The effect of sensory feedback on crayfish posture and locomotion: I.
Experimental analysis of closing the loop

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The effect of sensory feedback on crayfish posture and locomotion: I. Experimental analysis of closing the loop. J Neurophysiol 113: 1763–1771, 2015. First published December 24, 2014; doi:10.1152/jn.00248.2014.—The effect of proprioceptive feedback on the control of posture and locomotion was studied in the crayfish Procambarus clarkii (Girard). Sensory and motor nerves of an isolated crayfish thoracic nerve cord were connected to a computational neuromechanical model of the crayfish thorax and leg. Recorded levator (Lev) and depressor (Dep) nerve activity drove the model Lev and Dep muscles to move the leg up and down. These movements released and stretched a model stretch receptor, the coxobasal chordotonal organ (CBCO). Model CBCO length changes drove identical changes in the real CBCO; CBCO afferent responses completed the feedback loop. In a quiescent preparation, imposed model leg lifts evoked resistance reflexes in the Dep motor neurons that drove the leg back down. A muscarinic agonist, oxotremorine, induced an active state in which spontaneous Lev/Dep burst pairs occurred and an imposed leg lift excited a Lev assistance reflex followed by a Lev/Dep burst pair. When the feedback loop was intact, Lev/Dep burst pairs moved the leg up and down rhythmically at nearly three times the frequency of burst pairs when the feedback loop was open. The increased rate of rhythmic bursting appeared to result from the positive feedback produced by the assistance reflex.

Although real-time sensory feedback is critical for the correct performance of nearly all behavior, its role in producing motor output from the central nervous system has been difficult to study (Fink et al. 2014). This is largely a technical problem: it is difficult to reversibly compare the effects of closed (i.e., intact) and open feedback loops on motor output in the same preparation. To address this problem in the present study, we developed a hybrid neuromechanical preparation in which an isolated crayfish ventral nerve cord drives the movements of a computational neuromechanical model of the crayfish thorax and leg. Movements of the leg then excite appropriate sensory afferents to provide real-time feedback to the nerve cord. A comparison of motor responses when the real-time feedback loop is intact to responses when it is open revealed that sensory feedback has large and immediate effects on rhythmic locomotor patterns. These results highlight the importance of sensory feedback in forming and shaping motor output.

Walking is a behavior that results from interactions between central pattern generators (CPGs), which produce a rhythmic motor pattern and sensory feedback, which modulates the pattern (Brown 1911; Sherrington 1910). For example, during stepping in cats, the stance-to-swing transition is promoted by the responses of sensory afferents to flexion of the hip and decreased tension in the ankle extensor muscle (Ekeberg and Pearson 2005; McVea et al. 2005; Pearson 2008). Similar reflex mechanisms regulate the stance-to-swing transition in stick insects (Bucher et al. 2003; Hess and Buschges 1999) and can entrain locomotor CPGs in crayfish (El Manira et al. 1991b; Elson et al. 1992).

The reflexes that entrain crayfish CPGs include both negative feedback, “resistance” reflexes that help resist leg perturbations, and positive feedback “assistance,” reflexes that enhance on-going movements (Ayers and Davis 1977; Skorupski and Bush 1992; Skorupski et al. 1994). However, it is difficult to determine experimentally in a dissected preparation how reflexes contribute to locomotion when the reflex feedback loop is open and motor discharges do not result in sensory feedback (Clarac et al. 2000). The difficulty can be overcome by using electromechanical devices to stimulate the appropriate sensory afferents in response to the motor output and thereby close the sensorimotor feedback loop (Daur et al. 2012; Weiland et al. 1986). Here we use a computational neuromechanical model of a crayfish leg to provide a virtual periphery for an in vitro crayfish ventral nerve cord and produce real-time sensory feedback to its motor output. With this hybrid preparation, we show how sensory feedback from elevation and depression movements of a crayfish leg modulates the levator/depressor CPG by regulating transitions between different phases of the step cycle.

We studied the reflex and CPG interactions in crayfish using an electronic interface that coupled an in vitro nerve cord preparation to a computational neuromechanical model of a crayfish leg. Levator (Lev) and depressor (Dep) motor nerve activity evoked up and down model leg movements that released and stretched a model stretch receptor, the coxobasal chordotonal organ (CBCO). The interface then transmitted the model CBCO length changes to a speaker-driven probe attached to the live CBCO, an elastic strand with afferents that respond to leg depression (stretch) and elevation (release) (Fig. 1). Excited CBCO afferents projected their activity back through the CBCO nerve to the ventral nerve cord.

