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J Neurophysiol 98:1685-1696, 2007. First published 27 June 2007; doi:10.1152/jn.00291.2007

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Intersegmental Coordination: Influence of a Single Walking Leg on the Neighboring Segments in the Stick Insect Walking System

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Submitted 15 March 2007; accepted in final form 17 May 2007

Borgmann A, Scharstein H, Büschges A. Intersegmental coordination: influence of a single walking leg on the neighboring segments in the stick insect walking system. *J Neurophysiol* 98: 1685–1696, 2007. First published June 27, 2007; doi:10.1152/jn.00291.2007. A key element of walking is the coordinated interplay of multiple limbs to achieve a stable locomotor pattern that is adapted to the environment. We investigated intersegmental coordination of walking in the stick insect, *Carausius morosus* by examining the influence a single stepping leg has on the motoneural activity of the other hemiganglia, and whether this influence changes with the walking direction. We used a reduced single leg walking preparation with only one intact front, middle, or hind leg. The intact leg performed stepping movements on a treadmill, thus providing intersegmental signals about its stepping to the other hemiganglia. The activity of coxal motoneurons was simultaneously recorded extracellularly in all other segments. Stepping sequences of any given single leg in either walking direction were accompanied by an increase in coxal motoneuron (MN) activity of all other segments, which was mostly modulated and slightly in phase with stance of the walking leg. In addition, forward stepping of the front leg and, to a lesser extent, backward stepping of the hind leg elicited alternating activity in mesothoracic coxal MNs. Forward and backward stepping of the middle leg did not elicit alternating activity in coxal MNs in any other hemiganglia, indicating that the influence of middle leg stepping is qualitatively different from that of forward front and backward hind leg stepping. Our results indicate that in an insect walking system individual segments differ with respect to their intersegmental influences and thus cannot be treated as similar within the chain of segmental walking pattern generators. Consequences for the current concepts on intersegmental coordination are discussed.

INTRODUCTION

Locomotion requires coordinated interplay of all involved body parts to achieve a stable and adapted behavior. This coordination depends on intra- and intersegmental coupling (Büschges 2005; Cruse 1990; Grillner 1981; Orlovsky et al. 1999), of which intersegmental coordination is particularly important to ensure the operation of the locomotor system. In legged animals, such as cat, crayfish, cockroach, or stick insect, it adjusts the stepping cycles of the individual legs coordinating leg movements to allow the expression of different gaits and allow walking through irregular environments (e.g., Cruse 1990; Delcomyn 1989; Dürr et al. 2004). The mechanisms underlying intersegmental coordination range from purely central interactions of neurons in the nervous system to sensory-driven interactions between the segments of a locomotor system. The role of the CNS has been well studied in swimming [crayfish (swimmerets): Paul and Mulloney 1986; Tshuluun et

al. 2001; leech: Weeks 1981; lamprey: Cohen and Wallen 1980; Grillner et al. 1981a,b; tadpole: Dale and Roberts 1984; Tunstall and Roberts, 1994], whereas sensory feedback has been best investigated in terrestrial locomotion [locust: Macmillan and Kien 1983; crayfish: Cattaert et al. 2001; Clarac 1985 (review); cat: Andersson and Grillner 1983; Andersson et al. 1978; Conway et al. 1987; Grillner and Zangger 1975; Lam and Pearson 2002 (review); Rossignol et al. 1981; Shik et al. 1966; Wetzell et al. 1976].

The sensory-motor control of walking has been well investigated in the stick insect. Each of the six legs is driven by its own pattern generator for walking (e.g., Foth and Bässler 1985a,b), which can be subdivided into at least three central pattern generators (CPGs)—one for each of the major leg joints (Bässler and Wegener 1983; Büschges et al. 1995). Intrasegmental coordination has been fairly well studied and relies heavily on sensory feedback that couples the activities of each leg's three joints (reviews in Bässler and Büschges 1998; Büschges 2005). In contrast, little is known about the neural mechanisms underlying intersegmental coordination among the individual legs. It is known that intersegmental coordination cannot be achieved by mechanical coupling (Epstein and Graham 1983; Graham and Cruse 1981) and that neural information exchange between ganglia is essential to generate a coordinated walking pattern (e.g., Dean 1989; von Buddenbrock 1921). Behavioral experiments have led to the proposal of six rules that phenomenologically predict the interactions among the different legs (Bässler 1979; Cruse 1979, 1985, 1995; Cruse and Schwartz 1988; Dean and Wendler 1983; Graham 1979a,b). These rules mainly serve to reestablish the stepping pattern in case of disturbances and it is suggested that these rules act similarly for all legs (Cruse 1995). It remains unclear, however, whether these behavioral rules apply to the ganglia of all legs interchanging similar information with each other or whether leg-specific differences in information transfer exist. One way to resolve this issue might be the investigation of how single leg stepping affects the other legs. Ludwar et al. (2005a) first addressed this issue by investigating the influence of a single stepping front leg on the ipsilateral middle leg. Front leg stepping induced alternating activity in antagonistic motoneuron (MN) pools of the ipsilateral mesothoracic hemiganglion with a clear coupling to front leg activity. However, for some motoneuron pools the coupling observed was variable, e.g., for depressor motoneurons (Ludwar et al. 2005a). Results on contralateral flexor motoneurons support this observation (Ludwar et al. 2005b). The results indicate that

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marked modulating influences from each walking leg act on its neighbors, as previously suggested by the coordination rules. We here extended this work by investigating what influence single front, middle, and hind leg forward and backward stepping has on motoneuronal activity in the other hemiganglia. We report that single leg stepping in either walking direction is associated with tonic activation in coxal motoneuron pools of all other segmental ganglia and that exclusively forward front leg and backward hind leg stepping alone are associated with alternating motoneuron activity in the ipsilateral mesothoracic ganglion.

METHODS

Experiments were conducted on adult, female Indian stick insects (*Carausius morosus*; Brunner 1907) 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.

We used a semi-intact preparation with one intact front, middle, or hind leg walking on a passive treadmill (Bässler 1993; Gabriel et al. 2003). All other legs were amputated at the middle of the coxa and the animal was fixed dorsal side up on a foam platform with dental cement (Protamp II, ESPE, Seefeld, Germany). The thorax was opened, the gut moved aside, and connective tissue was carefully removed to expose the various hemiganglia and their leg nerves for extracellular recording. The body cavity was filled with saline (composition according to Weidler and Diecke 1969). Extracellular recordings were made from leg nerves n12, which includes protractor coxae motoneurons (MNs), and n15, which includes retractor coxae MNs (nomenclature according to Graham 1985; Marquardt 1940), with monopolar hook electrodes (modified after Schmitz et al. 1991). The segmental ganglion from which leg nerves were recorded was completely deafferented by cutting or crushing the lateral nerves. Stepping sequences were elicited by gently touching the abdomen with a paintbrush. The paintbrush was removed as soon as the animal started a sequence of stepping movements.

The intact leg walked on a lightweight, low-friction treadmill (Gabriel et al. 2003). A DC motor attached to the treadmill served as a tachometer for treadmill velocity. In some experiments the activity of the stepping leg's flexor muscle was recorded as an EMG by inserting two thin (40- μ m) copper wires into the proximal femur. The animal accelerated the treadmill during the stance phase. Treadmill velocity therefore indicates stance phase. The start of the velocity increase was defined as stance beginning and the last maximum in the velocity trace before velocity decreased to zero was defined as stance end (Fig. 1).

Data were first analyzed with respect to walking leg step cycle, defined by the start of one stance phase to the start of the next, using circular statistics. Phase histograms were used to show the distribution of motoneuronal activity in the step cycle. Polar plots show the mean vectors of activity in the step cycle for each experiment. Those vectors that had significant lengths are marked with a star (Rayleigh test; Batschelet 1981). The vector length from most experiments was highly significant due to the high number of spikes. For the overall mean vector of all experiments no test of significance was done due to the varying number of spikes and steps in the experiments. A cross-correlation between protractor and retractor MN activities was done for the complete recording time including the time between stepping sequences. Protractor and retractor MNs are tonically active only between stepping sequences (Büschges and Schmitz 1991; Graham and Wendler 1981). The cross-correlation function therefore mirrors the episodic occurrence of the stepping sequences and, if it exists, a periodic coupling between protractor and retractor MN activities.

