Control of stepping velocity in a single insect leg during walking

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In the single middle leg preparation of the stick insect walking on a treadmill, the activity of flexor and extensor tibiae motor neurons and muscles, which are responsible for the movement of the tibia in stance and swing phases, respectively, was investigated with respect to changes in stepping velocity. Changes in stepping velocity were correlated with cycle period. There was a close correlation of flexor motor neuron activity (stance phase) with stepping velocity, but the duration and activation of extensor motor neurons (swing phase) was not altered. The depolarization of flexor motor neurons showed two components. At all step velocities, a stereotypic initial depolarization was generated at the beginning of stance phase activity. A subsequent larger depolarization and activation was tightly linked to belt velocity, i.e. it occurred earlier and with larger amplitude during fast steps compared with slow steps. Alterations in a tonic background excitation appear not to play a role in controlling the motor neuron activity for changes in stepping velocity. Our results indicate that in the single insect leg during walking, mechanisms for altering stepping velocity become effective only during an already ongoing stance phase motor output. We discuss the putative mechanisms involved.

Keywords: locomotion; motor pattern generation; walking speed; cycle period; afferent feedback

1. Introduction

In a diverse and often non-predictable environment, the motor output of an animal needs to be adjusted at all times to fulfil the current requirements of a behavioural task. In walking, for example, such adjustments include changing the direction and the speed of locomotion. At present, detailed knowledge exists on the generation of a basic locomotor output for a variety of locomotor behaviours, such as swimming (e.g. Friesen 1994; Arshavsky et al. 1998; Grillner 2003), walking (Bäsßler & Büschges 1998; Pearson & Gordon 2000) and flying (Robertson 2003). It is well established that the generation of locomotor patterns results from a close interaction between central pattern-generating (CPG) networks in the nervous system, local feedback from sensory neurons about movements and forces generated in the locomotor organs, and

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coordinating signals from neighbouring segments or appendages (Pearson 1995; Grillner 2003; Büschges 2005). The result of this interaction is a specific rhythmic pattern of synaptic inputs to motor neurons that, together with their intrinsic properties (e.g. Perrier & Hounsgaard 2000; Grillner et al. 2001), determines the timing of their activation and inactivation and the actual magnitude of their activity. However, when it comes to the neural mechanisms that underlie the patterning of motor neuron activity during modifications of the motor output, e.g. changing the speed of locomotion, the picture is less clear.

In principle, changes in the speed of locomotion can be accomplished either by a change in cycle period of the motor output, a change in the intensity of the power stroke or combinations of both. For swimming in vertebrates and invertebrates, considerable information is available on the mediation of changes in speed of locomotion. An increase in the swimming speed results from an increase in the frequency of the rhythmic tail, fin or body movements. The lamprey, for example, swims by means of undulatory trunk movements that are generated by alternating contractions of the myotomes along its body axis. Within each body segment, there is a CPG network that receives tonic glutamatergic excitation from reticulospinal (RS) neurons in the brainstem (Buchanan et al. 1987; reviewed in Grillner et al. 1997). The more tonic excitatory drive the CPG interneurons receive, the faster the networks oscillate, which, in turn, provide alternating excitation and inhibition to the motor neurons (Orlovsky et al. 1999). With increasing locomotor output not only the frequency, but to some extent also the magnitude of motor neuron activation and muscle contractions increases (Sirot et al. 2000), which in a freely moving animal would lead to an increase in the swimming velocity. The results on fictive swimming in the Xenopus embryo (Sillar & Roberts 1993; Roberts et al. 1998) and the marine mollusc Clione (Satterlie 1993; reviewed in Orlovsky et al. 1999) point in a similar direction.