We used this hybrid preparation to measure the effects of reafference in quiescent preparations, where only resistance reflexes could be evoked, and in active preparations, where both assistance reflexes and spontaneous Lev/Dep burst pairs occurred in addition to resistance reflexes. We found that the sensory feedback loop excited a rhythmic pattern of Lev/Dep...
burst pairs that ended immediately upon opening the feedback loop. In addition, we found that sensory feedback changed the structure of Lev/Dep burst pairs by making them shorter and shortening the duration of the Lev burst relative to the Dep burst. Both the higher burst pair frequencies and the restructured burst pairs appear to result from the positive feedback that mediates the assistance reflexes.

MATERIALS AND METHODS

In vitro preparation and extracellular recordings. Adult crayfish (Procambarus clarkii) of either sex were obtained from a commercial supplier (Atchafalaya Biological Supply, Raceland, LA) and maintained communally in laboratory aquaria until use. Experimental animals were gradually chilled to 3°C until immobile and unresponsive, when their thoracic and abdominal portions of the ventral nerve cord were prepared as before (Cattaert and Bevengut 2002; Cattaert et al. 1995). The distal segments of the right or left fifth walking leg were pinned out in a Sylgard-lined dish (Dow-Corning) with the proximal end of the CBCO (closest to the sensory cells) was fixed to the Sylgard bottom of the Petri dish, while the distal end was attached to a probe driven in real time by the electronic interface.

Electronic interface between the nerve cord and model. A custom electronic interface transmitted voltage signals between the in vitro nerve cord and the AnimatLab biomechanical leg model (Fig. 1, Interface).¹ A Spike2 sorting algorithm distinguished large phasic Dep and Lev motor neuron (MN) spikes from smaller phas-tonic and tonic MN spikes according to amplitude and generated large or small 2-ms voltage pulses in real time for each detected spike. The large and small voltage pulses were applied to the AnimatLab leg model to excite the phasic and tonic muscles, respectively, and cause the leg to move. The resulting down or up leg movements stretched or released the model CBCO. The digital length of the CBCO model was converted to an analog output, amplified by a DC power amplifier, and applied to drive a loudspeaker attached to the probe. Movements of the probe produced in real time the same length changes in the live CBCO as occurred in the model CBCO. Tests using a light beam interrupted by movement of the probe showed that the probe could follow faithfully the fastest movements commanded by the changing length of the model CBCO.

Biomechanical crayfish leg model. A model of a crayfish thorax and fifth walking leg was constructed in AnimatLab v1 (www.AnimatLab.com) to reproduce the biomechanical responses of a real crayfish leg. All the parameter values and structural arrangements can be seen in the model file, “Crayfish hybrid experimental model,” which is available in ModelDB (https://senselab.med.yale.edu/ModelDB/). The ModelDB accession number for this model is 150697. (A list of all parameter values is in the file Model Parameters Supplemental Material.xls in Supplemental Materials; see ENDNOTE.) The biomechanical crayfish leg and thorax model was constructed from optical scans and mass measurements of a 9-cm crayfish. The animal was killed after cold, and the cephalothorax and segments of the fifth walking leg were separated and individually weighed and optically scanned (NextEngine). The wireframe mesh files were imported to AnimatLab v1 and connected by hinge joints to recreate the fifth walking leg attached to the thorax (Fig. 1, Crayfish leg and body model). The density of each model leg segment was assumed to be

¹To distinguish model elements from their real counterparts, all model element names are italicized.
uniform. During simulation, the thorax was fixed under water and the leg was free to move vertically around the full extent of the CB joint (coxopodite-basipodite joint) subject to muscular forces, gravity, and drag, all of which were simulated in AnimatLab v1. Other leg joints were immobile. AnimatLab simulations use two solvers that solve simultaneously the underlying equations that describe the neural responses and the biomechanical interactions as they evolve in time. The neural responses are produced by a custom-built solver that uses an exponential Euler method for numerical integration (MacGregor 1987), while the biomechanics equations are solved with the Vortex simulator from CM-Labs (Montreal).