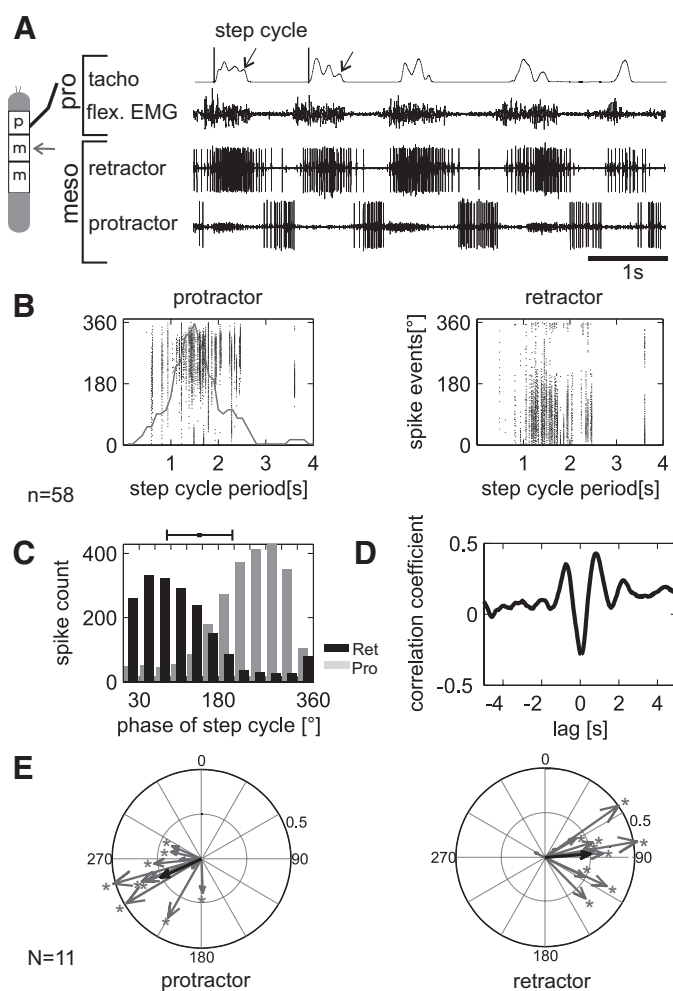


FIG. 1. *A*: mesothoracic retractor (Ret) and protractor (Pro) coxae motoneuron (MN) activities recorded from nerves n15 (Ret) and n12 (Pro) while the ipsilateral front leg performed walking movements on a passive treadmill. Front leg flexor electromyograph (EMG) and treadmill velocity were monitored. Beginning of stance is marked by the vertical solid line; end of stance is marked for 2 steps by black arrows. *B*: phase of protractor (*left*) and retractor (*right*) spikes in front leg step cycle (beginning of stance to beginning of next stance) plotted against step cycle period. Curve (*left plot*) shows the frequency of steps over the step cycle period. *C*, histogram: distribution of protractor (gray) and retractor (black) MN activities in front leg step cycle for 58 steps. Black square at *top* marks average end of stance phase; error bars are mean angular deviation. Protractor MN activity had a maximum between 270 and 300°. Retractor MN activity had a maximum during front leg stance phase between 30 and 60°. *D*: cross-correlation function showing alternating coupling between protractor and retractor MN activities. Similar coupling was observed in 9 of 11 experiments. *E*: polar plots of protractor MN activity (*left*) and retractor MN activity (*right*) in front leg step cycle for 11 experiments (gray arrows) and mean vector of all experiments (black arrows) (radius of cycle = 0.5). Each vector points in direction of the mean phase of spike activity in front leg step cycle. Consistently for all experiments protractor MN activity had an overall mean phase of about 246° (182–315°) and retractor MN activity of about 86° (58–135°). Stars mark significant vectors.

Circular statistics and cross-correlation analysis should answer two general questions. First, is there an influence of single leg stepping on motoneuronal activity in the other ganglia that can be related to the walking leg's step cycle? Second, are protractor and retractor MN activities coupled? One could expect in general different grades of influence. Stepping could induce a generalized activity increase in coxal MNs without any relationship to the steps. It could also lead to a generalized activity increase in coxal MNs and a phasic modulation with the steps or there could be clear bursting elicited by the steps.

Data were recorded using a Micro 1401 A/D converter and Spike2 data acquisition/analysis software (versions 3.13–4.12, Cambridge Electronic Design, Cambridge, UK). Data evaluation was done using custom-written scripts in Spike2 software and MATLAB 7.0 (The MathWorks, Natick, MA). In the text and figures, *N* is the number of experimental animals and *n* is the number of steps.

RESULTS

The animals walked with an intact front, middle, or hind leg on a passive treadmill while the activities of protractor and retractor coxae MNs were recorded in other hemisegments extracellularly. During forward walking protractor coxae MNs induce forward movement of the leg and are active mainly during swing, whereas retractor coxae MNs induce backward movement and are active mainly during stance (Graham 1985; Graham and Wendler 1981). The recordings (see, e.g., Fig. 2) show that in the resting animal in nerves nl2 and nl5 of all hemisegments small units were tonically active (Büschges and Schmitz 1991; Graham and Wendler 1981). With the beginning of a stepping sequence activity in both nerves increased (data not specifically shown but see Figs. 2 and 5). Shortly after the end of stepping the neural activity decreased to the low level of tonic activity of a few small units that was also present before the stepping sequence. We investigated the influence of front, middle, and hind leg stepping on motoneural activity of all other hemiganglia.

Influence of single front leg stepping

Past work showed single front leg stepping was associated with a general increase of activity in all mesothoracic leg MN pools (Ludwar et al. 2005a,b), with protractor MN activity decreasing and retractor MN activity increasing, during stance (Ludwar et al. 2005a). We extended this work by determining whether the occurrence of protractor and retractor MN activities in the front leg step cycle are phase or time dependent and whether a systematic coupling between protractor and retractor MN activities exists. Data and analysis are shown for one representative experiment (Fig. 1, A–D). Data have not been pooled.

Extracellular recordings from mesothoracic lateral nerves nl2 and nl5 containing protractor and retractor MNs showed alternating activity during front leg stepping (Fig. 1A). Plots of protractor and retractor MN spike phase against step cycle period (Fig. 1B) showed that, regardless of step cycle period, the protractor and retractor MNs were active in preferred phases of the front leg step cycle, as indicated by the broad dense horizontal bands in the plots. Protractor MN spikes occurred primarily between 180 and 360° of front leg step cycle and retractor MN spikes between 0 and 180°, independent of the actual step cycle period. The solid curve in the left plot (Fig. 1B) shows the frequency of steps over the step cycle periods. The curve was smoothed by a moving average. Most

