Task-dependent modification of leg motor neuron synaptic input underlying changes in walking direction and walking speed

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Rosenbaum P, Schmitz J, Schmidt J, Büschges A. Task-dependent modification of leg motor neuron synaptic input underly- ing changes in walking direction and walking speed. J Neurophysiol 114: 1090–1101, 2015. First published June 10, 2015; doi:10.1152/jn.00006.2015.—Animals modify their behavior constantly to perform adequately in their environment. In terrestrial locomotion many forms of adaptation exist. Two tasks are changes of walking direction and walking speed. We investigated these two changes in motor output in the stick insect Carcandelina impigra to see how they are brought about at the level of leg motor neurons. We used a semi-intact preparation in which we can record intracellularly from leg motor neurons during walking. In this single-leg preparation the middle leg of the animal steps in a vertical plane on a treadmill. Stimulation of either abdomen or head reliably elicits fictive forward or backward motor activity, respectively, in the fixed and otherwise deafferented thorax-coxa joint. With a change of walking direction only thorax-coxa-joint motor neurons protractor and retractor changed their activity. The protractor switched from swing activity during forward to stance activity during backward walking, and the retractor from stance to swing. This phase switch was due to corresponding change of phasic synaptic inputs from inhibitory to excitatory and vice versa at specific phases of the step cycle. In addition to phasic synaptic input a tonic depolarization of the motor neurons was present. Analysis of changes in stepping velocity during stance showed only a significant correlation to flexor motor neuron activity, but not to that of retractor and depressor motor neurons during forward walking. These results show that different tasks in the stick insect walking system are generated by altering synaptic inputs to specific leg joint motor neurons only.

ANIMAL LOCOMOTION is the result of a finely tuned output of the nervous system. Tuning motor output allows an animal to move through its often unpredictable environment, e.g., by changing movement direction or velocity. For example, an animal that encounters an obstacle has to change its walking direction; by encountering a predator the animal has to flee and therefore change its walking speed. But how does the nervous system or, more specifically, the leg-muscle control system generate these changes? The well accessible nervous and muscular system and a locomotor behavior that is easy to record make insects particularly well suited animals for studying adaptive neural mechanisms (e.g., Büschges 2012; Ritzmann and Büschges 2007). Changes of locomotor output have been studied for a variety of tasks, such as curve walking (e.g., Dürr and Ebeling 2005; Jander 1982), climbing obstacles (e.g., Theunissen et al. 2014; Wosnitza et al. 2013b), tunnel under objects (e.g., Harley et al. 2009), changes in walking speed (e.g., Gruhn et al. 2009; Wendler 1964), or changes in walking direction (Akay et al. 2007; Graham and Epstein 1985; Rosenbaum et al. 2010). Here, we examined changes in motor behavior by analyzing the generation of motor output and the synaptic input it receives in the stick insect walking system in varying conditions for locomotion as it climbs mainly in bushes and thus in a highly irregular environment (Bässler 1983).

Rhythmic leg motor neuron activity in the stick insect is based on tonic and phasic synaptic inputs. A tonic depolarization underlies motor neuron activity during stepping and is initiated prior to stepping and outlasts the stepping activity (Büschges et al. 2004; Ludwar et al. 2005). Rhythmicity of motor neuron activity results from additional phasic depolarizing and hyperpolarizing synaptic inputs. The phasic excitatory and inhibitory synaptic inputs arise from signals of leg sense organs on the same leg, signaling load, posture, and movement of leg joints (e.g., Akay et al. 2001; review in Büschges and Gruhn 2007). Such sensory input can be transmitted directly to leg motor neurons or via intercalated spiking and nonspiking interneurons (e.g., Burrows 1996; Sauer et al. 1997). Additional inhibitory synaptic inputs to leg motor neurons are likely to arise from the activity of central pattern-generating networks in the thoracic ganglia, so-called CPGs (Büschges 1998; Büschges et al. 2004; see Goulding 2009; Marder and Calabrese 1996 for general reviews on CPGs). However, all these insights have been collected in highly reduced preparations or in preparations in which walking direction and walking speed were not controlled for. An appropriate experimental preparation in which leg stepping in stick insects can be studied is the single-leg preparation (Bässler 1993). It offers the opportunity to obtain stable intracellular recordings from motor neurons in the thoracic ganglia supplying the stepping leg without phasic step cycle-dependent sensory signals from other walking legs (Fischer et al. 2001; Gabriel and Büschges 2007; Gruhn et al. 2009; Schmidt et al. 2001). In this setup, the leg is only able to move in the vertical plane. Although the thorax-coxa joint is deafferented and deafferented the activity of motor neurons innervating this joint is coupled to the movement of the two distal leg joints (Fischer et al. 2001) due to sensory signals of load sensing organs (trCS; Akay et al. 2004; Akay et al. 2007). Here, we present data on the synaptic drive leg motor neurons receive from premotor networks when walking direction or speed changes.
In mammals (Buford et al. 1990; Buford and Smith 1990; Pratt et al. 1996), as well as in insects (Fischer et al. 2001), forward and backward stepping is characterized by a modification of the coordination in activity between the proximal and the distal leg joints. These joints are the hip joints in mammals and the thorax-coxa (ThC) joints in insects, and, more distal, knee and ankle joints in mammals, and coxa-trochanter (CTr) and femur-tibia (FTi) joints in insects (Graham 1985; Rosenbaum et al. 2010). Such modification allows for the generation of opposite ground forces during leg stance necessary to invert stance direction. During forward stepping of a stick insect middle leg stance is generated by the simultaneous activity of retractor coxae (RetCx), depressor trochanteris (DepTr) and flexor tibiae (FlxTi). During backward stepping DepTr and FlxTi activity remains the same, now accompanied by protractor coxae activity (ProCx; Rosenbaum et al. 2010). How synaptic inputs to leg motor neurons change to achieve this change in motor output was examined in this study.

