the ground (Graham 1985; Orlovsky et al. 1999). For instance, in adult stick insects walking at moderate speeds, four legs are simultaneously in a stance phase forming a stepping pattern that corresponds to a tetrapod gait. With increasing speed, leg coordination shifts toward the stepping pattern of a tripod gait (Graham 1972, 1985; Wendler 1964).

This flexibility requires a sophisticated mechanism to coordinate the neural networks controlling leg movements. A basic component of those networks is an oscillatory neuronal circuit, a central pattern generator (CPG) that produces alternating activity in antagonistic motorneuron (MN) groups (Hooper and DiCaprio 2004; MacKay-Lyons 2002; Marder and Calabrese 1996; Pearson 1993, 2000). In stick insects, leg movements are generated by the activity of several CPGs, each of which controls the movement of individual leg joints (Bässler and Büschges 1998). The coordination among these CPGs required to produce coordinated joint movements in individual legs (intra-leg coordination) is believed to depend largely on afferent feedback arising within that leg (Akay et al. 2004; Bässler and Büschges 1998; Bucher et al. 2003).

With respect to inter-leg coordination, behavioral studies in which the position of individual legs of lobsters walking on a treadmill was perturbed showed that leg position influences the coordination of the remaining legs (Cruse and Müller 1986; Cruse et al. 1983). Similar behavioral experiments with stick insects (Bässler 1979; Cruse 1979, 1985; Cruse and Schwarze 1988; Dean and Wendler 1983; Graham 1979a,b) led to the proposition of six rules for coordination during walking (Cruse 1990; Cruse et al. 1998). Three of these rules are of particular importance for ipsilateral leg coordination: *1*) an ongoing swing phase inhibits the start of the swing phase in the next rostral leg, *2*) the start of a stance phase facilitates the start of the swing phase in the next rostral leg, and *3*) the posterior movement of a leg during its stance phase increasingly facilitates the start of the swing phase in the next caudal leg. Simulations using computer and electro-mechanical models showed that these rules are sufficient to produce patterned leg movements similar to those observed in walking insects (Cruse et al. 1995, 1996, 1998). Maintenance of these rules depends on interactions between segmental ganglia, because cutting the ipsilateral connectives disrupts inter-leg coordination (Dean 1989).

However, the nature of these pathways, in particular the relative importance of intersegmental afferent signals versus central CPG interconnections, is still not fully understood. Studies in stick insects described interneurons that receive afferent input, code position, and movement of single leg joints or complete legs and project to neighboring ganglia (Brunn and Dean 1994; Büschges 1989). In locusts, similar identified intersegmental interneurons form synaptic connections with leg MNs and nonspiking interneurons in the next posterior ganglion (Laurent 1986, 1987; Laurent and Burrows 1988, 1989a,b). However, in all these studies, the preparations were so reduced that the animals no longer walked, and thus, although these intersegmental interneurons are a potential pathway for intersegmental coordination of leg movements, it is unknown if they are in fact used to coordinate walking movement (Burrows 1996).

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We describe here an attempt to address this difficulty using a walking single leg preparation of the stick insect in which the body of the animal was fixed and all legs except a single front leg were amputated. This leg performed walking movements on a treadmill while MN activity was recorded from the deafferented and deefferented ipsilateral mesothoracic ganglion (the ganglion caudal to the ganglion that controls the walking leg). Thus this preparation provides a link between the behavioral studies performed in intact preparations and the electrophysiological data gathered from preparations, so reduced walking was impossible. Furthermore, because sensory input was limited to a single leg, the complexity of neural responses observed is much less than in a fully legged animal, which greatly facilitates studying the neural mechanisms underlying intersegmental coordination. This preparation thus provides "a reliable semi-intact preparation in which the function and physiology of central oscillators and sensory processes can be observed and manipulated, while the essential motor patterns are expressed" (Friesen and Chang 2001).

When the front leg performed walking movements, the activity of mesothoracic MN pools increased and produced rhythmic activity coupled to the front leg steps. In contrast, when the pro- and mesothoracic ganglia were isolated from the animal, mesothoracic MN activity was not coordinated with rhythmic activity of prothoracic leg MNs. In preparations in which a frontal leg sensory organ [the femoral chordotonal organ (fCO)] was left intact, fCO mechanical stimulation influenced mesothoracic MN activity. Taken together, these data suggest that sensory input from the frontal leg may underlie some of the intersegmental leg coordination observed in walking intact animals.

METHODS

The experiments were conducted with adult female Indian stick insects (*Carausius morosus* Brunner 1908) from a colony maintained at the University of Cologne. All experiments were carried out under daylight conditions and at temperatures between 18 and 24°C.

In experiments using a treadmill, all legs except the right front leg were amputated, and the animals were fixed dorsal side up on a foam platform using dental cement (Protemp II, ESPE). The thorax was opened to allow access for recording from mesothoracic leg nerves. The gut was moved aside, connective tissue was carefully removed to expose the mesothoracic ganglion, and the cavity was filled with saline (composition according to Weidler and Diecke 1969). Recordings were made from right side leg nerves nl2 (protractor coxae), nl5 (retractor coxae), C_1 (levator trochanteris), C_2 (depressor trochanteris), nl3 (extensor tibiae; nomenclature according to Graham 1985 and Marquardt 1940) with monopolar hook electrodes (modified after Schmitz et al. 1991). All mesothoracic nerves were crushed or cut to exclude sensory input in the mesothoracic segment. In three experiments, activity of mesothoracic flexor tibiae MNs was recorded as EMG activity. For these recordings, the main leg nerve nCr (*nervus cruris*) was left intact, and two thin copper wires were inserted into the proximal femur. To exclude local sensory signals as much as possible, the leg was firmly glued to the platform, and the receptor apodeme of the fCO was cut because its afferents share nCr with flexor efferents. An EMG from the prothoracic flexor muscle was also obtained by inserting two thin copper wires into the proximal femur. A lightweight low-friction treadmill (Gabriel et al. 2003) was positioned under the animal's right front leg to allow the unrestricted leg to perform walking like movements. A DC motor attached to the treadmill served as a tachometer for treadmill velocity. Data were analyzed with respect to the start of the front leg stance phase, the latter being defined as the time during which the treadmill was accelerated. To describe a modulation of spiking rate of mesothoracic MNs, we examined an interval of ±0.5 s around the time of the start of front leg stance. This interval was divided in bins of 33 ms, and spikes were summed in each bin for all analyzed steps of an animal. We determined the two bins representing the highest or lowest spike count prior to and during the front leg stance phase. The percentage given in RESULTS describes the difference in spike count with respect to the bin representing the higher spike count, thereby indicating the average depth of modulation.