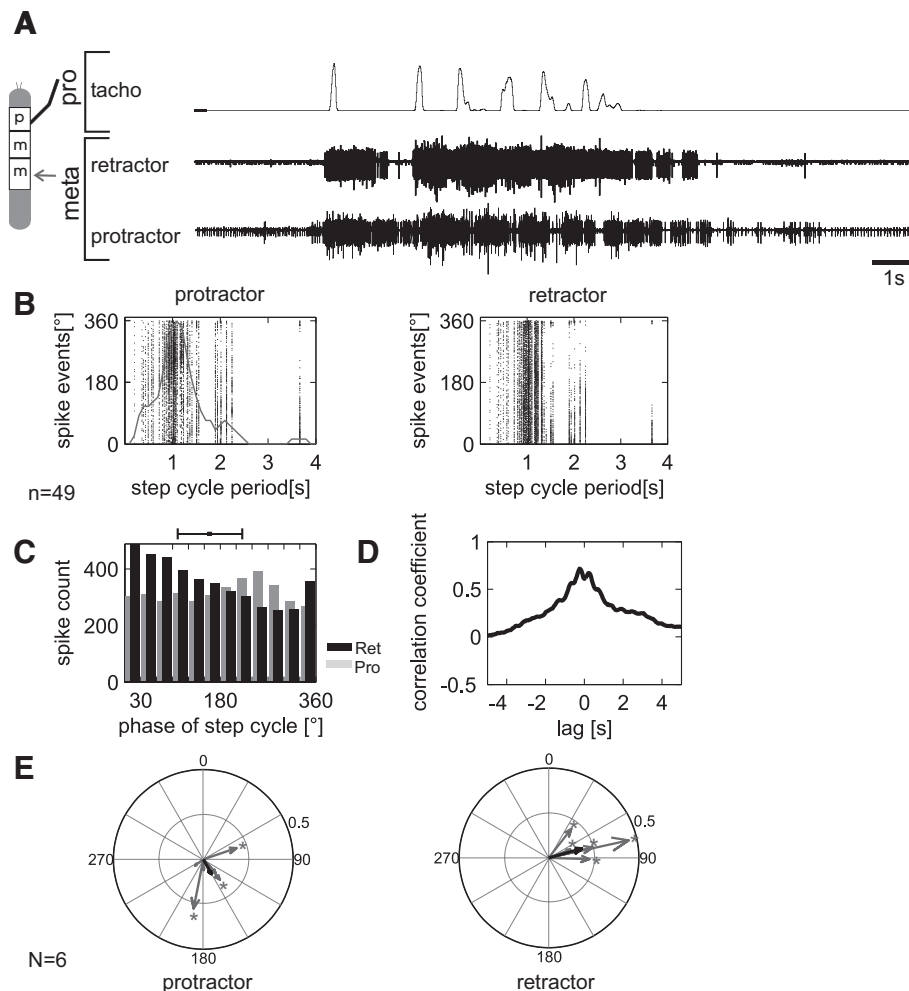


FIG. 2. A: mesothoracic protractor and retractor MN activities during front leg stepping. No clear bursts are visible but a modulation of the MN activities. B: phase of protractor (left) and retractor (right) spikes in front leg step cycle plotted against step cycle period. No obvious phase or time dependence of MN activity on front leg step cycle is present. Curve (left plot) shows the frequency of steps over the step cycle period. C: distribution of mesothoracic protractor and retractor MN activities in front leg step cycle for 49 steps. Retractor MN activity had a maximum between 0 and 30°. Protractor MN activity had a maximum between 240 and 270°. D: cross-correlation function is dominated by a broad peak around zero, indicating that activity in both MN pools increased together during front leg stepping sequences. No clear oscillation can be seen in the cross-correlation function, indicating that protractor and retractor MN activities were not permanently and systematically phase coupled with a constant phase shift. E: polar plots of mean phases of protractor (left) and retractor (right) MN activities in front leg step cycle for 6 experiments (gray arrows) and mean vector of all experiments (black arrows). Mean phases of protractor MN activity were variable between the experiments with an overall mean phase of 151° (71–240°). Retractor MN activity had an overall mean phase at 74° (38–92°) of front leg step cycle. Stars mark significant vectors.

of the steps had a step cycle period about 1.5 s. The histogram gives the distribution of protractor and retractor MN spikes in front leg step cycle phase for 58 steps of one animal (Fig. 1C). Retractor MN activity was maximal between 30 and 60° of front leg step cycle and sharply decreased at phase angles >180°. Protractor MN activity increased at 180° of step cycle and was maximal between 270 and 300°. The mean end of stance was 146° of step cycle, as indicated by the black square (error bars are mean angular deviation) at the top of the histogram. Retractor MNs were thus active primarily during front leg stance and protractor MNs during front leg swing.

To investigate whether a systematic relationship between protractor and retractor MN activities existed, activities of protractor and retractor MNs were cross-correlated (Fig. 1D). In the cross-correlation function a clear oscillation is visible with a maximal negative correlation at 0-s time shift, indicating an alternating coupling between protractor and retractor MN activities. Although this analysis is independent of step cycle phasing, this alternating coupling is not surprising considering the phase dependence of protractor and retractor MN activities on front leg steps and the fact that the distribution of step cycle periods had a relatively sharp peak at 1.5 s. Nevertheless, this is important for comparison with the following data. Coupling with 180° phase shift was observed in nine of 11 experiments. Figure 1E shows polar plots of the mean vectors of protractor and retractor MN activities in front leg step cycle for all 11 experiments ($N = 11$; $31 < n < 223$; mean: 76). The vectors point to the mean phase of spike occurrence in the front leg step cycle. The length of the mean vector of a unimodal distribution is an indicator for the variance of the data points (here, spikes). The plots summarize the data from the experiments not shown in detail and show that the results were consistent across the experiments. Each gray vector corresponds to one experiment and the black vector is the mean vector of all experiments. Each vector points in the direction of the mean phase of activity and its length is between 0 and 1. The radius of the circle is 0.5. A vector length of one would mean that all the data points coincide. Stars mark vectors with length significantly different from zero. The mean phases of protractor and retractor MN activities were consistent across the experiments. Protractor MN activity covered an angle from 182 to 315° with a mean phase for all experiments of 246°. Retractor MN activity covered a range from 58 to 135° with an overall mean phase of 86° of front leg step cycle.

In summary, front leg stepping was always accompanied by alternating activity in ipsilateral mesothoracic protractor and retractor MNs. Their activity pattern was phase coupled to the front leg step cycle. Middle leg retractor MNs were active during front leg stance and protractor MNs during front leg swing phase. Front leg stepping thus had a patterning influence on mesothoracic protractor and retractor MNs.

We next investigated whether and how single front leg stepping affected ipsilateral hind leg MN activity. During front leg stepping metathoracic protractor and retractor MN activities increased (Fig. 2A) ($N = 7$), although these activities were less clearly structured than those in the mesothoracic protractor and retractor MNs (compare Fig. 1). The phase plots show that the protractor and retractor MN spikes were widely distributed over front leg step cycle at all of step cycle periods (Fig. 2B) (no obvious horizontal "bands" are present; compare with Fig. 1B). The histogram (Fig. 2C) for 49 front leg steps reveals that

protractor MN activity was maximal between 240 and 270° of the front leg step cycle and retractor MN activity was maximal at the beginning of the stance phase between 0 and 30°. The cross-correlation function (Fig. 2D) is dominated by a broad peak around zero. This is explained by the fact that activity in both MN pools increased together during front leg stepping sequences. No clear oscillation can be seen in the cross-correlation function, indicating that protractor and retractor MN activities were not permanently and systematically phase coupled with a constant phase shift. The polar plots for the seven experiments show mean phases of activity for protractor and retractor MNs. The spike distribution for the different experiments is not normal but unimodal (e.g., Fig. 2C) and, as a consequence, the mean vector does not point exactly in the direction of the distribution maximum. Nevertheless this analysis shows the preferred phase of activity and the similarity between experiments. The polar plot reveals an overall mean phase for protractor MN activity at 151° (Fig. 2E), but the mean phases of the different experiments covered an angle from 71 to 240°. For retractor MNs the polar plot reveals an overall mean phase at 74° of front leg step cycle with mean phases between 38 and 92° for the different experiments ($N = 7$; $53 < n < 106$; mean: 68). Compared with the polar plots of mesothoracic protractor and retractor MN activities, vector direction is much more variable and vector length is generally shorter, indicating that each experiment's data points were less concentrated in one direction.

In summary, in metathoracic protractor and retractor MNs there was an increase in activity during front leg stepping. However, no alternating activity was present, although protractor and retractor MN activities increased with the beginning of a stepping sequence and were slightly modulated by the steps. No systematic coupling between protractor and retractor MN activities could be observed. Thus on the ipsilateral side the nature of the influence of front leg stepping appeared to change from rostral to caudal.

Front leg stepping likewise induced in the coxal MNs of all three contralateral hemiganglia a simultaneous activity increase that was slightly modulated with the steps. No systematic coupling between protractor and retractor MNs was observed for any of the contralateral hemiganglia. Only the polar plots for protractor and retractor MN activities in the front leg step cycle are shown. *Front leg*: protractor and retractor MN activities showed a consistent and nearly identical phase preference in front leg step cycle across the four experiments (Fig. 3A). The overall mean phase for protractor MN activity was 88° with the individual experiment mean phases covering angles between 39 and 125°. Retractor MN activity had an overall mean phase at 71° with individual mean phases between 38 and 111° for the different experiments ($N = 5$; $36 < n < 108$; mean: 74). *Middle leg*: contralateral mesothoracic protractor MN activity had an overall mean phase of 158° covering a range from 120 to 210° (Fig. 3B). The overall mean phase for retractor MN activity was 48° of front leg step cycle, with mean phases for the individual experiments between 357 and 138° ($N = 7$; $63 < n < 289$; mean: 150). *Hind leg*: metathoracic protractor MN activity had a mean phase of four experiments of 78° (73–187°). Retractor MN activity had an overall mean phase of 119° (21–114°) (Fig. 3C) ($N = 4$; $50 < n < 101$; mean: 73).