In slow walking animals like the stick insect, changes in walking speed are almost exclusively mediated by modifying stance motor output, as previously shown for FlxTi motor neurons (Gabriel and Büschges 2007; Rosenbaum et al. 2010; Wendler 1964). Is premotor synaptic drive to other stance leg motor neurons also modified, and if so are those modifications generated differentially for each leg joint or together for all joints by the premotor network?

Our results show for the first time in a locomotor system how the motor activities for different motor tasks are achieved by altering synaptic inputs to leg motor neurons. We show that fictive forward and backward walking is generated in the single middle leg preparation. Qualitative similar synaptic inputs, a tonic depolarization, phasic inhibitory and excitatory inputs, underlie the rhythmic modulation of all leg motor neurons during both forward and backward stepping. Only phasic synaptic inputs to ProCx and RetCx motor neurons change from swing to stance phase activity and vice versa during backward walking. Furthermore, stepping velocity on the treadmill is only significantly correlated with FlxTi motor neuron activity, but not with the activity of other stance phase motor neurons during forward walking. Our results show that modifications in walking direction and walking speed in the stick insect leg muscle control system are mediated in a joint-specific fashion.

METHODS

Preparation and Experimental Setup

Stick insects of the species Cuniculina impigra were used for all experiments, performed under dimmed light conditions at room temperature. Animals were provided by the breeding colony from the University of Cologne, reared in a 12:12-h light-dark cycle at 20°C, and fed with blackberry leaves Rubus fruticosus. Animals were provided by the breeding colony from the University of Cologne, reared in a 12:12-h light-dark cycle at 20°C, and fed with blackberry leaves Rubus fruticosus.

The single-leg preparation was first introduced by Karg et al. (1991), and modified in various subsequent studies for intracellular recordings from motor neurons and premotor interneurons in the thoracic ganglia (Fig. 1: Berg et al. 2013; Fischer et al. 2001; Gabriel and Büschges 2007; Schmidt et al. 2001; von Uckermann and Büschges 2009). The preparation allows the investigation of walking-like movements of a single leg without sensory information from movements and forces generated in adjacent legs. All legs except the left middle leg were cut at mid-coxa level and the animal was glued ventral side down on a foam platform with dental cement (Pro TempII, ESPE, Seefeld, Germany). The ThC joint was fixed with dental cement to allow only leg movements in the vertical plane. The thorax of the animal was opened with a cut along the dorsal midline. Gut, minor trachea, and fat tissue were removed in order to expose the mesothoracic ganglion. The body cavity was filled with saline (pH = 7.2, according to Weidler and Dicke 1969). ProCx and RetCx muscles were cut at their ventral thorax insertion. Stepping of the middle leg was allowed on a custom-made low-friction treadmill composed of two styrofoam drums with 40-mm diameter and a center distance of 50 mm, connected via a belt of crepe paper. Each of the two drums was mounted on a micro DC-motor (DC 1516, Faulhaber, Schönaich, Germany) of which one measured the belt velocity and the other supported the belt movement to give a low friction (for details, cf. Gabriel et al. 2003). The treadmill height was adjusted below the middle leg of the stick insect, so that the femur of the leg was parallel to the belt when the femur-tibia joint angle was about 90°. Forward walking was elicited by gently touching the abdomen of the animal with a brush, backward walking by touching/slightly pulling on the antennae or stimulating the head/prothorax region with a soft paint brush.