Active movement of the leg around the CB joint is produced in the crayfish by the levator and depressor muscles, which contain four and five muscle heads, respectively, including both phasic and tonic muscle fibers (Antonsen and Paul 2000). Separate muscle models were used to represent the tonic and phasic portion of each Lev and Dep muscle head (Fig. 1, Crayfish leg and body model). They were attached in parallel to a common origin and insertion in the model placed according to the origin and insertion of the corresponding muscle head in vivo (Antonsen and Paul 2000).

The biomechanical properties of the muscles were represented by Hill models, each of which contains a serial spring coupled in series to the parallel combination of a spring, a dashpot, and a force actuator (Shadmehr 2006). The length-tension curves and serial and parallel spring constants were adapted from an analysis of the crayfish leg extensor muscle (Chapple 1983; Zachar and Zacharova 1966). Phasic muscles were given spring constants and maximal actuator force values equal to five times those of tonic muscle models of the same cross-sectional area and from the same muscle head (Hoyle 1983). The spring constants and actuator values of each model were then set proportional to the cross-sectional area of the corresponding tonic or phasic portion of the crayfish muscle head that it represented (Antonsen and Paul 2000). The spring constants and forces of all the muscles were scaled again to enable the leg to move up and down in a fashion similar to those of live animals that are held by the thorax underwater (unpublished observations). The spring constants and dashpot constants of a Hill model help determine the muscle force time constants (Shadmehr 2006). Given those spring constants, the tonic and phasic muscle dashpot constants were set to produce muscle force time constants in the range of those found in other crustaceans, because they are unknown for crayfish (Chapple 1983). The electrical properties of the tonic and phasic muscle models were represented by single RC electrical compartments with a resting potential of −70 mV, and electrical parameter values were based on measurements of corresponding leg muscles of related decapod crustaceans (Chapple 1982). The actuator force of each muscle was set as a sigmoidal function of muscle membrane potential such that 10 to 90% of maximum force was generated by membrane potentials between −65 and −25 mV.

The large and small pulses produced by the interface in response to large and small levator and depressor motor nerve spikes were used to excite the model levator and depressor phasic and tonic muscles (Fig. 1). Each nerve’s pulses were applied to a levator or depressor input neuron, which transmitted only the large pulses directly to the phasic muscles to evoke large, nonfacilitating excitatory postsynaptic potentials (EPSPs). These EPSPs were modeled after similar EPSPs recorded in the phasic extensor muscle of the crayfish leg (Bradacs et al. 1997). Each input neuron also transmitted both large and small pulses to a levator or depressor tonic motor neuron. To prevent the tonic neuron from firing in response to the large pulses, the phasic muscle inhibited the tonic neuron for the duration of each phasic pulse. Consequently, the tonic neuron fired only in response to small pulses triggered by tonic MN spikes. The tonic neurons excited the tonic muscles through facilitating, conductance-based synapses that produced facilitating EPSPs modeled after those recorded in the tonic extensor muscles of the crayfish leg (Bradacs et al. 1997).

Experimental procedure. The in vitro preparation was bathed in continuously introduced (5.5 ml/min) chilled, aerated saline. Extracellular recording was continuous, with experimental trials separated by at least 2 min. Before the series of trials, the probe was adjusted to set the CBCO length to equal that of the model CBCO at rest, when the leg was horizontally extended at the beginning of a simulation. An experimental trial consisted of a simulation that began with the motor connection to the model open or closed. The model leg immediately dropped under its gravitational load at the beginning of a simulation; the fall of the leg stretched the CBCO. The CBCO stretch caused the real CBCO to be identically stretched; the stretch-sensitive CBCO afferents elicited reflex responses recorded from the motor nerves. Simulation periods typically lasted 60 or 120 s during which the model ran in real time, ±5%. Under open loop conditions, the recorded Lev and Dep motor nerve spikes had no effect on the model muscles and the leg remained depressed under gravity. Under closed loop conditions, Lev and Dep MN spikes evoked contractions in the corresponding model muscles, producing leg elevations or depressions. These produced corresponding releases and stretches of the CBCO and consequent CBCO afferent responses (Fig. 1, green and beige arrows, respectively). At the end of each simulation, the probe immediately returned to its starting position and the CBCO returned to its original resting length.

Oxotremorine application. Isolated nerve cords from seven animals were exposed to oxotremorine (O XO; Sigma), a muscarinic cholinergic agonist, by replacing the normal saline flow into the Petri dish with 10 or 50 μM O XO in chilled, aerated saline (Fig. 1). The flow was maintained for ~30 min and then replaced with a saline flow to wash out the O XO.