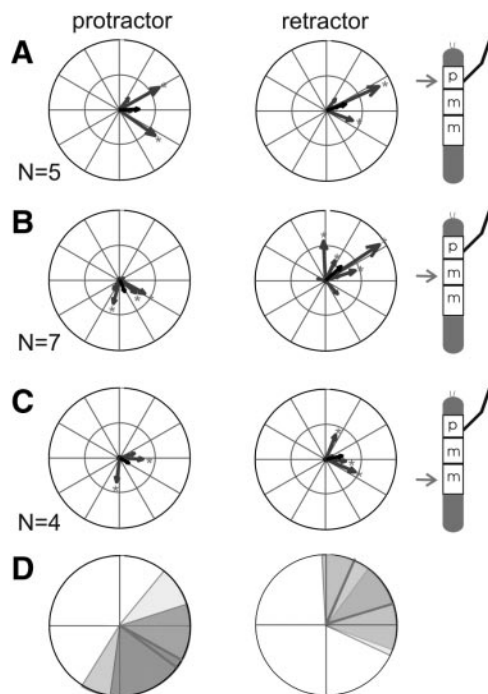


FIG. 3. Polar plots summarizing influence of front leg stepping on coxal MNs of the hemiganglia contralateral to the walking front leg. Radius of each circle is 0.5. Stars mark significant vectors. *A*: polar plots of mean phases of prothoracic protractor (*left*) and retractor (*right*) MN activities in front leg step cycle ($N = 5$). Overall mean phase of protractor MN activity was 88° ($39\text{--}125^\circ$). Overall mean phase of retractor MN activity was 71° ($38\text{--}111^\circ$). *B*: polar plots of mean phase of mesothoracic protractor (*left*) and retractor (*right*) MN activities in front leg step cycle ($N = 7$). Overall mean phase of protractor MN activity was 158° ($120\text{--}210^\circ$). Overall mean phase of retractor MN activity was 48° ($357\text{--}138^\circ$). *C*: polar plots of mean phases of metathoracic protractor (*left*) and retractor (*right*) MN activities in front leg step cycle ($N = 4$). Overall mean phase of protractor MN activity was 78° ($73\text{--}178^\circ$). Overall mean phase of retractor MN activity was 119° ($21\text{--}114^\circ$). *D*: ranges of mean phases for coxal MN activity in each of the contralateral hemiganglia (light gray: prothoracic contralateral hemiganglion; gray: mesothoracic; dark gray: metathoracic) and the sector where they overlap.

A comparison of the range of mean phases of protractor and retractor MN activities for the different contralateral ganglia shows that they overlap around 120° for protractor MN activity and around 60° for retractor MN activity (Fig. 3D). Taken together, these data show that front leg forward stepping was accompanied by alternating activity only in ipsilateral mesothoracic protractor and retractor MNs. In all other hemisegments front leg stepping was associated with a general activity increase that was only slightly modulated with the stepping activity.

Influences of a single walking middle leg

We next investigated the effect of middle leg stepping on the various hemiganglia. The single stepping middle leg experiments were performed with two different single leg preparations. In the first the middle leg stepped sideways on a treadmill perpendicular to the body axis, the traditional fashion for the single middle leg preparation (Fischer et al. 2001; Gabriel et al. 2003). In the second the treadmill was positioned parallel to the animal's body axis, resulting in walking resembling a more natural motion.

In the first preparation ($N = 5$), activity in ipsilateral metathoracic protractor and retractor MNs increased with middle

leg stepping but no clear rhythmic pattern was present (Fig. 4A). In some experiments, the increase in activity was stronger in one of the two MN pools than the other. In four of five experiments the activity increase in protractor MNs was stronger than that in retractor MNs. For instance in the 26 steps analyzed in Fig. 4B, protractor MN activity increased during stance phase with a maximum between 30 and 60° of middle leg step cycle (Fig. 4B), whereas retractor MN activity was about 75% lower than protractor MN activity and was widely distributed over the whole step cycle with a maximum between 0 and 30° . The polar plot of mean vectors for all five experiments shows a consistent phase preference for protractor MNs around 72° covering an angle of $45\text{--}107^\circ$ (Fig. 4C). For retractor MN activity the mean phases were highly variable between experiments with an overall mean phase of 9° (Fig. 4C) ($N = 5$; $24 < n < 50$; mean: 33).

Parallel stepping ($N = 6$) of the middle leg had comparable influences on metathoracic coxal MN activity. Although this walking pattern resembles more closely the natural forward walking pattern, no alternating activity was observed in metathoracic protractor and retractor MNs (Fig. 4D). Activity in both MN pools increased during walking sequences of the middle leg. The histogram for 24 steps shows an increase in protractor MN activity at the beginning of stance phase and a maximum between 120 and 150° of the middle leg step cycle. Retractor MN activity was maximal between 30 and 60° (Fig. 4E). The mean phases of protractor MN activity in the middle leg step cycle were similar for five of the six experiments with mean phases between 154 and 206° and an overall mean at 202° (Fig. 4F). The mean phases for retractor MN activity were again highly variable between experiments (Fig. 4F) ($N = 6$; $24 < n < 78$; mean: 43).

In summary, experiments with both preparations provided similar results. No alternating activity was observed in ipsilateral metathoracic protractor and retractor MNs in either preparation. The difference between the two was a 130° shift of the overall mean phase of protractor MN activity.

Experiments with both the "sideways" and "parallel" walking motion were also done to examine the influence of single middle leg stepping on prothoracic protractor and retractor MN activities (parallel stepping, $N = 2$; sideways stepping, $N = 5$). Because the results again were similar in both preparations, data from only one typical experiment are presented. Prothoracic protractor and retractor MN activities increased during middle leg sideways stepping but no alternating activity was observed (Fig. 5A). In this experiment protractor MN activity increased at the beginning of stance and was maximal between 90 and 120° . Retractor MN activity increased in the first quarter of the step cycle with a maximum between 60 and 90° (Fig. 5B). The cross-correlation function (Fig. 5C) is dominated by a broad peak around zero, showing that, although activity in both MN pools increased together during middle leg stepping, protractor and retractor MN activities were not permanently and systematically phase coupled. The polar plots with mean vectors for five experiments show that mean phases for protractor and retractor MN activities were consistent in all experiments and similar for protractor and retractor MNs (Fig. 5D). Protractor MN activity had an overall mean phase of 59° with a range from 344 to 90° and retractor MN activity had an overall mean phase of 41° with a range from 14 to 66° ($N = 5$; $37 < n < 80$; mean: 49).