In experiments investigating the role of CPG interaction alone, a pair of ganglia (pro- and mesothoracic or meso- and metathoracic) was removed from the animal. The ganglia were placed in a Sylgard-lined dish with two separate compartments to allow independent superfusion of each ganglion. The interganglionic connective was carefully placed in a slit connecting the compartments and sealed with petroleum jelly. In some experiments, the separation of the two compartments was verified by applying a small amount of the dye Janusgreen B (Eastman Chemical, Rochester, NY) next to the petroleum jelly barrier. While one ganglion was superfused with normal saline, the other ganglion was superfused with saline containing 5×10^{-4} M pilocarpine (Sigma, Taufkirchen, Germany). Pilocarpine is a muscarinic agonist that activates arthropod CPGs and evokes rhythmic alternating activity in antagonistic MN pools (Büschges et al. 1995). In the isolated pro- and mesothoracic ganglia, extracellular recordings were made from leg nerves nl2 (protractor coxae) and nl5 (retractor coxae) of both ganglia using monopolar hook electrodes. In the isolated meso- and metathoracic ganglia, recordings were made of nerves C_1 (levator trochanteris), C_2 (depressor trochanteris), nl2 (protractor coxae), and nl3 (extensor tibiae).

In experiments where the fCO was stimulated, the front leg walking preparation was used, but the right front leg was cut 3 mm distal of the femur-tibia joint. The fCO receptor tendon was clamped in an electromechanical stimulator (Hofmann et al. 1985). Mechanical stimulation to the fCO simulated femur-tibia joint flexion between 40 and 120°. The abdomen of the animal was touched with a paintbrush that elicited short bursts of MN activity, often including alternating bursts from antagonistic MN pools. fCO stimulation was triggered to occur during these bursts. Nerve recordings were made from the stumps of mesothoracic nerves nl2, nl5 (protractor and retractor coxae), C_1, C_2 (levator and depressor trochanteris), and nl3 (extensor tibiae). Activity of flexor tibiae MNs was recorded from fine branches of nerve nCr, which innervate the flexor tibiae muscle. In addition to recordings in the mesothoracic segment, extensor tibiae MN activity was recorded in the prothoracic segment from nerve F2. This allowed monitoring local reflex behavior during fCO stimulation (Bässler 1988). For comparison, nerve activity in an interval (1 s duration) prior to fCO stimulation was integrated. Activity was also integrated in an equally long second interval after the stimulation and divided by the first number. This provided a value describing spiking activity after fCO stimulation normalized to spiking activity. The same method was applied to data from control experiments with disconnected stimulation device. Percent values given in RESULTS refer to the difference between experimental and control values.

All data were recorded using a MICRO 1401 A/D converter and SPIKE 2 data acquisition/analysis software (versions 3.13–4.12, Cambridge Electronic Design, Cambridge, UK). Data evaluation was done using custom written scripts within the SPIKE 2 software and plotted with Grapher 4.0 (Golden Software, Golden, CO). In the text and figures, N is the number of animals and n is the sample size.

RESULTS

Activity of mesothoracic motorneurons during front leg walking

The animals walked with the right front leg on a treadmill while activity of mesothoracic MNs was recorded extracellularly. Tactile activation by touching the abdomen with a paintbrush typically elicited sequences of 8–15 consecutive

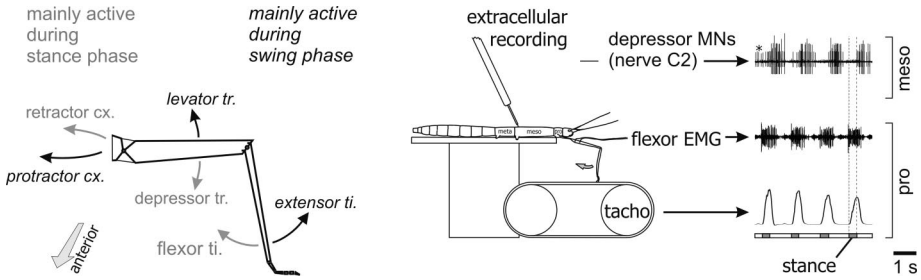

FIG. 1. Summary of the movements resulting from the activity of specific motorneuron (MN) pools is given on the *left* (cx., coxa; tr., trochanteris; ti., tibia). *Right*: schematic of the single leg walking preparation. The animal was fixed on a platform and performed walking movements with a single right front leg on a tread mill. The other 5 legs were amputated. Activity of the front leg's flexor tibiae muscle (flexor EMG), and the velocity of the treadmill (tacho) are shown for 4 consecutive steps on the *right*. A rising tachometer trace indicates treadmill acceleration that was used to define the stance phase of the leg (stance, gray boxes). Recording of mesothoracic nerve C_2 shows activity of slow (*) and fast depressor coxae MNs (spikes truncated).

steps lasting for 5–20 s. Spontaneous locomotor sequences also occasionally occurred. EMG recordings from the front leg flexor muscle monitored the stepping activity (Fig. 1). Shortly after the onset of flexor activity, the treadmill accelerated, indicated by a rising tachometer signal (1st dashed line). The onset of this rise was defined as the start of the front leg stance. Shortly before the end of the flexor burst, the leg lifted off the treadmill, and treadmill velocity decreased. The maximum treadmill velocity was defined as end of the stance phase (2nd dashed line). It is important to note that the decline in treadmill velocity after this peak does not give any information about leg movements, because during this time, the leg is lifted off the treadmill. The decline in treadmill velocity thus stems solely from the treadmill's mechanical properties. In the resting animal, mesothoracic slow MNs (small unit marked with asterisk) normally were spontaneously tonically active. When front leg walking began, activity of slow mesothoracic MNs increased and fast mesothoracic MNs (large unit) began to fire. In addition to this overall increase of firing rate, in most animals, MN activity was specifically modulated for each MN pool and correlated with front leg steps.

Protractor and retractor coxae motorneurons

Protractor coxae MNs control the forward movement of the leg and are active mainly during swing phase, while retractor coxae MNs control the backward movement and are active mainly during stance phase. During walking activity of the front leg, mesothoracic protractor MN activity was coordinately modulated in 9 of 10 animals (Fig. 2*A*). To quantify this modulation, the start of front leg stance was taken as a reference, and the number of protractor MN spikes was plotted in a time window ±0.5 s around this time. This was done for all steps of a sequence, and the plots were aligned so that each row represents one step cycle (Fig. 2*B*). This raster plot shows some variability in the firing pattern during different steps, but during all 116 steps shown in the plot, mesothoracic protractor activity is reduced during front leg stance (average duration