Statistical analysis of bursting. Open and closed loop conditions were randomly assigned to each trial of the series conducted during the hybrid preparation experiment on each animal. Experimental data were selected from sequential trials that included open and closed loop runs during experiments on each of six preparations. Motor bursts were defined using the Poisson Surprise method (Legendy and Salcman 1985) in DataView (see http://www.st-andrews.ac.uk/~wjlh/dataview/) based on threshold-detected phasic units of our extracel-

RESULTS

Resistance and assistance reflexes. In the isolated nerve cord, MNs driving the leg are usually tonically active at low frequencies, similar to the patterns recorded from a quiescent animal (Cattaert and Le Ray 2001). In a quiescent animal, an imposed lift or depression of the leg will evoke a resistance reflex in which release-sensitive or stretch-sensitive CBCO afferents monosynaptically excite Dep or Lev MNs, respectively, to resist the imposed movement (El Manira et al. 1991a). In closed loop trials of hybrid preparation experiments, lifting the model leg shortened the CBCO, which caused the loudspeaker probe to shorten the real CBCO by the same amount (Fig. 1). In seven preparations, an imposed upward force was applied to the leg 120 times and produced a movement that excited release-sensitive CBCO afferents and recruited Dep MNs as part of a resistance reflex on 105 of these leg lifts (Fig. 2, A, green background, and B, markers 1–4).
Fig. 2. Resistance reflex response of the quiescent hybrid experimental preparation to leg lift. A: responses to a 1-N upward force applied to the model leg for 0.4 s (vertical gray arrow). Traces are as labeled. Traces 3 (gray), 4, and 6 show neuronal activity as labeled; traces 3 (black), 5, and 7 show corresponding firing rates. The green vertical band identifies the depressor resistance reflex response; the brown vertical band identifies the levator resistance reflex response. The numbered gold markers correspond to those in B. B: depressor resistance reflex pathway. 1: Upward force (gray arrow) raises the leg (green leg) and releases the CBCO (green arrow). 2: Probe releases the CBCO and excites the release-sensitive CBCO afferents in the CBCO n. 3: afferent activity excites Dep motor neurons (MNs). 4: Recorded Dep n activity excites model Dep MNs and muscles. C: levator resistance reflex pathway. 5: Contraction of Dep muscles brings the leg down (red leg). 6: Downward leg movement (red leg) stretches the CBCO (brown arrow). 7: Probe stretches the CBCO and excites stretch sensitive afferents in the CBCO n. 8: Afferents excite Lev MNs. 9: recorded Lev n activity excites Lev MNs and muscle. 10: Lev muscle contraction slows the fall of the leg.

Upon termination of the applied force, a second resistance reflex was evoked as the leg was driven downward and excited stretch-sensitive CBCO afferents that excited Lev MNs (Fig. 2A, brown background, and C, markers 5–10). These chained reflex responses counteracted both phases of leg movement. In open loop (not shown), similar Dep responses occurred in response to leg lift, but the leg fell more slowly than in closed loop because the Dep muscles were not excited.

Application of the muscarinic cholinergic agonist OXO to a crayfish nerve cord can change the state of the nervous system from quiescent to active, where assistance reflexes, excite Dep motor neurons (MNs). In 11 trials run in 6 preparations, switching on the OXO exposure to the desheathed ganglion reversed the reflexes completely or partially: on 80 occasions an imposed leg lift elicited an assistance reflex alone or with a resistance reflex (Fig. 3C). In response to a leg lift, Lev MNs fired throughout the period of applied force and continued as the leg began to fall, creating chained assistance and resistance levator reflex responses (Fig. 3A, green background, C, markers 1–5). In a second assistance reflex response, Dep MN activity increased as leg continued to fall and peaked as the leg reached its lower limit (Fig. 3A, brown background, and D, markers 6–10).

In addition to simple reflex reversals, OXO exposure also enabled leg lifts to trigger pairs of Lev and Dep MN bursts (Fig. 3B). In these preparations, an imposed leg lift evoked an assistance reflex (Fig. 3C) that triggered a strong burst of Lev MN activity (Fig. 3, B, green background, and C, markers 1–5), and was immediately followed by a burst of Dep MN activity (Fig. 3, B, brown background, and D, markers 6–10). The abrupt rise in Dep MN activity as the leg moved rapidly downward (Fig. 3B, marker 10) suggests that the Dep MN burst may have been triggered by a Dep assistance reflex (Fig. 3D). These Lev/Dep burst pairs caused the leg to maintain its elevated posture for the duration of the Lev MN burst and then return to a fully depressed position (Fig. 3B, top).