In protractor and retractor MNs of all three contralateral hemiganglia, middle leg stepping was associated with a simultaneous activity increase that was slightly modulated with the steps. No systematic coupling between protractor and retractor MNs was observed for any of the contralateral hemiganglia. Experiments were performed with only the sideways stepping middle leg preparation. Only the polar plots for protractor and retractor MN activities in the middle leg step cycle are shown. *Front leg*: for prothoracic protractor and retractor MNs mean

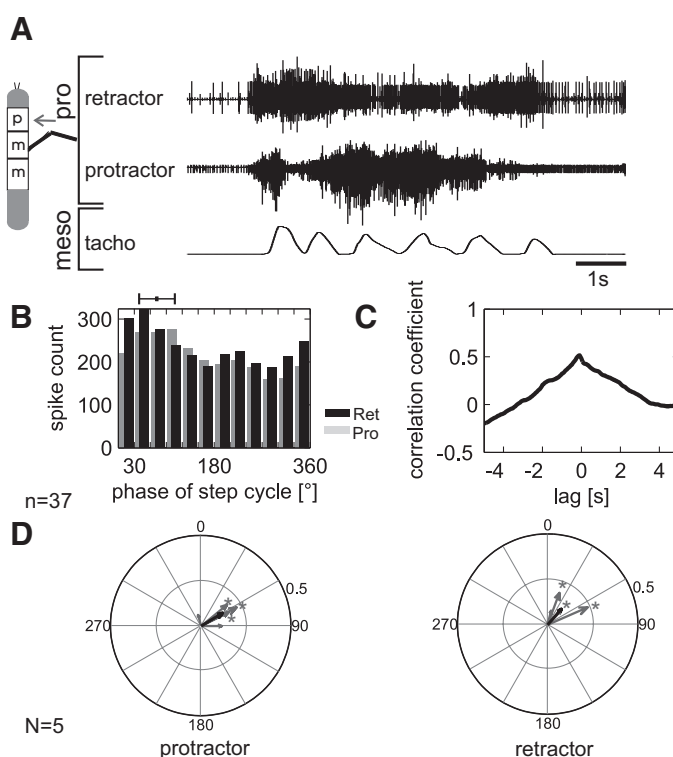
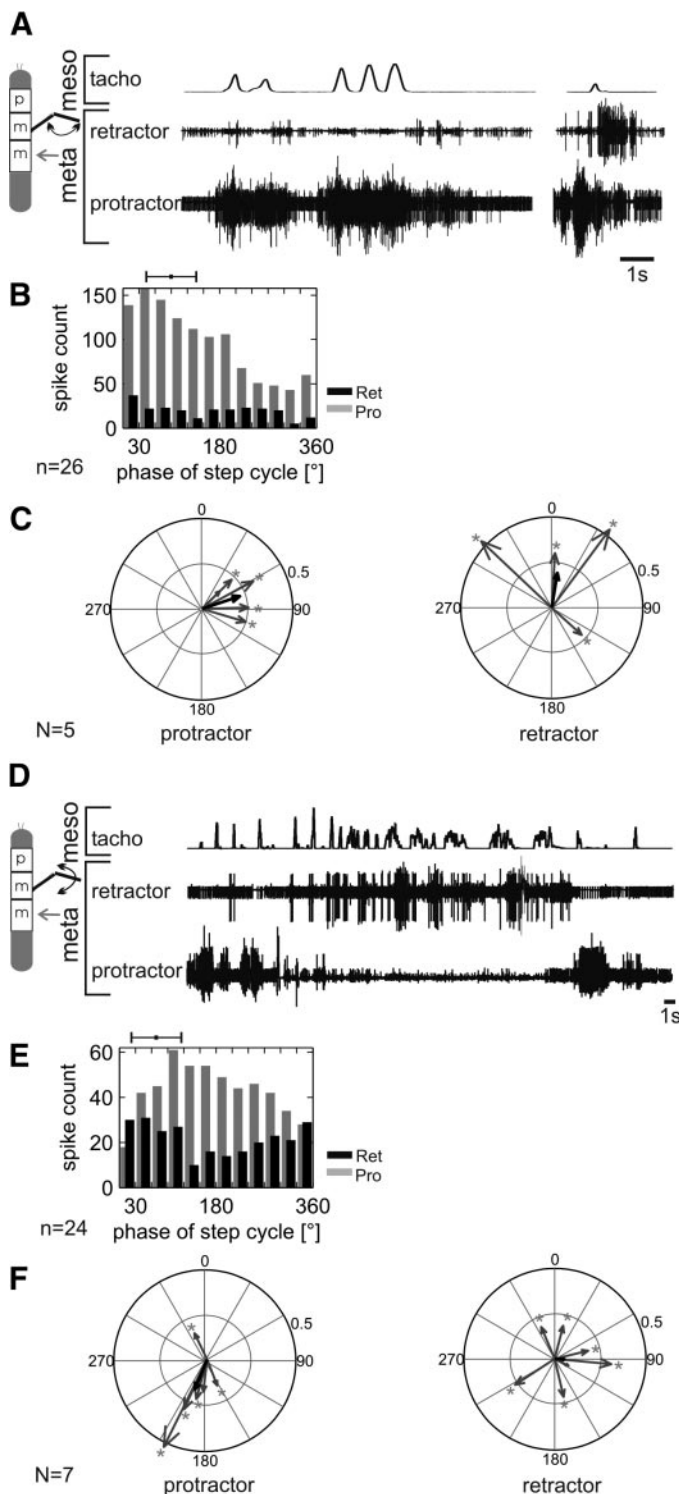


FIG. 5. *A*: prothoracic and retractor MN activities during middle leg sideways stepping; *B*: distribution of protractor (gray) and retractor (black) MN activities in the middle leg step cycle for 37 steps. Protractor MN activity was maximum between 90 and 120°. Retractor MN activity was maximum between 30 and 60°. *C*: cross-correlation function reveals no phase coupling between protractor and retractor MN activities. Broad peak is explained by the fact that protractor and retractor MN activities increased together with each stepping sequence. *D*: polar plots of mean phases of protractor (*left*) and retractor (*right*) MN activities in middle leg step cycle for 5 experiments (gray arrows) and mean vector of all experiments (black arrows). Overall mean phase of protractor MN activity was 59° (344–90°). Overall mean phase of retractor MN activity was 41° (14–66°). Stars mark significant vectors.

phases of activity were consistent across the experiments (Fig. 6A). Protractor and retractor MN activities had an overall mean phase of, respectively, 85° (63–102°) and 77° (60–98°) ($N = 6$; $40 < n < 135$; mean: 70). *Middle leg*: mesothoracic

FIG. 4. *A*: metathoracic protractor and retractor MN activities during sideways middle leg stepping. It was often observed that either protractor or retractor MN activity was much higher during a walking sequence. *B*: distribution of protractor (gray) and retractor (black) MN activities in the middle leg step cycle for 26 sideways steps. Protractor MN activity was maximal during middle leg stance phase between 30 and 60°. In this experiment retractor MN activity averaged 20% of protractor MN activity and was maximal between 0 and 30°. *C*: polar plots of mean phases of protractor (*left*) and retractor (*right*) MN activities for 5 experiments (gray arrows) and mean vector of all experiments (black arrow) for the sideways stepping preparation. Overall mean phase of protractor MN activity was 72° (45–107°). Retractor mean phase was highly variable, with an overall mean phase of 9°. Stars mark significant vectors. *D*: recording of metathoracic protractor and retractor MN activities during parallel middle leg stepping. *E*: distribution of protractor (gray) and retractor (black) MN activities in middle leg step cycle for 24 parallel steps. Metathoracic protractor MN activity was maximal between 90 and 120°. Retractor MN activity increased at the beginning and the end of the stepping cycle with a maximum between 30 and 60°. *F*: polar plots of mean phases of protractor (*left*) and retractor (*right*) MN activities in the middle leg step cycle for 7 experiments (gray arrows) and mean vector of all experiments (black arrow) for the parallel stepping preparation. Overall mean phase of protractor MN activity was 202° (154–206°). Mean phases of retractor MN activity were again highly variable between experiments.