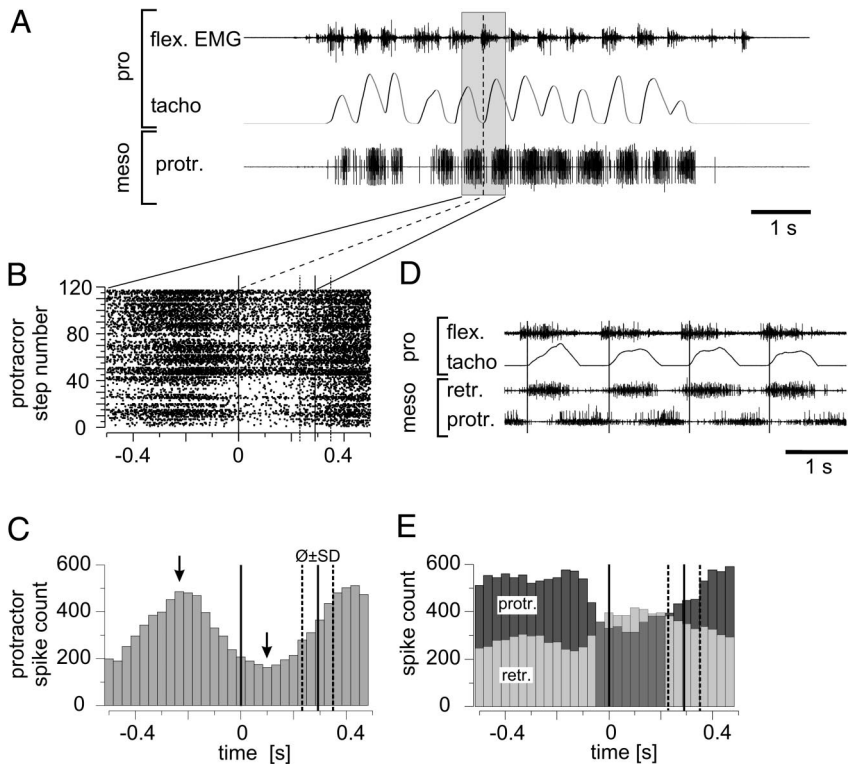

FIG. 2. *A*: mesothoracic protractor coxae MN activity was recorded from nerve nl2 (protr.) while the ipsilateral front leg performed walking movements on a treadmill. Dotted vertical line indicates start of front leg stance. Mesothoracic MN activity was analyzed in a time window of ±0.5 s around this start (shaded area). *B*: raster plot showing mesothoracic protractor activity during 116 front leg stance phases. Vertical lines mark start and average end of the stance phase. Mean spike frequency was reduced during front leg stance of all steps of the animal shown. *C*: histogram of mesothoracic protractor activity during the 116 front leg steps shown in *B* (bin width, 33 ms). Arrows mark bins with highest/lowest activity prior/during front leg stance. *D*: activity of mesothoracic retractor coxae MNs was antagonistic to that of protractor coxae MNs during front leg walking. *E*: histograms of protractor (dark) and retractor coxae (light) activity of 90 steps. Mesothoracic protractor activity decreased during front leg stance; retractor activity increased. In *B*, *C*, and *E,* average stance phase is indicated by vertical solid lines and SD by dashed lines. Difference in protractor MN activity prior to stance in *C* and *E* (protractor MN activity decreases at times ≤ −0.2 s in *C* but not in *E*) is due to the different step cycle period in the 2 animals (0.4 s for the animal in *A* and *C* and 1.4 s for the animal in *D* and *E*). Thus in the 1st animal, the decrease in protractor activity that occurs between stance phases is captured within the analysis time window, whereas in the 2nd animal, this decrease in activity occurs at times more negative than −0.5 s.

0.29 s; indicated in Fig. 2, *B* and *C,* by vertical lines). A summary of mesothoracic protractor MN activity during front leg stance is shown in the histogram (Fig. 2*C*). The bin representing lowest spike activity during front leg stance contains ~65% fewer spikes than the one representing highest spike activity prior to the stance phase (arrows in Fig. 2*C* mark the bins compared). Eight other animals showed a similar modulation of protractor MN activity during front leg walking, with depth of modulation in the range of 15–90%.

Recordings of mesothoracic retractor coxae MNs, the antagonists to protractor MNs, revealed that activity increased during front leg stance (Fig. 2*D*). Figure 2*E* shows retractor MN activity during 90 front leg steps (light bars) together with the simultaneously recorded activity of protractor MNs (dark bars). While in this animal retractor activity is ~25% higher during front leg stance, protractor activity is ~45% lower. A similar modulation of mesothoracic retractor MN activity during front leg stance was observed in all four animals tested.

Depressor and levator trochanteris motorneurons

Levator trochanteris MNs control the up movement of a leg and are active during the swing phase of a leg. The antagonistic depressor trochanteris MNs control the down movement during stance phase. We observed a front leg step correlated modulation of mesothoracic depressor MN activity in six of nine animals tested. In these six animals, depressor MN activity decreased in two animals (Fig. 3, *A* and *C*) and increased in four (Fig. 3*D*) during front leg stance. For a given animal, the modulation of depressor MNs with respect to front leg stance was similar during all steps, although the magnitude of the modulation varied from step to step (see raster plots). For instance, in Fig. 3*C2,* in steps 1–10, fast depressor MN activity was only weakly modulated, whereas in steps 20–60, it was almost completely abolished during front leg stance. The average depth of modulation ranged from 50 to 78% for slow depressor MNs and from 44 to 100% for fast depressor MNs.

In five animals, we recorded from mesothoracic levator trochanteris MNs, the antagonists to depressor MNs (Fig. 3*A*).