Closing the loop speeds bursting. In all of the preparations, exposure of the ganglion to OXO evoked low-frequency bursting in the Lev and Dep motor nerves when the leg simulation was not running, i.e., when the in vitro preparation was completely uncoupled from the leg model. During these periods, the CBCO was held at a constant length that corresponded to the leg being held in a fixed horizontal position (Fig. 4, N.s. = No simulation). The bursting was often irregular: Lev and Dep bursts could be long or short and occurred in a variable phase relationship, while assistance reflexes were present or absent. However, in six preparations where assistance reflexes were present, spontaneous Lev and Dep bursts occurred in pairs, usually with a Lev burst immediately followed by a Dep burst, infrequently at long, variable intervals.

In 11 trials run in 6 preparations, switching on the leg simulation in closed loop caused the initially extended model leg to fall and stretch the CBCO and thereby stretch the real CBCO (Fig. 4, Closed loop; see Hybrid experiment video.mp4 in Supplemental Materials; see ENDNOTE). The initial leg fall was immediately followed by rhythmic levator and depressor burst pairs. The levator burst occurred first (a Lev/Dep pair) in all but 3 of the 78 burst pairs recorded in the 6 preparations so that the leg was repeatedly raised and lowered in a pattern reminiscent of walking. The depressor burst occurred first (a Dep/Lev pair) in the remaining three burst pairs. The rhythmic burst activity stopped at the end of the simulation when the loop opened and the CBCO returned to its resting length. When
The leg simulation was run in the open loop configuration in seven trials in the same six preparations, the leg also fell at the beginning and stretched the CBCO. The CBCO stretch evoked reflex motor nerve responses but no subsequent leg movements because the connection between the motor nerve and muscle model was disabled (Fig. 4, Open loop). While the CBCO was stretched during these open loop trials, 21 Lev/Dep burst pairs were evoked and 0 Dep/Lev burst pairs.
To analyze the responses under open and closed loop conditions (Fig. 4), Lev and Dep bursts were identified separately and together as Lev/Dep burst pairs (see MATERIALS AND METHODS and Figs. 4, and 5A). The frequency and duration of Lev/Dep burst pairs, the interval between burst pairs, the durations of the Lev and Dep bursts in each burst pair, and the interval between bursts of a pair were measured for open and closed conditions for each animal and then compared statistically. The three Dep/Lev burst pairs were not counted.

We found that burst pair frequencies recorded in closed loop were significantly greater than those in open loop ($P = 0.030$; Fig. 5B). In open loop, the average burst pair frequency across animals was $0.04 \pm 0.03$ Hz, whereas in closed loop, the frequency was 2.5 times greater, $0.10 \pm 0.04$ Hz. The interval between burst pairs was also much shorter in closed loop than in open loop ($P = 0.052$; Fig. 5C). In closed loop, these intervals averaged $7.8 \pm 4.2$s, whereas in open loop they averaged $33.6 \pm 39.6$s. The durations of burst pairs in closed loop were shorter than those in open loop ($P = 0.065$; Fig. 5D). In closed loop, burst durations averaged $6.7\pm 3.4$s, two-thirds the average value in open loop, $9.9 \pm 2.9$s.

**Closing the loop changes the structure of burst pairs.** The structure of Lev and Dep bursts within each burst pair also differed in closed and open loop. In closed loop, the Lev burst was shorter than the Dep burst in five of the six animals, although the average Lev burst duration across animals did not differ from the average Dep burst duration ($P = 0.310$). In open loop, the Lev burst durations exceeded the Dep burst durations in only half the animals and the average Lev and Dep burst durations did not differ ($P = 0.818$; Fig. 5E). Moreover, in closed loop the Lev burst in each animal was consistently shorter than in open loop, although the average Lev bursts across animals in open and closed loop did not differ ($P = 0.394$). The Dep bursts did not differ in open and closed loop. Half the Dep bursts in closed loop were shorter than the Dep bursts in the same animal in open loop and half were longer, and there was no difference in their averages ($P = 0.485$; Fig. 5F). Finally, in four of the six animals, the interval between Lev and Dep bursts was shorter in closed loop than in open loop, while in the other two it was longer. However, because the variance in the closed loop intervals was so much smaller than the variance in the open loop intervals, the averages (closed loop: 0.6 $\pm$ 1.1 s; open loop: 3.5 $\pm$ 2.6 s; $P = 0.041$) differed significantly (Fig. 5G).