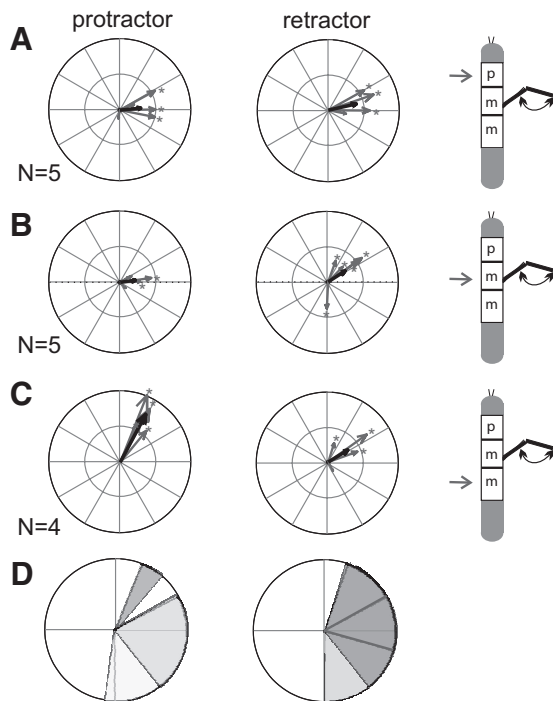


FIG. 6. Influence of middle leg sideways stepping on coxal MNs of the hemiganglia contralateral to the walking middle leg summarized in polar plots. Radius of each circle is 0.5. Stars mark significant vectors. *A*: polar plots of mean phases of prothoracic protractor (*left*) and retractor (*right*) MN activities in middle leg step cycle ($N = 5$). Overall mean phase of protractor MN activity was 85° ($63\text{--}102^\circ$). Overall mean phase of retractor MN activity was 77° ($60\text{--}98^\circ$). *B*: polar plots of mean phases of mesothoracic protractor (*left*) and retractor (*right*) MN activities in middle leg step cycle ($N = 7$). Overall mean phase of protractor MN activity was 82° ($61\text{--}135^\circ$). Overall mean phase of retractor MN activity was 57° ($19\text{--}182^\circ$). *C*: polar plots of mean phases of metathoracic protractor (*left*) and retractor (*right*) MN activities in middle leg step cycle ($N = 4$). Overall mean phase of protractor MN activity was 28° ($22\text{--}40^\circ$). Overall mean phase of retractor MN activity was 58° ($60\text{--}98^\circ$). *D*: plot shows the range of mean phases for coxal MN activity in each of the contralateral hemiganglia (light gray: prothoracic contralateral hemiganglion; gray: mesothoracic; dark gray: metathoracic) and the sector where they overlap.

protractor and retractor MN activities had an overall mean phase of, respectively, 82° ($61\text{--}135^\circ$) and 57° ($19\text{--}182^\circ$) of middle leg step cycle ($N = 5$; $39 < n < 75$; mean: 51) (Fig. 6*B*). *Hind leg*: metathoracic protractor and retractor MN mean phases of activity were similar in the four experiments performed ($N = 4$; $34 < n < 79$; mean: 55) (Fig. 6*C*). Protractor MN activity had an overall mean phase of 28° ($22\text{--}40^\circ$) and retractor MN activity of 58° ($60\text{--}98^\circ$).

A comparison of the range of mean phases of protractor and retractor MN activities for the different contralateral ganglia shows that they overlap at around 90° for retractor MN activity (Fig. 6*D*). In summary, middle leg stepping was associated with a general activity increase in protractor and retractor MNs of all other hemiganglia, ipsilateral and contralateral, that was slightly modulated by middle leg steps. The influence of middle leg stepping thus qualitatively differed from that of front leg stepping. Front leg stepping generates alternating activity of middle leg protractor and retractor MNs, but with middle leg stepping a rhythmic alternating activity pattern was not observed in any of the protractor and retractor MN pools.

Influences of a single walking hind leg

Experiments with the single hind leg preparation were very difficult to do because the intrinsic walking direction of the hind legs is backward (Bässler et al. 1985) and, consequently, forward walking only very rarely [two of 11 experiments ($n_1 = 52$, $n_2 = 20$)] occurred. In these experiments forward hind leg stepping was accompanied by a general increase in mesothoracic protractor and retractor MNs (Fig. 7). Due to the small number of experiments in which the animal stepped forward and the few stepping sequences available, no further quantitative and statistical evaluations were done.

Influence of walking direction

We next investigated whether walking direction played a role in the influence of single stepping legs using single backward stepping hind and middle legs. In the intact six-legged animal touching on the antennae elicits a few backward steps of the whole animal (Graham and Epstein 1985). In the single front leg preparation no or only sporadic backward steps were observed. Therefore the influence of backward stepping was not studied for the single front leg.

For the hind leg in three of nine experiments backward hind leg stepping elicited alternating activity in protractor and retractor MNs of the ipsilateral mesothoracic hemiganglion (Fig. 8*A*). The phase plots show no systematic phase dependence of protractor and retractor MN spikes on hind leg step cycle (Fig. 8*B*). The histogram for 24 hind leg steps shows that retractor MN activity increased at the beginning and end of the step cycle with a maximum between 30 and 60° . Protractor MN activity had a maximum around 180° of hind leg step cycle (Fig. 8*C*), thus bearing similarities to their activity during forward front leg stepping. The cross-correlation function shows a coupling of protractor and retractor MN activities with a phase shift of 180° indicated by a maximal negative correlation at 0-s time lag (Fig. 8*D*). In six of nine experiments hind leg backward stepping increased mesothoracic protractor and retractor MN activities without phase coupling between the motor nerves occurring. The polar plots (Fig. 8*E*) summarize the mean phases of activity for all nine experiments. The mean phase of protractor MN activity was very variable between the experiments ($N = 9$; $12 < n < 68$; mean: 33). The mean vectors of retractor MN activity revealed a consistent phase preference in all experiments with an overall mean phase of 90° and a range from 77 to 135° .

In summary, in one third of the experiments the backward hind leg stepping had a patterning influence on mesothoracic protractor and retractor MNs. This shows that the backward

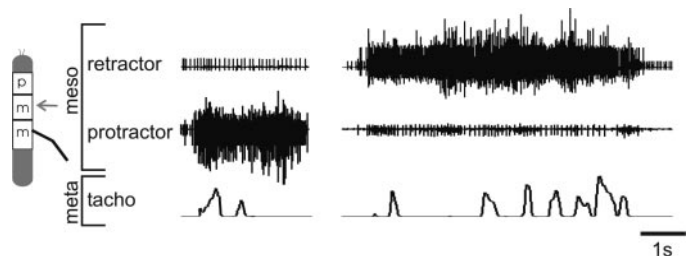


FIG. 7. Mesothoracic protractor and retractor MN activities during hind leg forward stepping. Protractor or retractor MN activity increased during stepping sequences.

hind leg stepping can be associated with alternating activity in mesothoracic coxal MNs. In contrast, during middle leg backward stepping a general increase in activity in ipsilateral metathoracic (Fig. 9A) ($N = 4$; $10 < n < 38$; mean: 26) and prothoracic (Fig. 9B) ($N = 4$; $17 < n < 54$; mean: 36) protractor and retractor MNs was present in four of four experiments. Thus the influence of single middle leg stepping did not depend on the walking direction. Both forward and backward middle leg stepping induced a generalized activity increase in coxal MNs of ipsilateral hemiganglia.