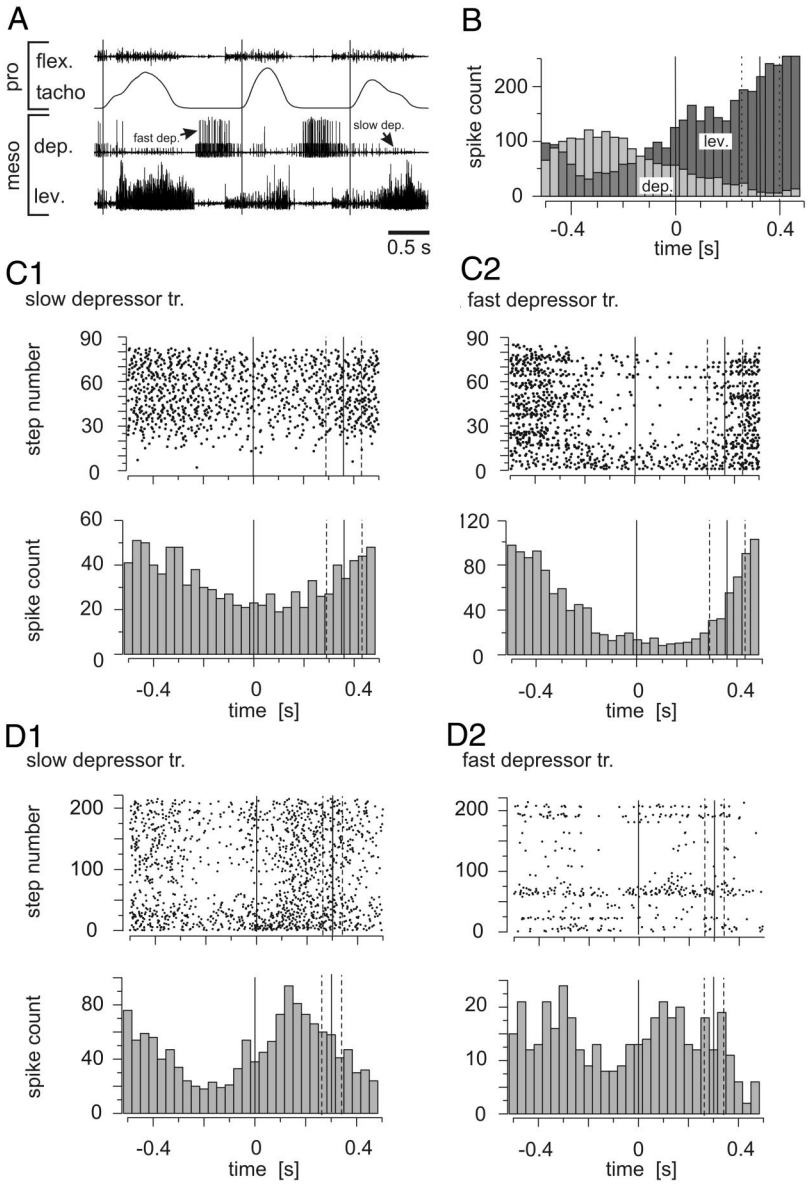

FIG. 3. *A*: mesothoracic levator and depressor trochanteris MN activity is correlated with front leg stepping. Prothoracic flexor tibiae EMG (flex.), treadmill velocity (tacho), and mesothoracic depressor (dep., nerve C_2) and levator (lev., C_1) MN activity were monitored. *B*: histogram of nerve activity shown in *A* for 31 front leg steps. In this animal, levator activity was greatest and depressor activity was least during and immediately subsequent to front leg stance. In 4 of 5 animals tested in which front leg stepping modulated levator MN activity, levator activity was always greatest during and immediately after front leg stance. *C* and *D*: phase of depressor modulation varied between animals. In 2 animals, mesothoracic slow (*C1*) and fast (*C2*) depressor MNs showed decreased activity during front leg stance (84 steps of 1 animal shown). In 4 animals, slow (*D1*) and fast (*D2*) depressor trochanteris MNs showed increased activity during front leg stance (209 steps of 1 animal shown). In *B–D,* average stance phase is indicated by vertical solid lines and SD by dashed lines.

In four of these recordings, levator MN activity was lowest with the start of front leg stance, increased during front leg stance, and reached maximum activity after the end of the stance phase (Fig. 3*B*, dark bars, shown with simultaneously recorded depressor MNs). The depth of modulation ranged 41–80%. In the fifth recording, no front leg step–correlated modulation was detected. In simultaneous recordings of depressor and levator MNs, depressor MN activity decreased during front leg stance; hence it was roughly antagonistic to levator MN activity (Fig. 3*B*).

Extensor and flexor tibiae MNs

The slow (SETi) and fast (FETi) extensor tibiae MNs and antagonistic flexor tibiae MNs control the movement of the femur-tibia ("knee") joint of the leg. Extensor MNs extend the tibia mainly during the swing phase of the leg, whereas flexor MNs flex it during the stance phase. In all four animals studied, FETi MNs showed a front leg step correlated modulation of activity. In three of four animals studied, SETi MNs showed such a modulation. Extensor MNs spike rate was lowest before front leg stance. It rapidly increased with the start of stance and reached its maximum at the end of the stance phase. SETi activity was modulated 34–50%, whereas FETi activity was modulated 64–100%.

It was difficult in this preparation to reliably identify the antagonistic flexor tibiae MNs in an extracellular recording because their axons share a nerve with those of tarsal MNs. We therefore used EMG recordings to study their activity during front leg walking. It was hence necessary to leave *nervus cruris*, which also carries afferents, intact. Through careful fixation of the middle leg stump and cutting of the fCO receptor strand, we minimized any local sensory input. In all three of these experiments, mesothoracic flexor MNs activity was modulated in phase with front leg stepping (Fig. 4*C1*). Mesothoracic flexor MN modulation was antagonistic to mesothoracic extensor MN modulation, with flexor MN spike rate being greatest before front leg stance and decreasing 90–100% during stance (Fig. 4*C2*).

In summary, all recorded mesothoracic leg MN pools showed a general increase of activity when the ipsilateral front leg performed walking movements on a treadmill. In addition, each MN pool exhibited a specific pattern of modulation correlated with the front leg cycle.

Prothoracic CPG activity and mesothoracic motorneuron activity

The information used for intersegmental coordination of movements could originate from prothoracic CPGs as well as