Electrophysiology

To monitor the walking direction, ProCx (nl2) and RetCx (nl5) motor nerves were recorded extracellularly by placing the nerves on to steel hooks of monopolar electrodes (modified after Schmitz et al. 1991). The nerves were then pinched distally to the recording site to abolish afferent and efferent signaling. Additionally, FlxTi muscle activity was recorded by inserting two twisted copper wires (49-μm diameter) into holes in the cuticle of the posterior portion of the proximal femur, fixed with dental cement (electromyogram, EMG). Extracellular nerve and EMG signals were amplified 1,000-fold and low-pass filtered (preamplifier MA101 and amplifier MA102, electronics workshop, Institute for Zoology, Univ. of Cologne). Fat tissue surrounding the mesothoracic ganglion was removed and the ganglion was lifted onto a wax-coated steel ganglion holder and positioned with a micromanipulator. To stabilize the ganglion, which was necessary to achieve long-lasting intracellular recordings from neurons in the ganglion during walking, it was fixed on the holder by placing cactus spines (Nopalea

Fig. 1. Schematic drawing of the single-leg preparation. The stick insect is glued to a platform and the thorax is opened dorsally. Middle-leg stepping is allowed only in the vertical plane on a treadmill belt; belt movement is monitored. A flexor tibiae (FlxTi) electromyogram (EMG) in the proximal femur serves as an additional stance phase indicator. Extracellular hook electrodes record the activity of the protractor coxae (ProCx) and retractor coxae (RetCx) motor nerves. The mesothoracic ganglion is held in place by a ganglion holder to allow stable intracellular recordings. Modified with permission from von Uckermann (2008).
dejecta) through the surrounding tissue. Lateral nerves on the contralateral side of the ganglion were pinched. Before recording intracellularly from the hemiganglion ipsilateral to the walking leg, the ganglion surface was treated with a proteolytic enzyme (Pro-nase E, Merck, Germany) to ensure an easy electrode penetration. Sharp microelectrodes with a resistance of 15–25 MΩ were pulled using a Sutter Micropuller (P-1000, Sutter Instruments, Novato, CA) and filled with a solution with 3 M KAc/0.1 M KCl. Signals were recorded in bridge mode using an intracellular amplifier (SEC-10L, npi electronics, Tamm, Germany).

Identification of Leg Motor Neurons

Motor neurons (MNs) were recorded from their neuropil arborizations in the hemiganglion ipsilateral to the walking leg. They were identified either by a 1:1 correlation of spikes in the intracellular recording with spikes in the extracellular recording (ProCx, RetCx, FlxTi, C11) or by an unambiguous movement response of the leg due to depolarizing current injection (extensor ExtTi, DepTr, levator LevTr MNs). In some experiments motor neurons were identified via dye fills. Neurobiotin tracer (5%, Vector Laboratories, Burlingame, CA) diluted in electrode solution was injected with depolarizing current pulses (1.5–2.5 nA, 400-ms pulse duration, 1 Hz) for 3–20 min. Thirty to forty-five minutes was allowed for tracer diffusion. The ganglion was removed from the animal and treated for 20 min with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) and 5% Triton X-100 (Fluka, Buchs, Switzerland), and then fixed for 2–14 h in 4% PFA. After washing 3 times with PBS, ganglia were incubated with the dye-coupled Neurobiotin antibody Streptavidin-Cy3 (1:500 in PBS, Sigma-Aldrich, St. Louis, MO) with 0.5% Triton X and 2–4% normal goat serum (Vector Laboratories, Burlingame, CA) overnight on a shaker at 4°C. After washing (3 times with PBS), ganglia were mounted in methyl-salicylate on an object slide and scanned with a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Data Analysis

Data were analyzed with Spike2 software (CED, Cambridge, UK) using custom-written scripts, Microsoft Excel 2007, and Origin 5. Figures were prepared with CorelDrawX4. For the statistical analysis of membrane potential and mean belt velocity regression, an ANOVA analysis, P < 0.05, in Origin 5.0 was performed.

Mean belt velocity was determined by dividing the integral of the treadwheel trace below the ascending slope (stance) by stance duration. By multiplying with a factor of 12.6 (treadwheel potential output × amplification × time/distance), mean belt velocity (in cm/s) was calculated.

Stance end was marked at the end of the ascending slope of the treadwheel trace. However, in some cases it was observed that at the end of stance the tarsus of the animal was clawed to the crepe paper of the treadwheel, and was pulling it towards the body. Some milliseconds later it disengaged from the belt to start a new swing phase. In most of the cases this could be clearly detected either by an artifact in the treadwheel trace at the time point in which the tarsus disengaged and/or by the switch from RetCx to ProCx motor neuron activity during forward walking or vice versa during backward walking. Steps in which switching from RetCx to ProCx motor neuron activity or vice versa could not be determined unequivocally were discarded from the analysis because they did not allow for the determination of the walking direction.

Throughout the text we will identify quantitative measures as follows: N = number of animals, n = number of steps.