**DISCUSSION**

The results described here highlight the importance of sensory feedback in the generation of motor patterns. The timing and selection of particular afferents by the movement itself ensure that the centrally generated motor patterns will be shaped from moment to moment by current conditions to produce well-adapted movements and behaviors. This is apparent in these experiments even though the feedback was from only one of several sensory organs that affect the leg’s motor patterns.

The effects of proprioceptive sensory inputs on rhythmic motor outputs have been studied primarily in anesthetized, restrained, and dissected nervous system preparations where limb or body movement that could produce sensory feedback is limited or absent. Spontaneous rhythmic bursting with variable frequencies and duty cycles have been seen in isolated crayfish nerve cord preparations after application of muscarinic agonists like OXO (Charchri and Clarac 1987, 1990). Imposed proprioceptive sensory inputs can evoke rhythmic alternating resistance reflex responses from antagonist MNs in quiescent preparations (El Manira et al. 1991b) and can entrain rhythmic bursting in active preparations (Le Ray and Cattaert 1997; Leibrock et al. 1996; Sillar et al. 1986). In our preparations, OXO induced a variety of active rhythmic patterns; to study the

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**Fig. 5. Identification of Lev/Dep burst pairs and burst pair measurements and statistics.** A: Lev/Dep burst pair with the bursts and durations identified. The thresholds (“T”) for identification of the large (phasic) Lev and Dep MN spikes are shown with a dashed line. Identifications of the Lev burst, Dep burst, and Lev/Dep burst pairs are shown below. The durations of burst and burst pair intervals are identified below that; burst identification procedures are explained in MATERIALS AND METHODS. B–G: box whisker plots for various statistics on burst and burst pairs under closed loop (CL) and open loop (OL) conditions. The data points, which are average values from single animals, are shown for CL and OL conditions linked by line segments. Data for individual animals are represented by the same colors across the plots. Each box shows the median (middle line) and 25th and 75th percentiles (box bottom and top) of the data distribution. Each plot is labeled at top; the parameters are identified in A. *Statistically significant difference between CL and OL responses.
effect of closing the feedback loop on motor activity, we focused on those preparations in which reflex reversal occurred and a leg lift triggered a Lev/Dep burst pair.

By artificially closing the feedback loop, the role of reafference can be studied in a stable preparation (Daur et al. 2012; Weiland et al. 1986). The hybrid experimental preparation provides the isolated nervous system with a virtual periphery that generates reafferent responses from the CBCO in real time to movements evoked either by external perturbation or by central motor commands. The neuromechanical leg model and hybrid preparation provides opportunities for producing reafferent responses to leg movements around other joints, including the thoracic-coxa joint that mediates forward/backward leg movement, and ground contact with the distal end of the leg. In the present experiments, the nature and timing of the resulting CBCO feedback resembled the natural feedback that a freely behaving animal would experience in response to its own movements. Moreover, the ability to interrupt the feedback experimentally has helped to reveal its significance for the normal function of the nervous system in posture and locomotion.

Resistance reflex responses. Resistance reflexes in crayfish, stick insects, and other animals act through negative feedback loops to stabilize a postural stance (Clarac et al. 2000). In our tonically active, closed loop hybrid preparations, resistance reflexes created a chained response to a leg lift perturbation by first exciting the release-sensitive CBCO afferent-to-Dep MN pathway that countered the lift and pushed the leg down. A second resistance reflex excited the stretch-sensitive afferent to Lev MN pathway to counter the rapid downward leg movement.

Assistance reflexes trigger bursts during rhythmic bursting in closed loop. Assistance reflexes use positive feedback to reinforce imposed movements and to promote rapid transitions between locomotor phases (Ayers and Davis 1977; Duysens et al. 2000; Le Ray and Cattaert 1997). Application of OXO in the hybrid preparation enabled assistance reflexes to help the applied force to lift the leg (Fig. 3, A and C). Once the raised leg began to fall, however, Lev MNs continued to fire, suggesting that they were excited by a resistance reflex that opposed the fall. Activation of muscarinic receptors is known to strengthen the resistance reflex responses of MNs in a voltage-dependent fashion (Le Bon-Jego et al. 2006), so that both assistance and resistance reflexes may be enhanced by the OXO exposure.