For legged locomotion, i.e. walking, less is known about the neural mechanisms in charge of modifying the activation pattern of motor neurons. In general, walking systems are multi-legged with two, four, six or more limbs. With changing speed, interlimb coordination changes. In quadrupeds, for example, there is a change in gait from walk to trot to gallop, when the speed of locomotion increases (summary in Orlovsky et al. 1999). The walking pattern of the single limb can contribute to an increase in locomotor speed in different ways, e.g. by an increase in step length, a decrease in cycle period or a combination of both. Although, for example in the cat, stride length of the stance phase increases to some extent with faster speeds of locomotion, it is primarily the decrease in cycle period that is responsible for the increase in speed (Halbertsma 1983; Yakovenko et al. 2005; review in Orlovsky et al. 1999). The same is true for walking in arthropods, e.g. in crayfish (Clarac & Chasserat 1986) and stick insects (Wendler 1964; Graham 1972; Graham & Cruse 1981). In turn, the decrease in cycle period is generally achieved by a decrease in stance phase duration, while swing duration varies little or not at all (stick insect, Wendler 1964; locust, Burns 1973; cat, Halbertsma 1983; reviewed in Orlovsky et al. 1999). In insects, it has been suggested that descending drive from the brain provides tonic excitation, the magnitude of which can influence walking speed (Roeder 1937; Ridgel & Ritzmann 2005). Similarly, in the cat, the cycle period of the walking motor output changes with the tonic background excitation from the brainstem. It is noteworthy that these descending signals do not directly control cycle period but instead determine the intensity of
muscle contractions (Shik et al. 1966), suggesting that the changing afferent feedback from the limb is responsible for changing the cycle period (Orlovsky et al. 1999; Yakovenko et al. 2005). How exactly the motor neurons and their activity pattern in a walking animal are affected in the course of changes in walking speed, in particular, in vivo is, however, still a largely unknown issue.

We set out to address this question in a preparation that allows us to analyse the activity pattern of leg motor neurons intracellularly during the execution of stepping movements in a single leg of a walking insect, i.e. the semi-intact single middle leg preparation of the stick insect (Bässler 1993; Fischer et al. 2001). Compared with the investigations reported previously, this more intact preparation endogenously expresses a locomotor rhythm varying in cycle period, speed or strength. The findings provide insights into the neural mechanisms that may be responsible for the generation of different walking speeds in vivo, which also has implications for the design of robots using biological principles of locomotion (Ritzmann et al. 2004). The results may allow conclusions on the modifications in the premotor network that contribute to changes in walking speed and enable us to develop specific hypotheses that can be tested in subsequent sets of experiments under more reduced conditions.

2. Material and methods

(a) Preparation

The experiments were performed on adult female individuals of the stick insect species Cuniculina impigra (syn. Baculum impigrum) from a colony maintained at the University of Cologne. The preparation was performed according to the established procedures (Fischer et al. 2001; Gabriel et al. 2003). In short, all legs except one middle leg were severed at the level of the mid-coxa. The thorax was glued to a platform dorsal side up. Pro- and retraction of the remaining leg was blocked with glue around the subcoxal joint. A window was cut dorsally into the cuticle and the gut was moved aside to expose the mesothoracic ganglion and the lateral nerves. The lateral nerves 2 and 5 on the side of the remaining leg, innervating the pro- and retractor coxae muscles, respectively, were crushed. The mesothoracic ganglion was lifted on a platform and the surrounding connective tissue was pinned down. To improve electrode penetration, small crystals of a proteolytic enzyme (Pronase E, Merck, Darmstadt, Germany) were placed on the ganglionic sheath for 60–90 s. The enzyme was thoroughly washed out and the thoracic cavity was filled with ringer (in mM: NaCl 180; sucrose 30; HEPES 10; CaCl₂ 5; KCl 4; MgCl₂ 1).

(b) Treadmill

The treadmill consisted of two styrofoam drums (diameter 40 mm; width 28 mm), each mounted on a micro-DC motor (centre distance 50 mm), one of which was used as a tachometer (for details, see Gabriel et al. 2003). The belt was made from crepe paper. The treadmill was positioned below the leg perpendicularly to the longitudinal axis of the animal. The height was adjusted so that the angle of the joint between femur and tibia was approximately 90° in mid-stance (figure 1a).