RESULTS

Fictive Forward and Backward Stepping Motor Activity Generated in the Single Middle Leg Preparation

In a first series of experiments we analyzed the activity of ThC motor neuron pools during stepping in the single middle leg preparation, in which the ThC joint is fixed and its muscles are denervated. To monitor the activity of these motor neurons, extracellular recordings were taken from the mesothoracic motor nerves nL2 and nL5 that contain the axons of the ProCx (nL2) and RetCx motor neurons (nL5; Fig. 2A). In addition, activity of the FlxTi muscle was recorded as a stance phase indicator. In the freely walking stick insect forward walking is known to be elicited by touching the animal with a brush at the abdomen, and backward walking by pulling on the antennae or stimulating the antennae/head region with a brush (Graham and Epstein 1985; Rosenbaum et al. 2010). During single-leg stepping we found a clear dependence of ThC motor neuron activity on the stimulus location (Fig. 2). Tactile stimulation of the abdomen induced single-leg stepping with well-coordinated activity in ThC motor neurons (Fig. 2A, left panel). ProCx motor neurons were active during leg swing along with LevTr and ExtTi. RetCx motor neurons were active during leg stance along with DepTr and FlxTi, an interjoint coordination pattern resembling forward stepping (Rosenbaum et al. 2010). When the antennae were stimulated (Fig. 2A, right panel) ThC motor neuron activity showed a phase change such that now ProCx activity was coupled to leg stance, while RetCx activity was coupled to leg swing. This activity pattern resembled the motor activity during backward walking in a six-legged animal (Rosenbaum et al. 2010). The coordination between ThC motor neuron activity and stepping movements of the distal leg joints was highly reliable: 97% of the steps (N = 7, n = 112), elicited by abdominal stimulation, led to ProCx and RetCx motor neuron activity resembling “fictive” forward stepping (Fig. 2B). Antennal stimulation induced backward stepping activity in 86% of the steps (n = 116, Fig. 2B).

The dependence of motor coordination on stimulus location became even more obvious from evaluating the mean activity of ProCx and RetCx as a function of phase in the step cycle (Fig.2, C and D). During single-leg stepping induced by tactile stimulation of the abdomen, ProCx activity was confined almost exclusively to leg swing and RetCx activity to leg stance (Fig. 2C). The opposite was true when leg stepping was induced by tactile stimulation of the antennae (Fig. 2D). For the remainder of the manuscript we will refer to the fictive walking direction indicated by the relation of the extracellularly recorded motor neuron activity of the deafferented motor nerves nL2 and nL5 to the leg movement during abdominal or antennal stimulation when talking about forward and backward walking. In the following experiments we used these two conditions in order to study the synaptic inputs to leg motor neurons in the generation of a forward and backward motor output for leg stepping in the stick insect.

Modulation in Leg Motor Neuron Membrane Potential in Forward and Backward Stepping

Retractor and protractor coxae motor neurons. RetCx (N = 10) and ProCx (N = 5) motor neurons were recorded intracellularly from their neuropil arborizations in the hemiganglion ipsilateral to the walking leg (Goldammer et al. 2012). Both
motor neuron types were identified by comparing intracellularly and extracellularly recorded action potentials. Figure 3A shows an example of a RetCx motor neuron recording during forward and backward stepping. In agreement with the analysis of the extracellular recordings (Fig. 2), RetCx depolarized and generated action potentials in stance during forward and in swing during backward stepping, while it was hyperpolarized in swing during forward and in stance during backward stepping (Table 1; see also investigation of synaptic inputs below). Furthermore, a tonic depolarization of the membrane potential was present. We analyze this later in more detail (see below and Fig. 6).

As for RetCx motor neurons phasic synaptic drive to ProCx motor neurons changed during forward and backward stepping. They were depolarized in swing during forward stepping and in stance during backward stepping (Fig. 3B). Similar to RetCx motor neurons a tonic depolarization was present during walking in both directions.

**Levator and depressor trochanteris motor neurons.** To test whether phasic and tonic synaptic inputs change in motor neurons controlling more distal leg joints we recorded the membrane potential modulations of trochanteral motor neurons during forward and backward walking. Figure 4, A and B, shows the reliability of response due to abdominal or antennal stimulation in black, forward-directed steps; in dark gray, backward-directed steps; in light gray, uncoordinated steps. N = 7 animals, n steps abdominal stimulation = 112, n antennal stimulation = 116. C and D: analysis of ProCx and RetCx nerve activity during forward (C) and backward walking (D). Peristimulus time histograms of motor nerve activity over the phase of the step cycle are shown. The gray box on the left side of the plots marks the average stance phase duration; the black bar above shows the SD of the stance duration.
leg swing in single-leg stepping (see also Fischer et al. 2001; Schmidt et al. 2001). In general, the strongest activity with highest spike frequency was observed during stance. The leg is actively depressed at the end of swing and the DepTr motor neurons are active during stance to support the body weight. At the start of swing, when the leg has to be lifted off the ground, DepTr motor neurons stop firing. Injection of hyperpolarizing current revealed the presence of a tonic depolarization during stepping in both directions (see below). In LevTr motor neurons the modulation in membrane potential depended on the phase of the step cycle but was independent of the walking direction (Fig. 4B). In the generation of both stepping conditions a tonic depolarization of the LevTr motor neurons was apparent (Fig. 4B). LevTr motor neurons were depolarized at swing onset and repolarized prior to the start of the next stance. This was true for forward and backward stepping and nicely corroborates earlier observations of leg muscle activity (Rosenbaum et al. 2010).