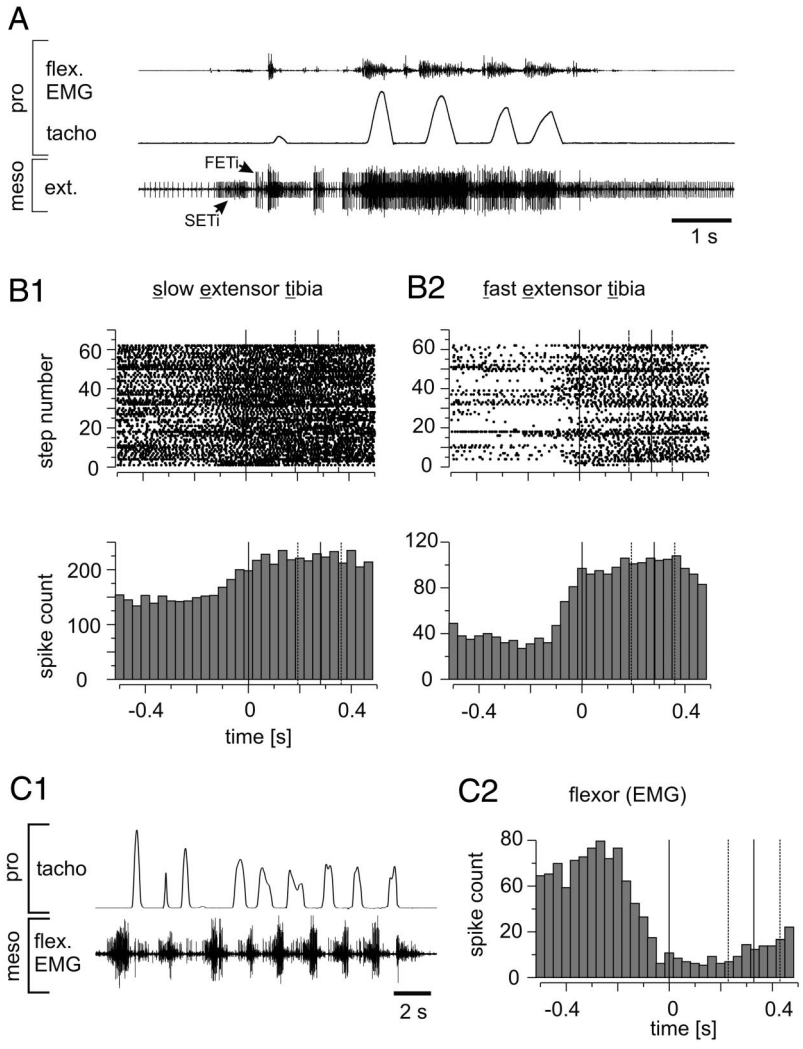

FIG. 4. *A*: recording of mesothoracic fast (FETi) and slow (SETi) extensor tibiae MN activity from nerve nl3 during walking activity of the front leg on a treadmill (tacho). SETi MN was spontaneously active prior to the walking sequence. Both SETi (*B1*) and FETi (*B2*) activity were increased during front leg stance (62 steps shown). This increase of activity outlasted the duration of front leg stance. *C1*: recording of the mesothoracic flexor tibiae muscle shows step coupled activity modulation of flexor tibiae MN activity during front leg walking. *C2*: histogram of mesothoracic flexor activity during 57 steps of a representative animal shows decreased activity during front leg stance. In *B* and *C2*, average front leg stance is indicated by vertical solid lines and SD by dashed lines.

from sense organs associated with the front leg. To examine the influence of prothoracic CPG activity on mesothoracic MN output, we isolated the pro- and mesothoracic ganglia and recorded protractor and retractor coxae activity from nerve stumps. The prothoracic ganglion was superfused with saline containing 5×10^{-4} M pilocarpine to activate locomotor CPGs and produce alternating rhythmic motor activity (Büschges et al. 1995). Unlike in previous preparations of the stick insect, which revealed no strong intersegmental coordination (Büschges et al. 1995; Ryckebusch and Laurent 1994), the adjacent mesothoracic ganglion was isolated by a petroleum jelly barrier and not exposed to pilocarpine. Slow units of both pro- and mesothoracic protractor and retractor MNs were usually spontaneously active at a constant spike rate. Shortly after pilocarpine superfusion, spike rates increased in the pharmacological activated prothoracic ganglion and, to a smaller extent, in the adjacent mesothoracic ganglion. In the prothoracic ganglion, the tonic activity subsequently became rhythmic, and in 19% of the experiments, a stable, alternating rhythm was established. In these preparations mesothoracic protractor and retractor MNs continued in all cases to fire tonically (Fig. 5; protractor: $N = 4$, retractor: $N = 3$). To detect subtle spike rate modulation, the starts of prothoracic retractor bursts were taken as a reference point, and mesothoracic MN activity was plotted as histogram in a ± 1 s interval around these times. No correlation between pro- and mesothoracic MN activity was found (Fig. 5, *B* and *C*).

We performed similar experiments with isolated pairs of meso- and metathoracic ganglia in which either the meso- or metathoracic ganglion was made rhythmically active by pilocarpine application. Rhythmic activity was monitored by recording from levator and depressor trochanteris MNs. In 58% of the experiments, a stable, alternating rhythm occurred in the ganglion to which pilocarpine had been applied. In the adjacent ganglion, we monitored the activity of protractor MNs (meso: $N = 3$, meta: $N = 3$), levator MNs (meso: $N = 2$, meta: $N = 4$), or extensor MNs (meso: $N = 5$, meta: $N = 4$). In no case was a modulation of spike rate correlated with the rhythmic activity of the MNs in the adjacent ganglion observed (data not shown).

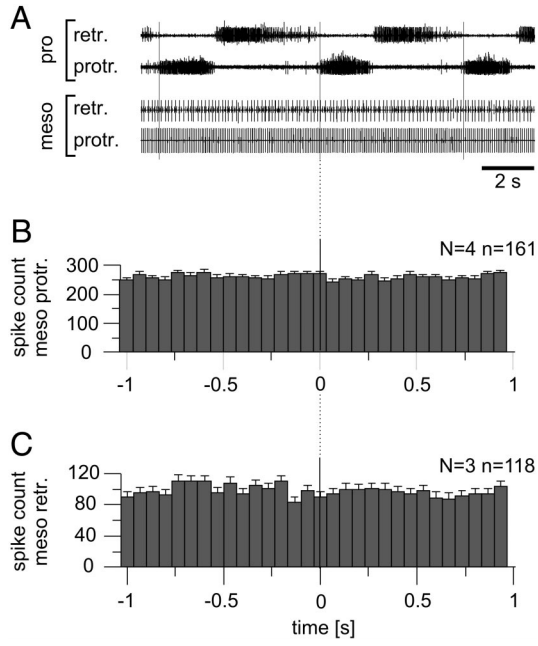

FIG. 5. *A*: prothoracic (pro) ganglion of an isolated pair of ganglia was pharmacologically activated by application of pilocarpine (5×10^{-4} M). This evoked alternating rhythmic activity of retractor (retr.) and protractor (protr.) MNs (recorded from stumps of nerves nl5 and nl2, respectively). The adjacent mesothoracic ganglion (meso) was separated by a petroleum jelly barrier and not in contact with pilocarpine. Its spontaneously active slow retractor (retr.) and protractor (protr.) MNs did not show any modulation of activity. These data were also analyzed by plotting the spike activity of mesothoracic protractor (*B*) or retractor (*C*) MNs in a time window of ± 1 s around the starts of prothoracic protractor bursts (*time 0*, vertical line); no correlated activity changes were present.

In summary, we found no evidence for influences of an activated CPG on MN activity in adjacent ganglia in isolated preparations.

Influence of front leg femoral chordotonal organ stimulation on mesothoracic motorneuron activity

Because CPG activity in an adjacent ganglion did not seem to be sufficient to provide signals for intersegmental coordination of locomotor activity, we studied intersegmental influences of a sensory organ, the fCO. The fCO senses the position and movement of the femur-tibia joint and could thereby provide signals suitable for leg coordination during walking. Its physiology and its influence on networks in the segmental ganglion are well studied (e.g., Bässler and Büschges 1998; Hofmann et al. 1985). Information about the femur-tibia joint provided by the fCO is used for the coordination with other joints of the leg. In an "active" animal, fCO signals can evoke transitions between levator and depressor trochanteris MN pools (Hess and Büschges 1999). Bucher et al. (2003) presented evidence that position signals from the fCO have access to the CPGs controlling MNs of the coxa-trochanter joint.