Phil. Trans. R. Soc. A (2007)
Figure 1. (a) Photograph of the single middle leg on the treadmill, with curved arrows indicating movement around the coxa–trochanter joint (depression, levation) and femur–tibia joint (flexion, extension). (b) Intracellular recording of a fast flexor (fFlex) MN during a stepping sequence with electromyogram of the flexor muscle (EMG Flex) and belt velocity. The horizontal dashed line always indicates the MN membrane potential when the animal was at rest; black and white bars mark the stance and the swing phase, respectively. (c) Changes in cycle period and belt velocity (maximum and mean) during stepping sequences ($N=3$, $n=51$). Often during long stepping sequences, cycle period increased ($p=0.0021$) and maximum and mean belt velocity decreased ($p=0.0061$ and 0.0023, respectively). (d) Cycle period and mean belt velocity showed a negative correlation in 10 out of 13 experiments ($N=13$, $n=289$, $p$-values from 0.0377 to $<0.0001$). The grey lines are the regression lines for individual experiments. The black line represents the regression line of the pooled data ($p<0.0001$).
(c) Electrophysiology

Recordings were carried out under dimmed light at room temperature (20–22°C). Electromyograms (EMGs) of the flexor tibiae (flexor) muscle were recorded by inserting two copper wires (diameter 50 μm, insulated except for the tips) closely together through the cuticle of the proximal femur. Muscle potentials from the flexor were recorded together with extensor tibiae (extensor) potentials, which could be distinguished by their smaller amplitude and correlation with leg movements (Fischer et al. 2001). All extracellular recordings were amplified and bandpass filtered (50 Hz–10 kHz).

Intracellular recordings of motor neurons (MNs) were made from their arborizations in the neuropil of the mesothoracic ganglion. Glass micropipettes (GB100-TF8P, Science Products, Hofheim, Germany) were pulled on a P-97 filament puller (Sutter Instruments, Novato, USA) and filled with 3 M KAc/0.05 M KCl tip solution (electrode resistance 15–25 MΩ). The signals were amplified with a SEC-10L intracellular amplifier (npi electronics, Tamm, Germany) in bridge or discontinuous current-clamp (DCC) mode. During recordings in DCC mode, switching frequency of more than 12 kHz was used and electrode potential was monitored on an oscilloscope. MNs were identified by leg movements after injection of depolarizing current that caused a firing of action potentials and by correlation with muscle potentials in the EMG recordings (for details, see Gabriel et al. 2003).

(d) Data recording and evaluation

The electrophysiological data and the voltage output of the tachometer were digitized with a Micro1401 A/D converter and recorded with SPIKE2 software (both Cambridge Electronic Design, Cambridge, UK) on a personal computer. For further data evaluation, custom SPIKE2 script programs were written. Before averaging the membrane potential, action potentials were eliminated from the intracellular recordings by substituting a straight line (maximal 5 ms before to 5 ms after the peak of action potential).

(e) Statistics

Regression analyses were performed with ORIGINPro (MICROCAL, v. 7.5), a correlation was assumed at $p<0.05$. The following symbols show the level of statistical significance: $-$, not significant; *, $0.01 < p < 0.05$; **, $0.001 < p \leq 0.01$; ***, $p \leq 0.001$. In the text, $N$ gives the number of experiments or animals while $n$ gives the sample size. Values are given as mean±s.d.

3. Results

(a) Description of walking movements

After the animal was positioned above the treadmill, it was usually resting. Walking episodes could be elicited by a brief puff of air or tactile stimulation with a soft paintbrush to the abdomen or antennae (Bässler 1983). Upon stimulation, the animal started to perform walking movements with the middle leg and showed other signs of arousal (searching movements of the antennae, bending of the abdomen). After walking movements were initiated, the stimulation was
terminated. A photograph of the middle leg is shown in figure 1a, with curved arrows symbolizing the movement around the leg joints responsible for single-leg stepping. During stance, the belt was moved by flexion of the tibia. Stance phase was defined as the time of flexor tibiae (flexor) MN activity recorded with the

Figure 2. (Caption opposite.)
Figure 2. (Opposite.) (a) Example of three steps with different mean belt velocities (8.3–4.4 cm s\(^{-1}\)) recorded within a stepping sequence from a slow flexor (sFlex) MN. During the stance phase of fast steps, the neuron showed a larger depolarization and was firing more action potentials (note instantaneous AP frequency) than during slow steps. (b) Quantification of peak and trough potential, spike frequency and belt velocity during the stepping sequence from (a); asterisks mark the steps shown there. Along with the maximum and mean belt velocity, the maximum and mean spike frequency decreased during this stepping sequence, as did the peak membrane potential during stance. The trough membrane potential during swing remained constant. (c) Example of three steps with different mean belt velocities (2.5–0.5 cm s\(^{-1}\)) recorded within a stepping sequence from a slow extensor (SETi) MN. The depolarization and action potential activity was similar during all steps. (d) Quantification of peak and trough potential, spike frequency and belt velocity during the stepping sequence from (c); asterisks mark the steps shown there. The maximum and mean belt velocity decreased during this stepping sequence, while the maximum and mean spike frequency changed only slightly. Neither peak nor trough potential showed the same variation as belt velocity.