**Flexor motor neurons.** Figure 4C shows the recording of a fast FlxTi motor neuron during forward and backward stepping. FlxTi motor neurons (N = 5) were depolarized early in leg stance in the generation of the motor output for both walking directions and hyperpolarized at the onset of leg swing. Injection of hyperpolarizing current revealed the presence of the tonic depolarization in both stepping directions, similar to the situation in RetCX and DepCx motor neurons (see below). In the forward walking sequence (Fig. 4C, left) two different stepping velocities and different depolarization amplitudes of the flexor membrane potential are emphasized by dashed lines. There was an apparent correlation between stepping velocity on the treadwheel and FlxTi motor neuron spike activity, which will be further analyzed below.

**Common inhibitor motor neurons.** In arthropods, leg muscles are not only controlled by excitatory motor neurons, but also by inhibitory motor neurons, so-called common inhibitors (CI, Ballantyne and Rathmayer 1981; Pearson and Bergman 1982). The presence of a tonic depolarization of the LevTr motor neurons during stepping is consistent with the presence of common inhibitor motor neurons (CI).

### Table 1. Resting membrane potential, amplitude of phasic modulation during forward and backward stepping, change in motor activity between forward and backward stepping, and correlation between stepping velocity and motor neuron activity

<table>
<thead>
<tr>
<th></th>
<th>Protractor</th>
<th>Retractor</th>
<th>Levator</th>
<th>Depressor</th>
<th>Flexor</th>
<th>CI1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential, mV</td>
<td>−62.1 ± 7.5</td>
<td>−63.2 ± 3.8</td>
<td>−62.8 ± 4.2</td>
<td>−60.3 ± 5</td>
<td>−63.2 ± 8.2</td>
<td>−59, −59</td>
</tr>
<tr>
<td>Mod fwd, mV</td>
<td>21.9 ± 4.9</td>
<td>15.1 ± 5.6</td>
<td>15.9 ± 4.7</td>
<td>15.3 ± 4.2</td>
<td>15.6 ± 2.8</td>
<td>11.6 ± 1.5</td>
</tr>
<tr>
<td>Mod bwd, mV</td>
<td>15.6 ± 5.3</td>
<td>18.3 ± 3.5</td>
<td>14.1 ± 2.3</td>
<td>15.7 ± 4.7</td>
<td>12.8 ± 3</td>
<td>10.6 ± 1</td>
</tr>
<tr>
<td>Change fwd/bwd</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Corr velocity</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>n fwd/bwd</td>
<td>43/30</td>
<td>91/24</td>
<td>50/17</td>
<td>108/31</td>
<td>93/27</td>
<td>22/13</td>
</tr>
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</table>

Values are means ± SD. Mod, modulation; fwd, forward; bwd, backward; Corr, correlation.
Today it is clear that the function of these motor neurons is to release muscle fibers from tonic forces and to allow the execution of fast movements (Bässler and Stein 1996; Wolf 1990). In a final set of experiments, we recorded intracellularly from the CI1 motor neuron (N/H11005, Fig. 5), one of three inhibitory motor neurons that innervates specifically proximal leg muscles, i.e., ProCx, RetCx, DepTr, LevTr, and the ExTi (Bässler and Storrer 1980; Goldammer et al. 2012; Graham and Wendler 1981; Schmitz 1986). CI1 was identified by comparing spikes in the intracellular trace with spikes in the extracellularly recorded ProCx and RetCx motor nerves. In Fig. 5 intracellular recordings of CI1 during forward and backward walking show a similar modulation with a depolarization in stance during forward and backward walking. The thick dashed lines in the bottom two traces on the left side help to distinguish the different amplitudes of the FlxTi membrane potential and the treadmill speed during the first two steps; thin dashed lines in A–C mark resting membrane potentials.

Fig. 5. Intracellular recording of a common inhibitor1 (CI1) motor neuron during forward and backward walking. Flexor EMG and treadmill mark the stance phase (gray bars). ProCx and RetCx nerve recordings indicate the walking direction. The intracellular recording of CI1 (4th trace) shows modulation with a depolarization and spikes in swing during both forward and backward walking.

Fig. 4. Intracellular recordings of depressor trochanteris (DepTr), levator trochanteris (LevTr), and FlxTi MNs during forward and backward stepping. A: slow DepTr MN activity during forward and backward walking is unchanged (4th trace). The MN shows action potentials during stance and most of swing, only at the onset of swing it stops firing. FlxTi EMG (1st trace) and treadmill trace (5th trace) serve as stance indicator, marked by gray bars. Extracellular ProCx (2nd trace) and RetCx (3rd trace) recordings indicate walking direction. B: an intracellular recording of a LevTr MN (4th trace) that is depolarized at the onset of swing during both walking directions. FlxTi, ProCx, and RetCx as in A. C: intracellular recording of a fast FlxTi MN (4th trace) during forward and backward walking shows a similar modulation with a depolarization in stance during forward and backward walking. The thick dashed lines in the bottom two traces on the left side help to distinguish the different amplitudes of the FlxTi membrane potential and the treadmill speed during the first two steps; thin dashed lines in A–C mark resting membrane potentials.
backward stepping are shown. CI1 was tonically depolarized throughout the stepping sequences. Action potentials were confined to leg swing during both walking directions. During leg stance the neuron was inhibited. The highest spike frequency occurred at the start of swing.