We used a preparation similar to the front leg walking preparation, but the right front leg was fixed and cut distal to the femur-tibia joint. The other five legs were amputated to exclude uncontrolled sensory input. Tactile stimulation of the animal often evoked alternating bursts of antagonistic MN pools in the mesothoracic ganglion (Fig. 6, *A1* and *B1*). The front leg's fCO was stimulated mechanically during such bursts to simulate femur-tibia joint flexion. When the stimulation occurred during a burst of mesothoracic protractor coxae MNs, it shortened the protractor burst and increased retractor coxae MN activity (Fig. 6*A1*). As the effect of the stimulus was often masked by the naturally varying protractor burst lengths, we compared the pooled data with data from a control situation in which the stimulus device was unplugged. Prothoracic fCO stimulation during a mesothoracic protractor MN burst reduced protractor MN activity by 35% and increased retractor MN activity by 61% (Fig. 6*A2*; protractor: $N = 11$/control: 9, $n = 161$/control: 148; retractor: $N = 9/5$, $n = 112/59$). A similar effect was observed when the stimulus was applied during extensor tibiae MN bursts (Fig. 6*B1*). Although extensor activity did not change significantly, activity of flexor tibiae MNs increased by 94% (Fig. 6*B2*; flexor: $N = 8/4$ $n = 88/76$; extensor: $N = 7/6$, $n = 146/101$, extensor data not shown). No influence of prothoracic fCO stimulation was found on mesothoracic depressor and levator trochanteris MNs (depressor: $N = 7/5$, $n = 143/81$; levator: $N = 11/3$, $n = 200/59$, data not shown).

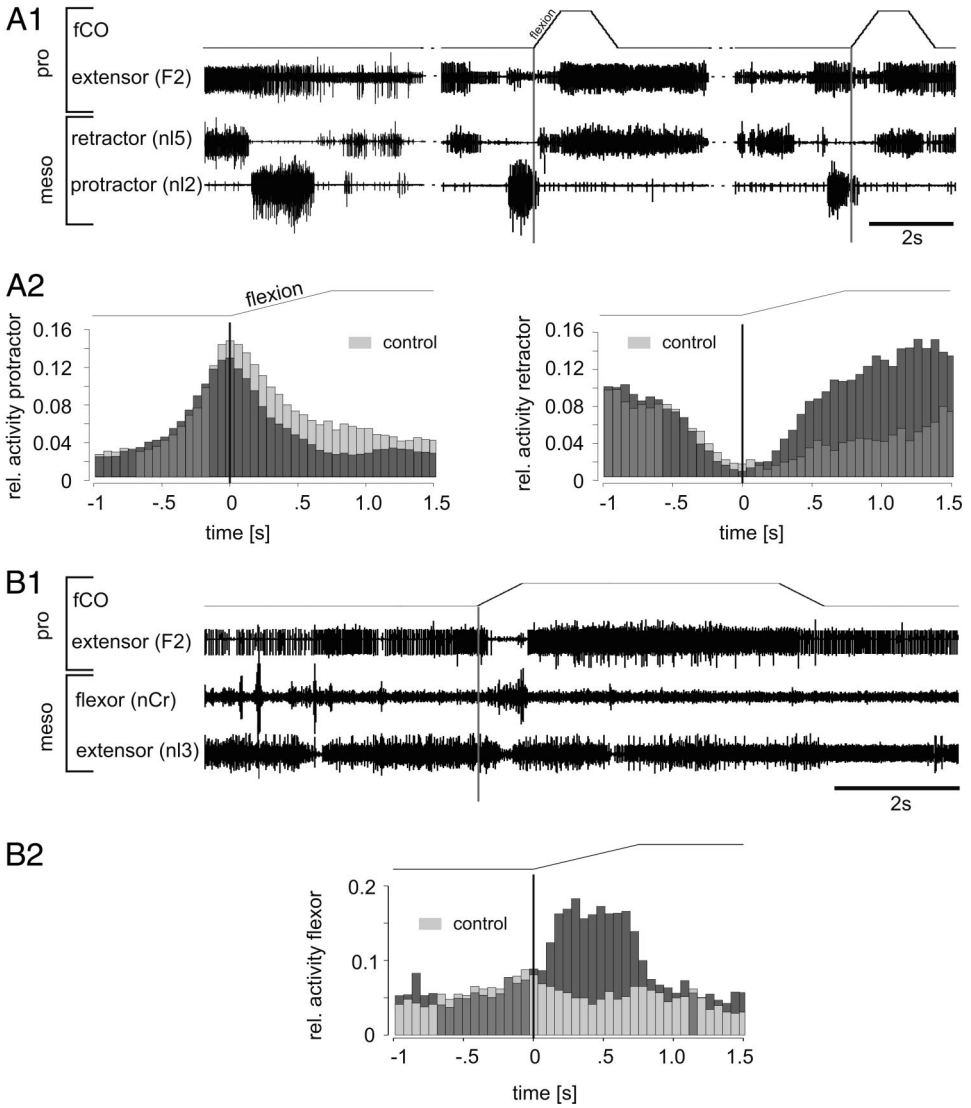

FIG. 6. *A1*: front leg chordotonal organ (fCO) was stimulated, simulating a flexion of the femur-tibia joint (rising ramp). Local reflex activity of extensor tibiae MNs (recorded from nerve F2) was monitored, and lack of a resistance reflex suggests an active state of the animal. Shortly after the onset of stimulus (vertical lines), a switch from protractor to retractor coxae MN activity occurred in the deafferented mesothoracic ganglion. *A2*: stimulation during a mesothoracic protractor burst (*left*, dark bars, $N = 11$, $n = 161$) decreased protractor activity compared with the control situation, in which the stimulation device was unplugged (*left*, light bars, $N = 9$, $n = 148$). When the stimulation occurred during a retractor burst, it increased retractor activity (*right*, dark bars, $N = 9$, $n = 112$) compared with control (*right*, light bars, $N = 5$, $n = 59$). *B1*: similarly prothoracic fCO stimulation had an effect on mesothoracic flexor tibiae MN activity. *B2*: stimulation during a flexor burst strongly increased flexor activity (*right*, dark bars, $N = 8$, $n = 88$) compared with control (*right*, light bars, $N = 4$, $n = 76$).

fCO stimulation elicits either a local resistance or assistance reflex, depending on the state of the animal ("active reaction") (Bässler 1988; Bässler and Büschges 1998). The transmission of intersegmental information could thus depend on the state of the animal, reflected by the occurrence or absence of a resistance reflex. We therefore analyzed the data separately with respect to the occurrence (or absence) of a local resistance reflex in the prothoracic segment. No correlation between the occurrence (or absence) of a prothoracic resistance reflex and the strength of effects on mesothoracic MNs was detected.

In summary, signals from the prothoracic fCO, indicating a flexion of the front leg, promote mesothoracic retractor coxae and flexor tibiae activity, while they repress protractor coxae activity.