Control of stepping velocity in an insect leg

Phil. Trans. R. Soc. A (2007)

EMG. It should be noted that the onset of EMG activity occurred approximately 30 to several hundreds of milliseconds before the contraction force of the flexor muscle was strong enough to move the belt (for details, see Gabriel et al. 2003). Swing phase was defined as the rest of the step cycle. During swing, the leg was lifted and the tibia was extended. Extensor tibiae (extensor) MN activity started at the beginning of swing phase. Sometimes there was a short pause between the last extensor action potential and the beginning of the next stance phase (Fischer et al. 2001; see below). Walking episodes typically consisted of 3–15 steps. The longest walking episode that was recorded consisted of 39 consecutive steps. A total of 25 recordings of flexor MNs (15 slow, 4 semi-fast and 6 fast) and 15 recordings of extensor MNs, i.e. 10 of the slow extensor tibiae MN (SETi) and 5 of the fast extensor tibiae MN (FETi), were performed.

(b) Correlation of cycle period and belt velocity

A section of a stepping sequence that consisted of a total of 12 steps is shown in figure 1b. The activity of the flexor muscle during stance is visible from the EMG and the intracellular recording of a fast flexor (fFlex) MN. During long stepping sequences, cycle period often increased while mean and maximum belt velocity decreased (figure 1c). In other cases, belt velocity did not only decline, but also increased within a sequence (cf. figures 4a and 5a). In general, there was a negative correlation between cycle period and mean belt velocity (figure 1d). In freely walking animals, both a short cycle period and a high mean belt velocity, i.e. a stronger and faster stance muscle contraction, would contribute to a high walking velocity. Therefore, steps with a short cycle period and a high mean belt velocity will be called ‘fast’ steps, while steps with a long cycle period and a low mean belt velocity will be called ‘slow’ steps.

(c) Amplitude of membrane potential modulation and spike frequency

In the stick insect, the membrane potential modulations of flexor MNs are generated by phasic excitatory and inhibitory synaptic inputs during walking (Schmidt et al. 2001). In addition, leg MNs are depolarized tonically throughout stepping sequences (figures 1b, 2a,c and 4a; see also Büschges et al. 2004; Ludwar et al. 2005b). During stance, flexor MNs are excited and their suprathreshold activity is responsible for the belt movement (Fischer et al. 2001), while they are
inactivated by inhibition during swing (Schmidt et al. 2001). Thus, the maximal depolarization (peak potential) in flexor MNs occurs during stance and the minimal (trough potential) during swing. When comparing the steps of different velocity, it was obvious that more flexor MNs were recruited during faster steps.
(figure 4b) and that activation of flexor MNs increased (figure 2a). Flexor MNs had a more depolarized peak potential and were firing more action potentials during fast steps than during slow steps (figure 2a,b). The values of peak potentials showed the same modulation as both maximum and mean belt velocity throughout the stepping sequence. Trough potential was not modulated and appeared to be independent of variations in mean belt velocity (figure 2b). This was true for all recorded flexor MNs.

The membrane potential of extensor MNs reached a peak during swing and a trough during stance. Contrary to the flexor MNs, the activity of extensor MNs was independent of the mean belt velocity during the previous stance phase (figure 2c). Neither peak nor trough potential showed systematic changes in parallel to maximum and mean belt velocity during the stepping sequence (figure 2d). The same was true in all recorded extensor MNs.

The relationship between mean belt velocity and peak and trough potential in flexor MNs is drawn in figure 3a. The recordings from four slow flexor MNs were selected that included a large number of steps over a large mean belt velocity range. There was a positive correlation between peak potential of flexor MNs and mean belt velocity, while trough potential was correlated with mean belt velocity only in one out of four neurons. In addition, the maximum and mean action potential frequency of flexor MNs was correlated with mean belt velocity (figure 3b). This was observed in all the 25 recorded flexor MNs; no difference between slow, semi-fast and fast MNs was detected.