**Synaptic Drive to Leg Motor Neurons for Forward and Backward Stepping**

How does the synaptic drive to motor neurons compare between both stepping directions? Previous work showed that leg motor neurons receive a tonic depolarization plus alternating excitatory and inhibitory synaptic drive (Büsches et al. 2004; Schmidt et al. 2001). An example of this synaptic drive is shown for a recording from a FlxTi motor neuron during forward stepping in Fig. 6A. In this experiment depolarizing current was injected until the neuron spiked tonically. Then the abdomen was stimulated to elicit walking. Here, the FlxTi receives inhibitory inputs during leg swing (Fig. 6Ai). A marked tonic depolarization was revealed by the injection of hyperpolarizing current (Fig. 6Aii), outlasting the stepping sequence. This was true for ProCx, RetCx, LevTr, and DepTr motor neurons (not shown). Upon hyperpolarizing current injection the amplitude of the phasic synaptic excitation during stance increased compared with the walking sequence without current injection (cf. Fig. 4C). Finally, when injecting more hyperpolarizing current a reversal of the inhibitory synaptic drive to the motor neuron became apparent (Fig. 6Aii, arrows in inset).

From the intracellular recordings during forward and backward stepping the question arose how the switch in ThC motor neuron activity is achieved. To test whether the synaptic inputs cause this switch tonic current was injected during leg stepping in both directions (\(N = 5; \) Fig. 6, B and C). When depolarizing current was injected into a RetCx motor neuron until it was spiking tonically, a distinct hyperpolarization during swing was revealed (Fig. 6Bi). This demonstrated that the RetCx motor neuron received inhibitory synaptic inputs in leg swing during forward stepping. Hyperpolarizing the RetCx motor neuron led to increased amplitudes of excitatory inputs (Fig. 6Bii) compared with the regular condition (cf. Fig. 3A). A large tonic depolarization was obvious throughout the stepping sequence, and additional phasic depolarizations were detectable during stance in forward stepping. During backward stepping the phase of phasic excitatory and inhibitory inputs was reversed, now showing an inhibition during stance and depolarizing inputs during swing (Fig. 6C). Interestingly, the same tonic depolarization was detectable throughout the backward stepping sequence (Fig. 6, B and C). These recordings provide evidence that during backward walking RetCx motor neurons receive phasic inhibitory synaptic drive during leg stance and excitatory drive during leg swing.

In summary, all motor neurons showed a tonic synaptic depolarization and phasic excitatory and inhibitory synaptic drive during swing...
inputs. Importantly, a switch in phasic inputs in dependence on walking direction was joint specific and occurred exclusively in ProCx and RetCx motor neurons.

Velocity Dependence of Leg Motor Neuron Activity during Single Leg Stepping

Another example for a modification of leg motor output is a change in walking speed. In the middle leg, FlxTi motor neurons determine the stepping velocity on the treadmill by the magnitude of their activity, whereas no change was observed for ExtTi motor neuron activity (Gabriel and Büschges, 2007). Changes in FlxTi motor neuron activity are generated during ongoing stance only, pointing toward an important contribution of sensory feedback (Gabriel and Büschges 2007). It is important to note that in single-leg stepping the prime contribution to leg kinematics is arising from the activity of tibial motor neurons and muscles, with the FlxTi muscle pulling the treadmill during leg stance (von Uckermann and Büschges 2009). We were interested whether changes in stepping velocity are accompanied by alterations in stance motor neuron activity of the more proximal leg joints, i.e., the CTr- and the ThC-joint during forward stepping, or if this modification in motor output is joint-specific, similar to the change in walking direction.

Our initial recordings from FlxTi motor neurons confirmed the previous results by Gabriel and Büschges (2007). Figure 7A shows the correlation between stepping velocity and peak-to-trough amplitude of the FlxTi membrane potential modulation, and the mean spike frequency during stepping. In all stepping sequences the velocity is significantly correlated with FlxTi motor neuron membrane potential modulation (black solid lines). Also, the mean spike frequency of FlxTi motor neurons correlated with belt velocity.

Next, we compared the activity of other stance motor neurons to the stepping velocity on the treadmill, i.e., DepTr and RetCx motor neurons. DepTr motor neurons are active in stance and most of swing during single-leg stepping. In Fig. 7Bi the peak and trough changes in membrane potential compared with resting potential of one exemplary DepTr motor neuron recording are plotted vs. the mean belt velocity. For reasons of clarity, in Bii only 5 forward stepping sequences are shown. Asterisks mark significance (ANOVA, $P < 0.05$).
forward stepping. Peak-to-peak amplitude generally did not correlate with belt velocity. Only in one of five stepping sequences a negative correlation of belt velocity to peak-to-peak amplitude change of the membrane potential modulation was found (Fig. 7Bi, black lines). An analysis of the mean spike frequency during single steps showed no correlation.