DISCUSSION

In the front leg walking preparation, ipsilateral mesothoracic MN firing rate increased when stepping began. Furthermore, the firing rate was modulated in phase with individual front leg steps: mesothoracic retractor coxae, levator trochanteris, and extensor tibiae MN activity increased during front leg stance, while the activity of the antagonistic protractor coxae, depressor trochanteris (in two experiments), and flexor tibiae MNs decreased (Fig. 7*A*). Modulation of depressor trochanteris MN activity was variable in that in four other experiments MN activity increased during front leg stance (depressor tr. II in Fig. 7*A*). During front leg stance, mesothoracic MN activity increased or decreased—multiple activity bursts, as present after amputation of a middle leg in cockroaches (Delcomyn 1991b), were not observed. Since the mesothoracic ganglion was deafferented, these spike rate modulations reflect influences of intersegmental signals from the prothoracic ganglion on the mesothoracic ganglion. These data thus strongly suggest that these interganglionic signals help coordinate leg movements during walking.

Variability of intersegmental modulation of mesothoracic motorneuron activity

Although the modulation seen in each mesothoracic MN pool was similar in all animals studied, step-to-step variability was observed in individual animals. For example, mesothoracic protractor MNs showed reduced activity during front leg

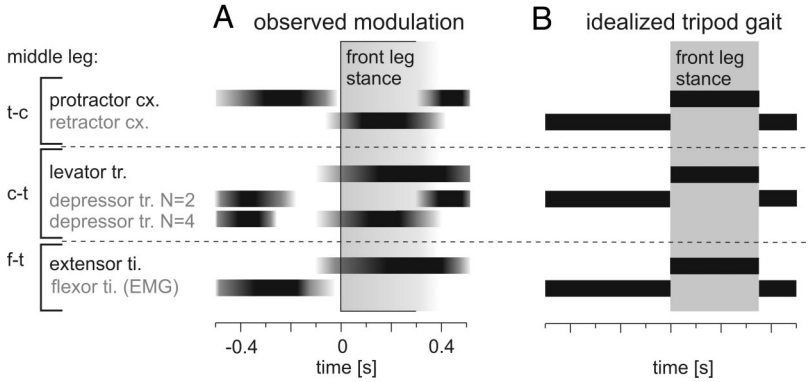

FIG. 7. *A*: schematic summary of observed modulation of mesothoracic MN activity during front leg walking. Black bars represent times of increased activity of MNs controlling the thoraco-coxa (t-c), coxa-trochanter (t-c), and femur-tibia (f-t) leg joint. Gray shaded area indicates front leg stance. *B*: schematic summary of mesothoracic MN activity that would be expected for an idealized tripod gait.

stance in nine animals. The variation seen between steps included variations in underlying firing rate (e.g., Fig. 2*B,* steps 1–40 compared with 40–70) and in modulation depth (e.g., Fig. 3*C2,* steps 1–10 compared with 20–60). This variation was still observed with other reference points in the walking cycle (e.g., stance end, peak front leg flexor activity) and thus is not due to using the front leg stance as reference to analyze mesothoracic MN activity. Similarly, this step to step variation also remained when the data were re-analyzed with respect to phase within in the step cycle (i.e., dividing all burst durations and delays by step cycle period). The source of this variation is unknown but is comparable to that observed in animals walking in similarly regular environments, e.g., on treadmills (e.g., Fischer et al. 2001; Foth and Bässler 1985; Gabriel et al. 2003; Graham 1985; Graham and Wendler 1981). Interestingly, in *Aplysia* feeding under conditions in which environmental variation was also deliberately reduced as much as possible, wide variation in bite parameters was again observed (Horn et al. 2004). These authors ascribed this variation to an inherent variability in CPG activity and suggested that such inherent variability is functionally advantageous in dealing with a varying environment (in this case, different foodstuffs). Whether the variations observed in stick insect walking arises, at least in part, from an inherent variability in the system is unknown, but natural substrates do show wide variation, and it is thus possible that the variation we have observed in our preparations arises from inherent system properties. Regardless, despite this variability in individual step activity, the modulation in mesothoracic MN activity induced by front leg stepping was nonetheless present in all steps.

Comparison with the step pattern of an intact animal

Stick insect walking (Graham 1985) and searching (Dürr 2001) movements are generally described with respect to leg movement; relatively little data are available on MN activity. This distinction is important because of a significant difference between the timing of MN activity and leg movement (Watson and Ritzmann 1998). For example, the delay between the onset of flexor EMG activity and treadmill movement in our experiments was typically 100 ± 50 ms—similar to the delay of 126 ± 151 ms reported by Gabriel et al. (2003). Unfortunately, the magnitude of delays is not known for all MN types, and it is therefore difficult to predict MN activity from recordings of whole leg movement. Prior work that examined MN activity directly was performed in preparations in which protraction and retraction were prevented (Fischer et al. 2001; Schmidt et al. 2001), and thus these data also cannot be compared with those presented here. Furthermore, MN coordination seems to be strongly influenced by treadmill mechanical properties (friction, inertia) (Gabriel et al. 2003), and comparison of data using different treadmills is thus also problematic.

As a result of these difficulties and of the variety of step patterns that result in a tetrapod gait, we therefore chose to instead compare our data to an "idealized" tripod gate in which protractor, levator, and extensor MNs are active throughout the stance phase and a 180° phase shift exists between neighboring legs (Fig. 7*B*). A similar step pattern was observed under conditions of reduced proprioceptive feedback such as walking on a mercury surface ("gait I"; Graham and Cruse 1981). Nevertheless, it should be kept in mind that the tripod gait is only one of several step patterns shown by stick insects (Graham 1972, 1985; Wendler 1964). For an idealized tripod gait, it would be predicted that middle leg protractor, levator, and extensor MN activity should be greatest, and retractor, depressor, and flexor activity least, during front leg stance. These predictions are borne out for our extensor/flexor and levator data (Fig. 7*B*). With respect to the protractor/retractor MNs (although they do correctly fire in antagonism with each other), relative to front leg stance, their activity is almost in anti-phase to that predicted and would thus produce not forward, but backward, movement of the middle leg during its swing phase. With respect to the depressor MNs, in two of nine animals, the observed modulation was consistent with that predicted, but in the other four animals that showed modulation, the activity was again significantly out of phase with that predicted and would incorrectly depress the leg during its swing phase. Clearly, these protractor/retractor and (in the 4 animals) depressor MN activities are inconsistent with functional middle leg movement. This is true not only for a tripod gait, but also if a tetrapod gait would be chosen for comparison.

An explanation for the disparity is that, in the intact animal, local proprioceptive input as well as afferent signals from the contralateral front and the middle and hind legs results in appropriate phase shifts of protractor/retractor and depressor MN activities. The MN pools controlling the most proximal leg joint, protractor coxae, and retractor coxae MNs seem to be in-phase with those in the prothoracic segment. Such in-phase coordination has been described for front legs of decapod crustaceans, while the body weight is supported by only the back legs and the front legs are lifted above ground (Duysens et al. 2000). Similarly, leg stumps of rock lobsters show in-phase coordination after leg autotomy (Clarac 1981). Clarac (1981) suggested that peripheral proprioceptive inputs could

shift this in-phase coordination toward the alternating pattern observed in the intact walking animal.