In nearly all the recordings, stepping sequences started with a stance phase and ended with a swing phase (not shown). We compared the activity of extensor MNs with the mean belt velocity of the previous stance phase. The recordings from four extensor MNs (two slow and two fast) were selected that included a large number of steps, three of which included steps that covered a large mean belt velocity range. There was no consistent correlation of peak or trough potential (figure 3c) with mean belt velocity. This was observed in all the 15 recorded extensor MNs; no difference between SETi and FETi MNs was detected. In addition, the maximum and mean action potential frequency during swing was not correlated with the mean belt velocity during the previous stance.
Figure 4. (Caption opposite.)
phase (figure 3\textit{d}). This is supported by FETi recordings in the closely related stick insect species \textit{Carausius morosus} (von Uckermann 2004).

In walking, flexor and extensor MNs receive phasic inhibition during the activity of their respective antagonistic MNs (Schmidt et al. 2001; Gabriel & Büschges unpublished observation). As stated previously, there was no correlation of trough potential of extensor MNs with mean belt velocity and therefore not with flexor activity (figure 3\textit{c}). Only when comparing steps with very high flexor activity with those with very low flexor activity could we observe that the strength of inhibition of extensor MNs during stance, as concluded from the level of hyperpolarization, was somewhat correlated to the level of flexor activity (figure 3\textit{e}).

\textbf{(d) Time course of membrane potential modulation in flexor motor neurons}

The activity of a flexor MN during a walking sequence with steps of varying velocity is shown in figure 4\textit{a}. In order to analyse the changes in the time course of membrane potential modulation that accompanied variations in belt velocity, we compared steps with a high mean belt velocity (figure 4\textit{b}, left) with those with a low velocity (figure 4\textit{b}, right). When the intracellular traces were aligned at the
time of the beginning of flexor EMG activity (figure 4c), it became apparent that flexor MNs were depolarized in a similar way at the beginning of stance during both slow and fast steps (see arrows in figure 4b–d). After this initial depolarization, the membrane potential quickly depolarized to its maximal value during fast steps, while the depolarization ebbed before a second depolarization to the maximal value could be observed during slow steps. The same was true when comparing the average membrane potentials recorded during the 10 steps with the highest mean belt velocity with those that showed the lowest mean belt velocity for a given recording (figure 4c, bottom left). At the end of the flexor EMG activity (i.e. the transition from stance to swing phase), the membrane potential rapidly hyperpolarized both in fast and slow steps (figure 4c, top right; averages bottom right). Note that the depolarization at the transition from swing to stance was, in general, slower than the hyperpolarization at the transition from stance to swing. The results described previously were true for all flexor MN recordings.

The depolarization at the beginning of stance phase was further analysed by averaging the membrane potential of flexor MNs during steps with intermediate mean belt velocity. In all the cases, the time course of the initial depolarization was similar, while its amplitude appeared to be slightly smaller during slow steps as compared with faster steps. During slow steps, the second depolarization to the maximal value appeared late and with a slow time course. During faster steps it appeared sooner, had a faster time course, and a larger amplitude.

As mentioned previously, flexor MNs are tonically depolarized throughout stepping (Ludwar et al. 2005b; see §3c) and phasically inhibited during swing (Schmidt et al. 2001). We were interested to know whether the initial depolarization could be the result of the termination of this inhibition. In figure 4e, an intracellular recording of a flexor MN is shown, together with the extensor EMG (see §2). Note that extensor activity was the highest during the first half of swing and then slowly decreased. The membrane potential of the flexor MN showed little modulation during swing. Injection of short hyperpolarizing current pulses revealed that while extensor activity decreased, the input resistance of flexor MNs increased (N=13). Figure 4f shows quantitatively that input resistance steadily increased during the end of swing and the beginning of stance phase. The solid vertical line marks the beginning of the initial depolarization (0.07±0.061 s before the beginning of stance; N=4, n=128) and the dashed vertical line marks the standard deviation. The stereotypic initial depolarization occurs during an increase in input resistance, indicating that a release from inhibition may be involved in its generation. This is supported by the fact that the amplitude of the underlying tonic depolarization (3.9±2.2 mV; range 1.3–9.5 mV; observed in N=18 out of 25 MNs, n=28) was also stereotypic and not correlated with mean belt velocity (figures 1b, 2a and 4a).