RetCx motor neurons are active in stance during forward walking (Fig. 3A). Only little variation in the membrane potential peak and trough amplitudes at different belt velocities could be observed in an exemplary experiment (Fig. 7Ci). Furthermore, when comparing the mean belt velocity with peak-to-trough amplitudes and mean spike frequencies, no overall correlation with walking speed during fictive forward stepping was found (Fig. 7Cii, one exception for spike frequency).

The activity of ProCx, LevTr, and CI1 motor neurons was not correlated with the treadmill velocity (ProCx $N = 4, n = 30$; LevMN $N = 5, n = 50$; CI1 $N = 2, n = 22$, data not shown).

In summary, the velocity of a single leg stepping on the treadmill is significantly correlated only with FlxTi motor neuron activity during forward walking, showing a joint-specific modulation of motor neuron activity similar to the change of phasic inputs to coxal motor neurons during backward walking.

DISCUSSION

In the present study we have analyzed the synaptic drive from premotor sources onto motor neurons during forward and backward stepping in the stick insect. We were able to show that tactile stimulation depending on its location can initiate a specific leg motor output for forward and backward middle leg stepping. Despite the lack of self-movement-related sensory feedback, the direction-specific phasing of ThC motor neurons was typical for forward and backward stepping. Intracellular recordings from all motor neuron pools revealed that the activity pattern for forward and backward stepping motor output is generated by similar synaptic drive onto leg motor neurons, with an underlying tonic depolarization and excitatory and inhibitory synaptic drive patterning motor neuron activity.

Interestingly, we found that under experimental conditions in which only FlxTi motor neurons are the main determinants of stance performance, only these motor neurons receive a synaptic drive that is modified with stepping velocity.

Fictive Forward and Backward Walking

We found that in addition to a “forward walking” motor output, elicited by stimulation of the abdomen (Fischer et al. 2001), also a fictive “backward walking” motor output can be elicited by stimulating the head of the animal in the single-leg preparation. During fictive backward walking the activity of ProCx and RetCx motor neurons is changed by a switch of phasic inputs. This result shows that sensory initiation of forward and backward walking depends on stimulus location, i.e., stimulation of head or abdomen. In the denervated ThC-joint the coupling of ThC motor neuron activity to the activity of distal motor neurons does not rely on its own movement, but on a walking direction-dependent modification of activity elicited by intrasegmental signals from trochanteral campaniform sensilla (trCS, Akay et al. 2007). According to previous studies one may call the motor activity observed in response to the two different tactile stimulation sites as “fictive forward stepping” and “fictive backward stepping” (Grillner and Zanger 1979).

Our study furthermore shows that eliciting a walking direction-specific coordination pattern for single middle leg stepping does not depend on walking activity of other legs.

Synaptic Inputs to Leg Motor Neurons

We have found that synaptic inputs to leg motor neurons are qualitatively very similar during the generation of forward and backward walking. For both directions motor neuron activity was determined by a tonic depolarization and phasic excitatory and inhibitory synaptic inputs. For trochanteral and tibial motor neurons no difference was found between both stepping directions. Only in the ThC motor neurons, i.e., ProCx and RetCx motor neurons that switch phase in the step cycle with stepping direction, the sign of phasic synaptic drive was changing with walking direction. The tonic depolarization of insect motor neurons (see also Ludwar et al. 2005) can be compared with the effect of glutamatergic neurons in the brain stem of vertebrates (e.g., Shefchik and Jordan, 1985). These descending inputs provide excitation to motor neurons and interneurons in the spinal cord, in which central pattern generators (CPGs) are responsible for the coordinated motor output of the motor neurons (e.g., Grillner et al. 1995; Grillner 2003; Kiehn 2006; Roberts et al. 2008). Also the inhibitory motor neuron CI1 innervating all proximal leg muscles showed no change in activity. CI1 was activated at the transition from stance to swing in both stepping directions. This extends earlier findings by Büschges et al. (1994), who showed that CI1 is active in swing during forward walking. Wolf (1990) showed for CI1 activation in the locust that its activity allows for fast activation and speed of the leg during the swing movement. There is no need for CI1 to switch phase when walking direction changes. CI1 will always affect the ThC swing phase muscle because it innervates both RetCx and ProCx muscles.