Earlier work identified a disynaptic pathway to mediate the leg depression assistance reflex, in which stretch-sensitive CBCO afferents excite an assistance reflex interneuron (ARIN), and it excited Dep MNs to assist the downward movement of the leg (Bevengut et al. 1997; Le Ray and Cattaert 1997). We presume that the circuit is symmetric, such that our experimental leg elevation excited release-sensitive afferents that then excited release-sensitive ARINs (Cattaert and Le Ray 2001). The ARINs would then have excited Lev MNs to assist the leg rise, while the resistance reflex is blocked or reduced by presynaptic inhibition of the monosynaptic connection between the release-sensitive afferents and Dep MNs (Le Bon-Jego and Cattaert 2002).

OXO exposure helps induce an active state in which the excitability of the ARINs and the Lev and Dep CPG, which includes some MNs, is increased (Cattaert and Le Ray 2001). We found that the assistance reflex evoked by an imposed leg lift often triggered a Lev/Dep burst pair that caused the leg to remain raised during the levator burst and then rapidly depress in response to the Dep burst (Fig. 3, B and C). Once the Lev/Dep CPG became spontaneously active, closing the loop should enable the assistance reflex to trigger a Lev/Dep burst pair in response to leg movements produced by the CPG.

This mechanism is illustrated in Fig. 6, which compares an open loop burst pair to a closed loop burst pair. In open loop, the Lev/Dep half-center CPG generated Lev/Dep burst pairs at a low rate. The Dep and Lev half-centers are linked through mutual inhibition (Cattaert and Le Ray 2001), so that the Lev MNs were strongly inhibited by each Dep burst (Fig. 6A, red arrowhead and marker 1). The Lev MNs’ firing rates then gradually increased before triggering their own burst (Fig. 6A, marker 2). The Lev burst produced strong inhibition that silenced the Dep MNs (Fig. 6A, marker 3, blue arrowhead). Towards the end of the Lev burst, Dep MNs began to fire rapidly after having been inhibited (Fig. 6A, marker 4). They quickly formed a Dep burst that strongly inhibited the Lev MNs, leaving only the common inhibitor MN active (Fig. 6A, markers 5 and 1, and red arrowhead) (Cattaert et al. 1993). The Dep firing rate then slowed and allowed the Lev firing rate to increase again in another cycle.

It is apparent that the open loop burst frequency depended on the intrinsic dynamics of the CPG and on the effects of mutual inhibition between half-centers. In closed loop, the effects of the intrinsic dynamics and mutual inhibition on the half-center burst frequency were supplemented by positive feedback provided by the assistance reflexes (Fig. 6B). As in open loop, the Lev firing rate increased from having been strongly inhibited...
by the Dep burst. The Lev firing rates were initially too low to raise the leg. However, as their firing rates increased, they evoked an upward leg movement (Fig. 6B, marker 1, light blue arrowhead) that excited release-sensitive CBCO afferents (Fig. 6B, marker 2, green arrowhead). The afferents are likely to have excited the release ARIN and produced a levator assistance reflex that triggered a burst in the Lev MNs (Fig. 6B, marker 3). As in open loop, the Lev MNs strongly inhibited the Dep MNs (Fig. 6B, marker 3, blue arrowhead) until some Dep MNs began to increase their firing rates (Fig. 6B, marker 4). They inhibited the Lev MNs while they excited the Dep muscle and caused the leg to move down (Fig. 6B, marker 4, pink arrowhead). This is likely to have excited stretch-sensitive CBCO afferents and a depressor assistance reflex (Fig. 6B, marker 5, brown arrowheads). The assistance reflex excited a depressor burst that then inhibited the Lev MNs (Fig. 6B, marker 6, red arrowhead) and increased depressor muscle tension.