(e) Time course of membrane potential modulation in extensor motor neurons

All the 17 recorded extensor MNs rapidly depolarized at the transition from stance to swing phase and fired action potentials within 5–30 ms (figure 5a,b). The membrane potential modulation recorded during steps with a high mean belt
velocity (figure 5b, left) was compared with that during steps with a low mean belt velocity (figure 5b, right). Both the hyperpolarization at the beginning of stance and the depolarization at the beginning of swing were similar during fast and slow steps (figure 5c, top), with the hyperpolarization being slower than the
depolarization. The averaged membrane potential modulation during the four steps with the highest and the four steps with the lowest mean belt velocities recorded from this neuron (figure 5c, bottom) confirms this finding. The results described previously were true for all extensor MN recordings.

In 50% of the steps at the end of swing phase, there was a pause between the last extensor spike and the onset of flexor activity during which the neuron remained depolarized (figure 5b; duration 0.42 ± 0.360 ms; 33 out of 66 steps recorded in six animals). The duration of stance phase (i.e. flexor activity) and swing phase (i.e. the phase of extensor depolarization that lasted from the first extensor spike until the next flexor activity) was evaluated more closely (figure 5d). As shown before in figure 1d, cycle period is negatively correlated with mean belt velocity. While the duration of the stance phase also showed a negative correlation, the duration of swing phase was independent of mean belt velocity, which shows that extensor activity was more stereotypic than flexor activity with respect to both magnitude and duration.

4. Discussion

We analysed the changes in step parameters and motor neuronal activity that occurred during variations in stepping velocity of the stick insect middle leg on a treadmill. As reported previously for the intact walking animal (Wendler 1964; Graham 1972), changes in stepping speed were accompanied by changes in cycle period, such that fast stepping velocities were correlated with short cycle periods. Intracellular recordings revealed that during fast steps, flexor MNs showed a faster depolarization and a stronger activation. Interestingly, alterations in the time course of membrane potential related to changes in stepping velocity were almost exclusively limited to stance phase (i.e. flexor) MNs, while there were no systematic alterations detectable in swing phase (i.e. extensor) MNs. This indicates that stepping velocity is mediated by phasic neural mechanisms effective only during the generation of the stance phase motor output. Most importantly, we did not detect any evidence for both MN pools being affected in a similar way during changes in stepping velocity; for example, by a tonic background excitation that increases excitability of MNs during a high motor output. Therefore, it appears that the mechanisms responsible for generating changes in stepping velocity specifically affect the neural subsystem generating stance phase motor output, but not (or only subtly) the part of the premotor network, which generates the swing phase in the single middle leg.

(a) Cycle period of single leg stepping movements and varying stepping velocity

During steps with a high mean belt velocity, both the cycle period (figures 1d and 5d) and the duration of flexor MN activity (i.e. stance) were short, while the duration of extensor activity (i.e. swing) remained unchanged (figure 5d). Also in freely moving animals, a short cycle period contributes to high walking speeds, and the decrease in cycle period is mainly due to a decrease in stance duration (Wendler 1964; Graham 1972).

We have shown that swing duration did not correlate with mean belt velocity. Furthermore, as we have shown that mean belt velocity in the single middle leg correlates with cycle period, our data imply that swing phase duration is
independent of cycle period. This result differs from Fischer et al. (2001), who concluded on the basis of EMG recordings only that the duration of both stance and swing phases depended on cycle period (their fig. 3d). At present, we cannot give a conclusive explanation for this difference, because a simple comparison of the results of both investigations is not possible; Fischer et al. (2001) did not monitor the velocity of their treadmill, so it is not possible to estimate the range of belt velocities that were generated. The treadmill used in their investigation had a higher inertia, and since it has been shown that slower steps are generated on high inertia treadmills (Gabriel et al. 2003), it is probable that belt velocities were shifted towards lower values.