Our recordings have shown that all synaptic inputs to leg motor neurons seem also to be present during backward walking and that only the signs of phasic inputs to ProCx and RetCx motor neurons are reversed when a backward stepping motor output is generated. How do the segmental premotor networks generate the change in synaptic drive to ThC motor neurons? At present a walking direction-dependent influence of sensory feedback from load sensors on ThC motor neurons is known (Akay et al. 2004; Akay et al. 2007). Load signals from the leg support stance motor neuron activity in both stepping directions, i.e., RetCx motor neuron activity in forward stepping and ProCx motor neuron activity in backward stepping (Akay et al. 2007). This may indicate that the sign of influence of local sensory feedback on the ThC joint CPG is under the control of intersegmental and descending pathways (see below). Subsequent studies will need to unravel the topology and operation of those neural networks underlying the task-dependent control of ThC motor neuron activity. The composition of synaptic drive to leg motor neurons found for stick insect forward and backward stepping is reminiscent of that in other animals, in which a tonic depolarization and phasic inhibitory and excitatory inputs have been found to underlie rhythmic locomotor activity (locust flight: Hedwig and Pearson 1984; crayfish: J Neurophysiol • doi:10.1152/jn.00006.2015 • www.jn.org

Our finding on the activity of leg motor neurons and its synaptic origin during forward and backward walking is not a foregone conclusion. A recent study in the nematode C. elegans showed that two populations of motor neurons are coactive during forward locomotion, but only one of those is active during backward locomotion, suggesting that there are motor neurons which are dedicated to forward locomotion only (Haspel et al. 2010). In the lamprey, descending drive from groups of reticulospinal neurons in the brain stem discriminates between swim directions (Zelenin 2011).

At present, we cannot determine whether the tonic depolarization in the stick insect has the same source during both walking directions.

In insects, it is presently largely unknown whether necessary neural operations for the switch in walking direction signals originate from the head ganglia or from the thoracic nerve cord. One potentially relevant structure involved in the generation of adaptive locomotor behavior is the central complex in the insect brain. The central complex was particularly well investigated in the fruitfly Drosophila melanogaster (Strauss and Heisenberg 1993; Strauss 2002) and has been shown to contribute to the control of turning behavior (Guo and Ritzmann 2013). A recent study on Drosophila walking (Bidaye et al. 2014) shows that two newly described pairs of descending command neurons from head ganglia initiate and together with a pair of ascending neurons in the ventral nerve cord can maintain backward stepping. In our experiments the initiation of forward or backward walking was dependent on the stimulus location. We do not know, however, whether in case of abdominal stimulation sensory information directly influences thoracic motor centers or is relayed via similar descending neurons in the head ganglia.

**Velocity Dependence of Leg Motor Neuron Activity in the Single-Leg Preparation**

Stick insects, as many other insects, alter their walking speed primarily by a change of stance phase duration (Gabriel and Büschges 2007; Wendler 1964; Wosnitza et al. 2013a). In the single-leg preparation, stepping velocity is significantly correlated only with FlxTi motor neuron activity during forward stepping. Other stance motor neurons, DepTr and RetCx, did not show a systematic correlation with stepping velocity. Alterations in FlxTi activity underlying the generation of stance velocity were only detected with already ongoing stance phase activity (Gabriel and Büschges 2007). Here, no sensory information from the ThC-joint is available, and the FTi-joint, but not the CTr-joint, shows large angular changes during single-leg stepping (von Uckermann and Büschges 2009). This suggests that local sensory feedback from the femoral chordotonal organ (fCO) is relevant for mediating modifications in stepping speed of a single leg (cf. Bässler 1993). The ICO is also known to exert a powerful inter-joint influence on CTr motor neurons (Bucher et al. 2003; Hess and Büschges 1997; Hess and Büschges 1999). However, in contrast to FTi-joint motor neurons CTr-joint motor neuron activity was not changed. Load signals from trCS, necessary for the coupling of ThC-joint motor neuron activity to those of the more distal leg joints (Akay et al. 2004), and signals from the femoral campaniform sensilla (fCS), influencing activity in the motor neuron pools of the CTr and FTi-joints (e.g., Akay et al. 2001; Akay and Büschges 2006), are present in our preparation but are apparently not involved in the control of stepping velocity since only FlxTi activity is changed. Based on these indications it would be interesting to investigate the influence of proprioceptive feedback from the ThC-joint, e.g., from the ventral coxal hairplate (Büschges and Schmitz 1991) in the single-leg preparation by electrical or mechanical stimulation and the effect on RetCx motor neuron activity with changing stepping velocity.

In summary, we have shown that modifications in walking direction and walking speed in the stick insect leg muscle control system are mediated in a joint-specific fashion. ProCx and RetCx motor neurons are subject to a change in phasic synaptic inputs during backward walking. FlxTi motor neurons exclusively contributed to a change in stepping velocity in the single-leg preparation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: P.R., J. Schmitz, J. Schmidt, and A.B. conception and revised manuscript; P.R. drafted manuscript; P.R., J. Schmitz, J. Schmidt, and A.B. edited figures; P.R. prepared and revised manuscript; P.R., J. Schmitz, J. Schmidt, and A.B. interpreted results of experiments; P.R. performed experiments; P.R. analyzed data; P.R., J. Schmitz, J. Schmidt, and A.B. interpreted results of experiments; P.R. prepared and revised figures; P.R., J. Schmitz, J. Schmidt, and A.B. edited and revised manuscript; P.R., J. Schmitz, J. Schmidt, and A.B. approved final version of manuscript.

**REFERENCES**