Assistance reflexes reset the CPG early in each cycle to accelerate the Lev/Dep rhythm. The shorter closed loop interval between burst pairs (Fig. 5C) suggests that the levator assistance reflex is triggered early in each cycle of the closed loop rhythm relative to the cycle of the open loop rhythm. By triggering the burst, the assistance reflex should reset the closed loop rhythm, so that the rhythm would be reset early in every cycle by proprioceptive feedback from the leg movement. It appears likely that a depressor assistance reflex is also triggered at the end of the Lev burst of each burst pair and that this triggers the Dep burst (Fig. 6B, marker 5). This would account for the shorter closed loop interburst duration in four of the six animals (Fig. 5G) and would indicate that the Dep assistance reflex resets the Dep rhythm at each cycle. Together, the early resettings shorten the burst pair periods and speed the motor rhythm.

CBCO feedback also restructured the Lev/Dep burst pairs. The burst pairs were shorter when the feedback loop was closed (Fig. 5D), because the Lev bursts were shorter (Fig. 5F), and in four of six animals, because the interval between Lev and Dep bursts was shorter (Fig. 5G). If the depressor assistance reflex helped to advance the onset of the Dep burst, this may account for both of these changes in the burst pair structure. The advance of the Dep burst would shorten the interval between Lev and Dep bursts and cause an earlier inhibition of the Lev MNs that would shorten their burst. CBCO input may also increase the Lev/Dep burst pair frequency by increasing the excitation of central neurons linked to the CPG, as input from wing stretch receptors does for the flight system of locust (Pearson and Ramirez 1990).

Regulating the step cycle in the context of walking. A step cycle can be defined as lasting from the onset of the swing phase until the end of the stance phase. In our closed loop experiments, where sensory feedback is provided only by the CBCO, the transitions are triggered by an assistance reflex response to the moving leg, which resets the CPG each cycle and thereby accelerates the rhythm. However, in freely walking animals, the transitions must be coordinated with the overall movement of the leg and its changes in load as it supports and propels the animal.

In several animals, interjoint reflexes assist the transition to the next phase of the step cycle when the leg becomes unloaded and ready to shift from stance to swing. In the cat, a fall in the tension of ankle extensors at the end of the stance phase disinhibits leg flexors, while hip sensors that signal hip extension also help trigger the stance-to-swing transition (Ekeberg and Pearson 2005; Pearson 2008). In the stick insect, leg elevation is promoted by receptors that respond to flexion at the femur-tibia joint, which occurs as the leg pulls the animal forward (Bucher et al. 2003; Hess and Buschges 1999). In crustacea, several sensory pathways exist for signaling readiness for a stance-to-swing transition. The funnel canal organs on the dactyl are sensory afferents that respond to leg contact with the substrate and can reset and coordinate the walking rhythm in the crab (Libersat et al. 1987a,b). Cuticular stress detectors, which respond to stress developed in the cuticle of the proximal segments of the crayfish leg, can also entrain both promotion/remotion and elevation/depression movement rhythms of the leg around the adjacent thoracic-coxopodite and CB joints, respectively (Leibrock et al. 1996). When the nervous system is in an active state, the thoracic-coxopodite muscle receptor organ reports remotion of the leg and, through reflex reversal, excites leg remotors and inhibits promotors (Elson et al. 1992; Skorupski and Sillar 1986; Skorupski and Sillar 1986). During walking, these different receptors may act to signal when the leg is unloaded and ready to make a stance-to-swing transition. Their responses are likely to help silence the depressors and excite the levators in an interjoint reflex (Ayres and Davis 1977) and thereby reverse the downward torque on the leg needed to support the body. This condition will produce the upward leg movement that triggers the assistance reflex and levator burst response observed in our experiments. It seems likely that this assistance reflex mechanism, perhaps together with a similar one that excites leg promotors (Skorupski and Bush 1992; Skorupski et al. 1992, 1994), produces the stance-to-swing transition during each step of normal walking.

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ENDNOTE

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: B.C., J.B.-C., D.W.C., D.C., and D.H.E. conception and design of research; B.C., J.B.-C., D.W.C., D.C., and D.H.E. performed experiments; B.C., J.B.-C., D.W.C., D.C., and D.H.E. analyzed
data: B.C., J.B.-C., D.W.C., D.C., and D.H.E. interpreted results of experiments; B.C., J.B.-C., D.C., and D.H.E. prepared figures; B.C., J.B.-C., and D.H.E. drafted manuscript; B.C., J.B.-C., D.W.C., D.C., and D.H.E. edited and revised manuscript; B.C., J.B.-C., D.W.C., D.C., and D.H.E. approved final version of manuscript.

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