(b) Time course in membrane potential of flexor and extensor motor neurons

Our analysis proceeded to determine constant and variable features in the time course of membrane potential modulation of flexor and extensor MNs during walking movements. From our results it is clear that all neurons of the flexor motor pool contribute to the control of stepping velocity during stance phase. In all flexor MNs recorded, the membrane potential depolarization as well as the mean and maximum spike frequency was correlated with mean belt velocity (figure 3a,b). This indicates that during stance, flexor MNs receive common synaptic drive that contributes to the variations in stepping velocity. In this, the results are similar to the flexor MN activity under conditions of varying load (Gabriel et al. 2003).

Interestingly, there appear to be two distinct phases of depolarization in the activation of flexor MNs during stance phase, i.e. an initial depolarization at the beginning of flexor activity and a subsequent fast and large depolarization that changes with stance velocity (figure 4c,d). All flexor MNs recorded received a similar initial depolarization, which for some MNs (i.e. the slow flexor MNs due to their earlier recruitment; Gabriel et al. 2003) was sufficient to bring them above the action potential threshold and initialized the flexor burst. For individual MNs, the time course of this depolarization at the beginning of stance was similar during slow and fast steps. We could show that it occurred well after the maximal conductance in the flexor MNs during swing (figure 4e,f). This maximal conductance is generated by inhibitory synaptic inputs (Schmidt et al. 2001; Gabriel & Büschges 2005, unpublished work). In this phase, the input resistance in flexor MNs was still increasing (figure 4f). On the one hand, excitatory synaptic inputs are conceivable that are related to touch down of the leg, e.g. from tarsal receptors (Laurent & Hustert 1988) or campaniform sensilla on the leg (Newland & Emptage 1996; Akay et al. 2001). However, on the other hand, some arguments point towards a different origin. We believe that the initial depolarization is the result of an interplay between a release from the inhibition that was active during leg swing and the state-dependent tonic depolarization in leg MNs, which persists during stepping activity (Büschges et al. 2004; Ludwar et al. 2005b) for the following reasons: (i) the initial depolarization developed comparably slowly and, like the tonic depolarization, was independent of stance velocity (figures 1b, 2a and 4a), and (ii) it occurred although the input resistance of flexor MNs was still increasing from its minimal values during leg swing, indicating that it was not probably generated by an additional phasic synaptic conductance.
Even though we do not know the cause of the initial depolarization of flexor MNs at the beginning of leg stance, the fact that it is independent of stepping velocity has important implications for the control of stance velocity. It indicates that at this time during the step cycle, no neural inputs to the flexor MNs were active that caused a differentiation towards slow or fast stance phases.

The second depolarization of flexor MNs depended on stepping velocity, i.e. it occurred earlier and was larger for fast steps compared with slow steps (figure 4d). It was this depolarization by which fast flexor MNs were activated above the action potential threshold and that therefore contributed mostly to alterations of stepping velocity. There are different possibilities for how this second depolarization is generated. Previous investigations show that sensory signals from strain and movement sensors reinforce flexor MN activation during voluntary and locomotor movements (Bässler 1986; Akay et al. 2001; summary in Bässler & Büschges 1998), as has been shown in a variety of walking systems (for summary, see Pearson 1993; Büschges & El Manira 1998). At present, it is not known what other sources of synaptic inputs are contributing to the control of flexor MN activity. No evidence exists for a contribution of phasic excitatory synaptic drive from central pattern-generating networks of the femur–tibia joint. On the contrary, only phasic inhibitory synaptic inputs from central sources are known that could contribute to patterning of MN activity (Büschges 1998; Büschges et al. 2004). Given that some of the synaptic drive that controls flexor MN activation arises from sense organs, it is quite conceivable that changes in the effectiveness of these pathways may alter rate and amplitude of activation in flexor MNs. Such alterations will occur automatically with changes in stance velocity, e.g. by faster flexion of the joint or greater forces generated by the flexor muscle. However, a mechanism would still be needed through which the leg muscle control system can control the effectiveness or gain of these sensory feedback pathways to achieve a stronger motor output. For changing cycle period in walking, similar solutions via the sensory feedback from the limb have recently been discussed for the cat (Yakovenko et al. 2005). One possible mechanism is the presynaptic inhibition of sensory afferents, which has been discussed in great details with respect to its putative function in gain control (Burrows & Matheson 1994; Sauer et al. 1997; for summary, see Nusbaum et al. 1997; Clarac et al. 2000). It is conceivable that by a reduction in presynaptic inhibition, the gain of the reinforcing sensory pathways during stance increases and larger, more rapid, depolarizations of flexor MNs are generated. Further experiments involving recordings from the major sensory afferents, i.e. those of the campaniform sensilla and the femoral chordotonal organ, will be necessary for an unequivocal conclusion. In summary, our results strongly suggest that those synaptic inputs to leg motor neurons that determine the stance phase motor output and thereby the stepping velocity are only activated during an already ongoing stance phase motor output.