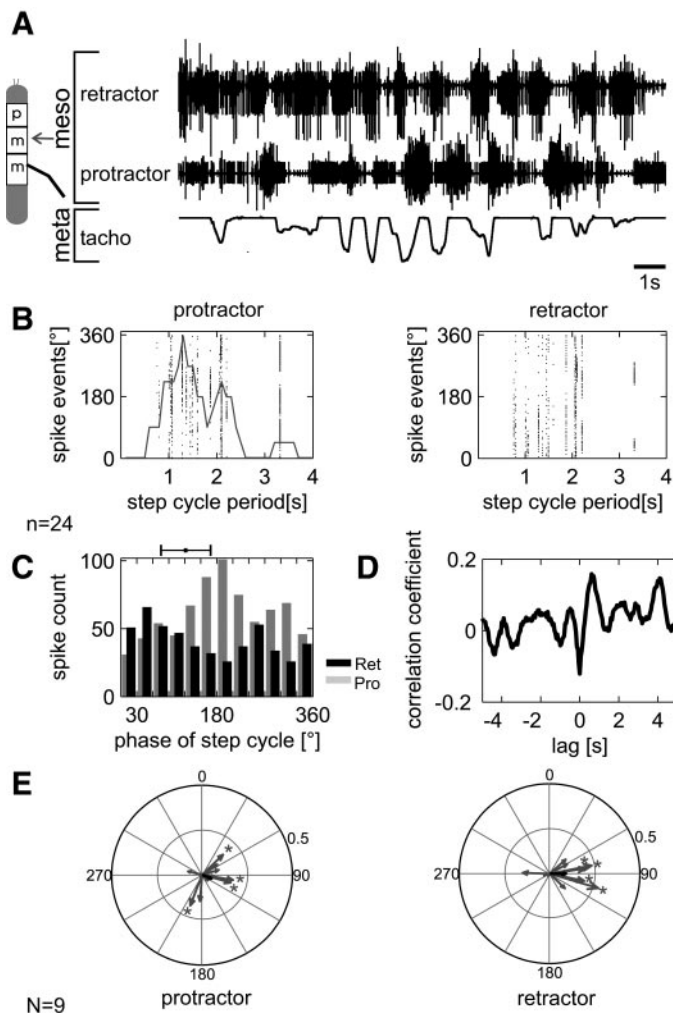


FIG. 8. *A*: mesothoracic protractor and retractor MN activities during hind leg backward stepping. In 3 of 9 experiments alternating activity occurred during hind leg stepping sequences. *B*: phase of mesothoracic protractor and retractor MN spikes in hind leg step cycle plotted against step cycle period. No clear phase dependence of protractor or retractor MN activity on hind leg step cycle was present. Curve (left plot) shows the frequency of steps over the step cycle period. *C*, histogram: distribution of protractor (gray) and retractor (black) MN activities in the hind leg step cycle for 24 steps. Protractor MN activity was maximum between 180 and 210°. Retractor MN activity had 2 maxima, one between 30 and 60° and the other between 240 and 270°. *D*: cross-correlation function reveals an alternating coupling between protractor and retractor MN activities. *E*: polar plots of mean phases of protractor (left) and retractor (right) MN activities in hind leg step cycle for 9 experiments (gray arrows) and mean vector of all experiments (black arrows). Mean phases of protractor MN activity were variable between experiments. Overall mean phase of retractor MN activity was 90° (77–135°). Stars mark significant vectors.

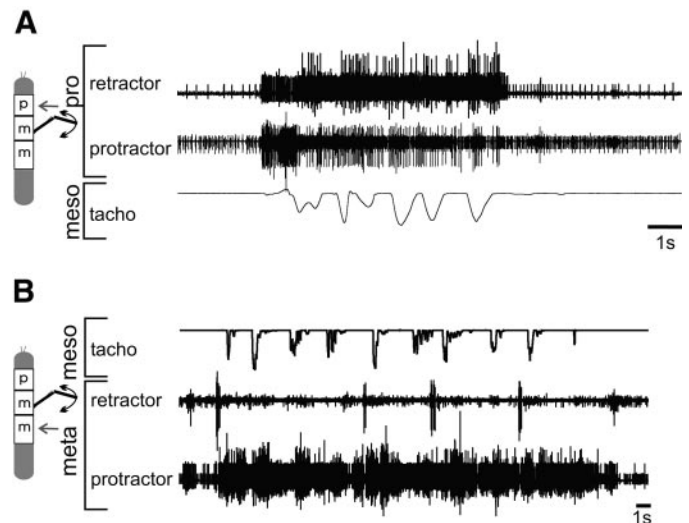


FIG. 9. *A*: prothoracic protractor and retractor coxae MN activities during backward middle leg stepping ($N = 4$). Protractor and retractor MN activities increased during stepping sequences. *B*: recording of metathoracic retractor and protractor coxae MN activities during backward middle leg stepping ($N = 4$). Protractor and retractor MN activities increased during stepping sequences. Walking direction did not alter the influence of middle leg stepping.

DISCUSSION

We have investigated the influence that single stepping leg has on motoneuronal activity in the other segmental ganglia.

Front, middle, and hind legs do not have the same influences on motoneuronal activity in adjacent segments

Single leg stepping was always accompanied in all hemisegments of the walking system by a general increase in motoneuron activity. Single front leg forward walking was associated with alternating activity in ipsilateral mesothoracic protractor and retractor MNs. Thereby, protractor and retractor MN activities were phase coupled to the front leg step cycle with retractor MNs active in the first half of the step cycle and protractor MNs active in the second. Hind leg backward stepping was accompanied by alternating activity in ipsilateral mesothoracic protractor and retractor MNs in about 33% of the experiments. In the remaining 67% of the experiments protractor and retractor MN activities were associated with a general activity increase in MN activity. From behavioral experiments it is known that backward walking is not attributed to levator and depressor phase shift, but instead arises from a general biasing of activity toward the rear of the animal (Graham and Epstein 1985). The front legs seem to give up their leading role, as indicated by their stepping more slowly than the middle or hind legs and showing a larger range of movement (Graham and Epstein 1985). The hind legs become functional front legs with kinematics more closely resembling those of the front legs during forward walking. This behavioral data showing that during backward walking hind legs can assume a functional role equivalent to that which the front legs play during forward walking may have relevance to our observation that hind leg stepping can be accompanied by alternating activity in mesothoracic MNs. By taking these previous results and our data together it appears quite conceivable that in the stick insect walking system stepping of the functional front leg is associ-

ated with activity in intersegmental neuronal pathways that leads, first, to alternating activity in coxal MNs of the functional next caudal segment and, second, to a phase coupling of the alternating activity in the ipsilateral mesothoracic hemiganglion with stepping of the functional front leg.

In contrast, single middle leg stepping was primarily associated with tonic activity in adjacent segments, but not with alternating activity in coxal MNs in any of its neighboring segments. These experiments were done under two different conditions. The first resembled the traditional single leg preparation (Fischer et al. 2001) in which the middle leg performs sideways steps on a treadmill positioned perpendicular to the body's length axis. In this preparation the thorax-coxa (TC) joint of the middle leg does not move and the kinematics of this walking condition is comparable to that of the inner middle leg during curve walking or, to a lesser extent, with front leg stepping. The restricted movement in one plane results only in a different activation of sense organs on the walking leg and thus might lead to a different influence of middle leg stepping on the neighboring ganglia compared with *in vivo* forward straight walking. Therefore, to overcome this restriction a second single leg walking position was used in which the middle leg performed stepping movements parallel to the body axis on a treadmill positioned parallel to the body. This walking situation resembled more closely the kinematics during *in vivo* forward straight walking (Cruse and Bartling 1995), specifically with respect to the activation of coxal sensors, like the ventral coxal hairplates and the hair rows (e.g., Bässler 1983; Büschges and Schmitz 1991). The results, however, were the same for both preparations. Single middle leg stepping caused a general activation of protractor and retractor MNs and only a slight modulation with the steps in all other hemiganglia; i.e., middle leg stepping did not induce alternating activity in adjacent segments.

The influence of middle leg stepping thus is qualitatively different from that of the forward walking front leg and backward walking hind leg. Therefore, the neural networks controlling front, middle, and hind legs might not be regarded as fairly symmetrical elements in a segmental chain of oscillators with respect to their influence on other segments (cf. Cruse et al. 1998). This finding on the neural level parallels a previous behavioral study (Bässler et al. 1985) that also reported differences between the three pairs of legs and their neural control in the stick insect walking system. In experiments with decerebrated animals, Bässler et al. (1985) previously showed that front and hind legs have different intrinsic walking directions when being the sole pair of legs present. When all other legs are amputated front legs tend to walk forward and, under the same conditions, hind legs tend to walk backward. However, middle legs have no preferred intrinsic walking direction. Furthermore they follow either front legs or hind legs when a second pair of legs is present. These observations support the conclusion that the middle leg influence is different from that of the front and hind legs, and they show that middle legs are dominated by the front and hind legs.

The influence that single front and middle leg stepping had on coxal MNs of the contralateral hemiganglia was similar. Associated with stepping a generalized activation of coxal MNs of the contralateral hemiganglia was generated. This corresponds to prior recordings from mesothoracic MNs contralateral to the walking leg. Ludwar et al. (2005b) showed that

on front leg stepping a tonic depolarization throughout the walking sequence occurred in contralateral mesothoracic flexor tibiae MNs. Only small-amplitude phasic modulation with variable coupling to the front leg step cycle was detectable in the intracellular recordings.

Activation of the stick insect locomotor system

Single front leg stepping was always accompanied by a general activation of protractor and retractor MNs of the two caudal ipsilateral hemiganglia and all contralateral hemiganglia. Similar results were observed for single middle leg stepping. A phase-coupled coordinated activity of coxal MNs was in addition generated in the ipsilateral mesothoracic segment on front leg stepping. Although it is clear that the source of the phasic influence on mesothoracic coxal MN activity is front leg stepping, the source for the generalized activation of protractor and retractor motoneurons in all hemisegments on stepping of one single leg is less obvious.