Amputation experiments with cockroaches led to the idea that "sensory input helps keep the legs synchronized with one another" (Delcomyn 1991a). In support of this hypothesis, local sensory signals [e.g., from the fCO (reviewed in Bässler and Büschges 1998) and the campaniform sensilla (Akay et al. 2004; Duysens et al. 2000; Ridgel et al. 2000)] are known to have an important function in the local control of leg movements. Further evidence of the importance of local sensory information in leg coordination comes from experiments by Graham (1985), in which he moved the coxal stump of an amputated middle leg while a stick insect walked on a double tread wheel. With the stump held still, retractor MNs of one leg showed weak rhythmic activity in a fixed phase relation to the activity of retractor MNs controlling other legs. When proximal sensory organs were stimulated by moving the stump, retractor MN activity was observed only if the movement occurred in a certain phase window with respect to the movements of the legs in front and behind it. This shows that local sensory input, which is not present in our single leg walking preparation, modifies motor output and contributes to the formation of the stepping pattern seen in a freely walking animal.

The experimental conditions (reduced sensory input) could induce searching in the mesothoracic segment. Searching movements are for example observed if the middle leg of a walking stick insect steps into a gap and thereby looses mechanical feedback (Dürr 2001). Searching movements are stereotypic, loop-like leg movements with simultaneous protraction, depression, and flexion (Dürr 2001). We observed roughly simultaneous activity of mesothoracic protractor, depressor (2 of 6 experiments), and flexor MNs (Fig. 7*A*). Although it is not possible to predict accurate leg movement from this MN activity, we do not want to exclude the possibility that the observed MN activity, in part, reflects searching movements.

Relevance of central coordinating mechanisms

MN activity that, in the absence of sensory input, is nonetheless relatively similar to that observed in intact animals (fictive motor patterns) can be induced by appropriate treatment in several multi-CPG systems (crayfish swimmeret system by application of the neuropeptide proctolin (Ikeda and Wiersma 1964; Mulloney et al. 1987), leech swimming by brief electrical stimulation of lateral nerve roots (Kristan and Calabrese 1976), and swimming in lamprey and tadpole by application of *N*-methyl-D-aspartate (NMDA) (Grillner and Wallén 2002; Skinner and Mulloney 1998). These data indicate that, although sensory signals play a role in modifying system output as necessary to maintain function, in several systems, the basic coordination among the different CPGs is to a large extent maintained by central mechanisms (Friesen and Chang 2001).

With respect specifically to walking, muscarinic agonists (pilocarpine or oxotremorine) induce "fictive locomotion" with slow cycle periods but basically correct leg coordination in crayfish (Chrachri and Clarac 1990). However, in stick insects, although pilocarpine application does induce alternating rhythmic activity of antagonistic motor pools, the activity of the MN pools controlling different leg joints is not correctly coordinated (Büschges 1995). In the work presented here, when CPG activity was pharmacologically induced in one ganglion of an isolated pair, no evidence that this activity influenced spontaneous activity of leg MNs of the neighboring ganglion was found. Similarly, previous work on ganglion chains in stick insects and locusts also found no evidence for a strong inter-CPG coupling when rhythmic activity was induced in the complete chain of ganglia, although a weak intersegmental coordinating influence could have been masked by strong local activity in these preparations (Büschges et al. 1995; Ryckebusch and Laurent 1994). It is, of course, possible that this lack of inter-joint and inter-leg coordination is due to incomplete activation of the neural networks underlying walking, and that in real walking, as observed in the swimmeret and leech, lamprey, and tadpole CPG systems, central connections mediate intersegmental coordination (Hill et al. 2003). Nonetheless, our data and the prior work mentioned above are also consistent with the alternative hypothesis that direct coupling of segmental CPGs is not involved in intersegmental coordination in stick insect walking.

It is important to note, however, that this lack of apparent central coordinating mediation is not present in all insects, because Johnston and Levine (2002) found coordinated, tripod-like activity of leg MNs in all three thoracic ganglia in pilocarpine-activated, isolated nerve cords in *Manduca sexta*. Although normal walking in the animals displays a more complex gait (Johnston and Levine 1996), these data nonetheless suggest that, in *Manduca,* central coupling at least partially underlies intersegmental coordination. This difference between *Manduca* and stick insects may be related to the different primary forms of locomotion the two animals use—*Manduca* mostly flies, whereas stick insects walk and climb. A more flexible control of walking, with a stronger influence of afferent feedback to better match walking movements with substrate variation, might therefore be advantageous for stick insects.

Relevance of sensory signals for intersegmental coordination

Early evidence for a role of sensory information in stick insect leg coordination came from experiments in which middle leg autotomy changed phase relations of the remaining legs (Wendler 1964). We show here an intersegmental effect of fCO afferents. This stimulus mimics flexion, which would occur at the start of the front leg stance phase during walking. The stimulus decreased mesothoracic protractor MN activity and increased mesothoracic retractor MN activity, which parallel the observations from the single leg walking preparation. Intersegmental fCO signals could therefore be at least partially responsible for the observed modulation of protractor and retractor MN activity. In contrast, prothroracic fCO stimulation caused an increase of flexor MN activity. This observation is contradictory to the decrease of flexor MN activity during front leg stance observed in the single leg walking preparation. Similarly, no evidence for intersegmental fCO modulation of mesothoracic extensor, levator, and depressor MN activity was found, although their activity was modified in the single leg preparation. These data thus suggest that, although fCO activity plays a role in intersegmental coordination, the combined activity of multiple front leg afferent pathways [e.g., signals

from the fCO and campaniform sensilla (Akay et al. 2001) or hair fields (Wendler 1964)] are required for correct inter-leg coordination.

fCO stimulation can locally (i.e., in the same segment) induce either an assistance or a resistance reflex, with the assistance reflex being commonly associated with the system being in an "active" state (Bässler 1988). It was therefore possible that the effect of fCO stimulation on mesothoracic MNs would vary depending on whether the system was in this active state or not. We tested this by simultaneously observing the effect of fCO stimulation on prothoracic and mesothoracic MN activity, and found no correlation between the mesothoracic effects and whether an assistance or resistance reflex was induced in the prothoracic ganglion. These data thus suggest that the changes in local networks that underlie the "active" state do not gate or otherwise alter transmission in fCO intersegmental pathways.

In summary, we show that, in a preparation with a single walking front leg, intersegmental signals modulate MN activity in the adjacent deafferented segment. Experiments with completely isolated nervous systems indicate that these effects are unlikely to be due to central connections. The prothoracic fCO was shown to be an intersegmental input that could contribute to the observed MN modulation. These data suggest that inter-leg coordination in the stick insect is primarily due to intersegmental pathways whose activity is altered by afferent input.

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