(c) Antagonistic synaptic drive to flexor and extensor MNs and organization of the leg muscle control system for the single leg

During the transitions between step phases, particular antagonistic features were observed in the membrane potential modulations of flexor and extensor MNs (figure 6). At the transition from stance to swing phase, the membrane
potential of both MN pools rapidly changed, i.e. flexor MNs were hyperpolarized and extensor MNs were depolarized rapidly. On the other hand, at the transition from swing to stance phase, both the depolarization of flexor MNs and the hyperpolarization of extensor MNs were generally slower.

There was no close correlation between mean belt velocity (which represents the level of flexor activity) and trough potential of extensor MNs during stance (figure 3c). This may be due to the small difference of the membrane potential from the reversal potential of the inhibitory conductance (Gabriel & Büschges in preparation). Therefore, both weak and strong inhibitions could hyperpolarize the extensor MNs to the same value. The antagonistic nature of the synaptic drive to flexor and extensor MNs is supported by two observations: (i) for gross differences, the flexor activation level during stance was correlated to some extent with the level of hyperpolarization of extensor MNs (figure 3e), and (ii) measurements of input resistance qualitatively show that the level of inhibition of flexor MNs during swing was correlated with extensor activity. Together, this raises the possibility that both antagonistic MN pools share common premotor elements that contribute to phase transitions. Indeed, in the stick insect, mesothoracic ganglion local premotor non-spiking interneurons exist that provide excitatory drive to one pool of MNs, e.g. the extensor MNs and the inhibitory synaptic drive to the antagonistic pool, e.g. the flexor MNs, and vice versa (Büschges & Schmitz 1991; Büschges 1995; Sauer et al. 1996). In another orthopteran insect (i.e. the locust), MNs of antagonistic pools have been shown to receive ‘mirror-image synaptic drive’ from certain premotor interneurons (Burrows & Horridge 1974; summary in Burrows 1996). In a functional context this may be useful, because co-contractions of antagonistic muscles are both energetically unfavourable and potentially harmful for the animal (Cruse 2002). In order to further analyse the nature of the antagonistic drive from premotor elements, paired recordings from flexor and extensor MNs will be necessary.

In the single middle leg preparation, a basic antagonistic nature of the synaptic drive to tibial MNs exists during the generation of stepping movements (see above and Schmidt et al. 2001). However, there appears to be no influence.
between stance and swing phase generations in such a way that the magnitude of motor output during stance would influence the subsequent swing phase. While flexor activation was strongly modulated with changing step velocity, no correlated alterations were found in the pattern of extensor MN activation. The time course of the transitions between the stepping phases were not affected by stance phase velocity either. These results are interesting in the light of current conclusions concerning the organization of the stick insect walking system, as derived from behavioural studies on intact walking animals (e.g. Cruse & Müller 1984; Schmitz et al. 2000; see also Cruse 2002). It has been reported in the stick insect that, depending on the actual magnitude of motor output during stance, i.e. comparing uphill versus downhill walking, the velocity of the subsequent swing phase was altered (Schmitz et al. 2000). This result corroborated considerations about a very close coupling of the neural networks in charge of generating stance and swing phases (Cruse 2002). Our intracellular analysis of leg motor neuron activity in single leg stepping indicates that such influence of the magnitude of motor output during stance and the subsequent swing phase may not be a property of the local pattern-generating networks of a leg, but rather arise from intersegmental sources. Both phasic and tonic influences are known to act between the legs of a walking stick insect (Cruse et al. 1998; Ludwar et al. 2005a, b). Thus, it may well be that the influence of stance phase on the subsequent swing phase arises from the cooperative action of the neural networks coordinating the walking movements of the six legs.

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