Different possible explanations exist. First, the general activation could be the result of a change in the behavioral state of the whole locomotor system of the stick insect (see following text). Second, the general activation could be the influence of the stepping front or middle leg on its neighboring segments. Given that a general activation of motoneurons in adjacent segments has also been found for other leg movements, like searching movements (Büschges et al. 2004), the latter possibility is less likely to be the exclusive and sole explanation. Furthermore, results on a variety of invertebrate and vertebrate locomotor systems favor the first possibility. These show that the initiation of a locomotor movement is mediated by descending signals from higher brain structures. Thereby the activation is either the result of the activity of individual descending command neurons (*Clione limacine*: Norekian and Satterlie 1996; Panchin et al. 1995; Satterlie and Norekian 1995; leech: Brodfuehrer and Friesen 1986a,b,c; crayfish: Bowerman and Larimer 1974a,b; Davis and Kennedy 1972a,b,c; Wiersma and Ikeda 1964) or the result of the simultaneous activation of neuron populations of descending pathways from higher brain structures (lamprey: El Manira et al. 1997; McClellan and Grillner 1984; Viana di Prisco et al. 1997; cat: e.g., reviewed by Mori et al. 1991). In both cases the activity of the underlying descending pathways is tonic and outlasts locomotor bouts. The descending pathways activate local networks downstream in the CNS on the segmental level of the locomotor organs (e.g., Panchin et al. 1995; Viana di Prisco et al. 1997). In insects the command system for walking initiation has not yet been identified. Data presented by Kien (1990) for the locust suggest that some of the 200 pairs of identified brain neurons descending to the thoracic ganglia are involved in walking initiation. Behavioral experiments on the stick insect have suggested that start and end of a stepping sequence, as well as the walking direction, are determined by descending pathways from the subesophageal ganglion (Bässler et al. 1985). Finally, it is quite conceivable that more intrinsic components such as sensory excitation could be the source. The finding that we observe a rather generalized activation in motoneurons could be an effect of sensory modulation or activation that is generated by the sensory signals from one walking leg. Such a generalized excitatory modulation has been observed in both invertebrates [e.g., crayfish

(STG): Beenhakker and Nusbaum 2004] and vertebrates (e.g., lamprey: Kiemel and Cohen 2001; human: Zehr et al. 2007).

In the present study single legs performed stepping movements. This implies that the pattern-generating networks for walking are activated in the hemiganglion of the stepping leg. Our data show that the central neural networks generating alternating rhythmic activity in MN pools of the other segments were not activated, except perhaps for the mesothoracic segment with front leg forward and hind leg backward walking. Ludwar et al. (2005a) showed that front leg stepping induced modest but clear alternating activity in all motoneuron pools of the ipsilateral mesothoracic ganglion, the source of which is not yet known. It would have been conceivable that such activation of alternating activity would have been present throughout the thoracic segments. However, our data show this is not the case. One walking leg is apparently not sufficient to activate an alternating motor output for antagonistic muscles in all other segments as one might have expected as being typical for the locomotor state of walking system.

This result clearly differs from that of activation of other well-investigated locomotor systems, where activation of one part of the system by the CNS of the animal itself was found to be sufficient to result in a coordinated motor output of the whole. Experiments *in vitro* on the leech (Debski and Friesen 1987) and *in vivo* and *in vitro* in crayfish (Cattaert et al. 1992; Davis and Kennedy 1972a,b,c; Larimer 1976; Wiesma and Ikeda 1964) and lamprey (Brodin et al. 1988) belong to these. In the crayfish swimmeret system, for example, stimulating one swimmeret's CBCO induces rhythmical activity in the whole multisegmental swimmeret system (Cattaert et al. 1992).

Taken together, the data presented emphasize a decentralized and modular structure of the stick insect walking system, a conclusion supported by previous behavioral studies starting from the finding that each leg possesses its own walking pattern-generating system, encompassing the necessary central circuitry and sensory feedback on the local leg to coordinate the motor output generated (Büschges et al. 1995; Foth and Bässler 1985a,b). Our results strongly suggest that the individual hemiganglia of the stick insect walking system are independently activated. Future work is needed to identify the specific activating pathways in the walking system and to address the question of whether these inputs actively participate in generating alternating activity in the individual hemiganglia.

Intersegmental influences of single stepping legs in the light of the coordinating rules governing stepping in the stick insect

What do the influences described earlier mean for intersegmental coordination, particularly in view of prior behavioral studies and the known behavioral rules for intersegmental coordination (e.g., Cruse 1990; Dürr et al. 2004)? In general, these single leg experiments did not reveal neural mechanisms underlying coordination. Single leg stepping was associated with an increase in activity in all other hemisegments of the thoracic nerve cord, except for the hemisegment posterior to a functional walking front leg, which exhibited rhythmic activity in coxal motoneurons. In these cases, however, the coxal MNs of the functional caudal neighboring segment exhibited in-phase activity with the leading front leg, a pattern of coordi-

nation not found *in vivo*. However, in several instances these influences that have been observed in behavioral experiments, e.g., during the stance phase of adjacent ipsilateral legs in the tetrapod gait (Bässler 1983; Graham 1985), are found on the motoneural level.

In amputation experiments in which the middle leg was cut off (Cruse 1983; Wendler 1964), the stump of the amputated leg produced in forward walking retraction and protraction movements in phase with the walking front leg. This corresponds to the activation of mesothoracic protractor and retractor MNs observed here during front leg stepping. Retractor MNs were active during the first half of the step cycle, especially during stance, and protractor MNs were active in the second half of the step cycle. Because retraction is performed in forward walking during stance, our data correspond to in-phase retraction and protraction of the front and "middle" legs. Furthermore, in behavioral experiments a weaker coupling between contralateral legs has been observed in stick insects and crayfish (Cruse 1990). This corresponds to our results in the way that single leg walking had in general no phasic influence on motoneural activity of contralateral hemiganglia.

In the intact walking stick insect there are seven behavioral rules known that describe the coordination of activity of ipsilateral neighboring legs (Cruse 1990; Dürr et al. 2004), three of which play the most important general role for coordination. Two of these rules describe a rostral effect, the first hindering an anterior leg from starting a return stroke while the posterior leg is performing its return stroke. The second assists the start of a return stroke in a rostral leg when the posterior leg starts a power stroke. The third influence is caudally directed and acts to advance induction of a return stroke with the ongoing power stroke of the rostral leg. These three rules are thought to function equally between ipsilateral neighboring legs (Dürr et al. 2004). From this one may expect that all ipsilateral neighboring legs have an equal influence on their ipsilateral neighbors on the neural level. It would thus be reasonable to expect middle leg stepping to be associated with alternating activity in coxal MNs of at least one of the ipsilateral neighboring hemiganglia. This was not observed in our experiments, and thus despite this behavior pattern (Cruse 1990), on the neural level the different segments do not exert qualitatively equal influences on their neighbors. Only one of the coordination rules formulated for the stick insect walking system is partially fulfilled by the changes in motoneuron activity in adjacent segments on single leg stepping, i.e., the coactivation rule number 5 (reviewed in Dürr et al. 2004). This rule describes that on encountering an increased resistance during stepping, such as when walking uphill or when loading the animal, the force output of all legs is enhanced. From our results it is quite conceivable that pathways underlying the observed activation of motor activity in adjacent segments related to single leg stepping can contribute to this coactivation influence among walking legs. However, this interpretation is hampered by the fact that both pro- and retractor coxae activities were enhanced on single leg stepping, indicating that this influence might also have another function, i.e., the general and unspecific activation of locomotor networks in adjacent hemisegments.

Subsequent experiments will have to clarify the following issues. 1) Does the neural activity of intersegmental coordinat-

ing pathway rely on the stepping of multiple legs? 2) What is the neural substrate of the rhythmic activity observed in the mesothoracic ganglion on ipsilateral front leg forward and hind leg backward stepping?

ACKNOWLEDGMENTS

We thank Drs. U. Bässler, H. Cruse, V. Dürr, S. L. Hooper, K. G. Pearson, and J. Schmidt for stimulating discussions in the course of the work. Special thanks are owed to S. Hooper and S. Meyen-Southard for style-editing the manuscript.

GRANTS

This study was supported by Deutsche Forschungsgemeinschaft Grant Bu857 to A. Büschges, the Institute for Advanced Study Berlin, and the Boehringer-Ingelheim Foundation.

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