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# Feedback From Peripheral Musculature to Central Pattern Generator in the Neurogenic Heart of the Crab *Callinectes sapidus*: Role of Mechanosensitive Dendrites

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Feedback from peripheral musculature to central pattern generator in the neurogenic heart of the crab *Callinectes sapidus*: role of mechanosensitive dendrites. *J Neurophysiol* 103: 83–96, 2010. First published October 14, 2009; doi:10.1152/jn.00561.2009. The neurogenic heart of decapod crustaceans is a very simple, self-contained, model central pattern generator (CPG)-effector system. The CPG, the nine-neuron cardiac ganglion (CG), is embedded in the myocardium itself; it generates bursts of spikes that are transmitted by the CG's five motor neurons to the periphery of the system, the myocardium, to produce its contractions. Considerable evidence suggests that a CPG-peripheral loop is completed by a return feedback pathway through which the contractions modify, in turn, the CG motor pattern. One likely pathway is provided by dendrites, presumably mechanosensitive, that the CG neurons project into the adjacent myocardial muscle. Here we have tested the role of this pathway in the heart of the blue crab, *Callinectes sapidus*. We performed “de-efferentation” experiments in which we cut the motor neuron axons to the myocardium and “de-afferentation” experiments in which we cut or ligated the dendrites. In the isolated CG, these manipulations had no effect on the CG motor pattern. When the CG remained embedded in the myocardium, however, these manipulations, interrupting either the efferent or afferent limb of the CPG-peripheral loop, decreased contraction amplitude, increased the frequency of the CG motor neuron spike bursts, and decreased the number of spikes per burst and burst duration. Finally, passive stretches of the myocardium likewise modulated the spike bursts, an effect that disappeared when the dendrites were cut. We conclude that feedback through the dendrites indeed operates in this system and suggest that it completes a loop through which the system self-regulates its activity.

## INTRODUCTION

Adaptive behavior is not the product of the nervous system alone but rather of the coupled system of both the nervous system and peripheral structures—of both the “brain” and the “body” (Beer 2009; Bernstein 1967; Brezina et al. 2005; Chiel and Beer 1997; Clark 1997; Cohen 1992; Nishikawa et al. 2007). Prominent among the reciprocal interactions that couple this larger system together is feedback from the periphery back to the CNS. In the case of cyclical, rhythmic behaviors—behaviors such as breathing, biting, chewing, scratching, walking, running, swimming, and flying—there is typically important feedback conveying information about the activity of the

peripheral motor apparatus back to the central pattern generator (CPG) that initiates, times, and patterns that activity (Büschges 2005; Büschges and El Manira 1998; Cohen 1992; Pearson and Ramirez 1997; Rossignol and Dubuc 1994; Wallén 1997). Such feedback to CPG networks can be difficult to study in the complex motor systems of mammals, but it is often more accessible to detailed investigation in simpler model systems (e.g., Beenhakker et al. 2005, 2007; Borgmann et al. 2009; Shetreat-Klein and Cropper 2004).

The neurogenic heart of decapod crustaceans is a self-contained, anatomically very simple, yet complete model CPG-effector system. The CPG of the system, the cardiac ganglion (CG), lies within the heart itself, on the inner dorsal wall of the single-chamber myocardium (Fig. 1A) (for reviews, see Cooke 1988, 2002). In most crabs and lobsters, the CG contains only nine neurons, strongly coupled to each other by electrical and chemical connections. Four of the neurons are local premotor interneurons that act as pacemakers of the system, initiating rhythmic waves of depolarization and bursts of spikes that, after being further patterned by the five motor neurons, propagate through the motor neurons' axons to the periphery of the system, the myocardium, to produce its rhythmic contractions (Alexandrowicz 1932; Anderson and Cooke 1971; Hagiwara 1961; Hartline 1967, 1979; Mayeri 1973; Tazaki and Cooke 1979, 1983a,b). The entire system is extensively modulated by numerous neuromodulators with multiple actions on both the CG and the myocardium (e.g., Cooke and Sullivan 1982; Fort et al. 2004, 2007a,b; Maynard 1960). With these properties, the crustacean cardiac system was early recognized as ideal for studies of how the “brain” interacts with the “body” to produce output that is robust yet responsive to changing physiological demands (Alexandrowicz 1932; Hagiwara 1961; Welsh and Maynard 1951; see Cooke 1988, 2002). Using the heart of the blue crab, *Callinectes sapidus*, we are carrying out such studies to understand the functional architecture of the cardiac system and the logic of its modulatory control (Fort et al. 2004, 2007a,b; Stern et al. 2007). This paper is a further installment of this project.

In our previous work in *Callinectes*, we found strong indications of peripheral feedback from the myocardium back to the CG. Notably, parameters of the motor pattern generated by the CG, such as the frequency of the motor neuron spike bursts, the number of spikes per burst, and the burst duration, differed markedly in the isolated CG and when the CG remained embedded in the myocardium (Fort et al. 2004). The

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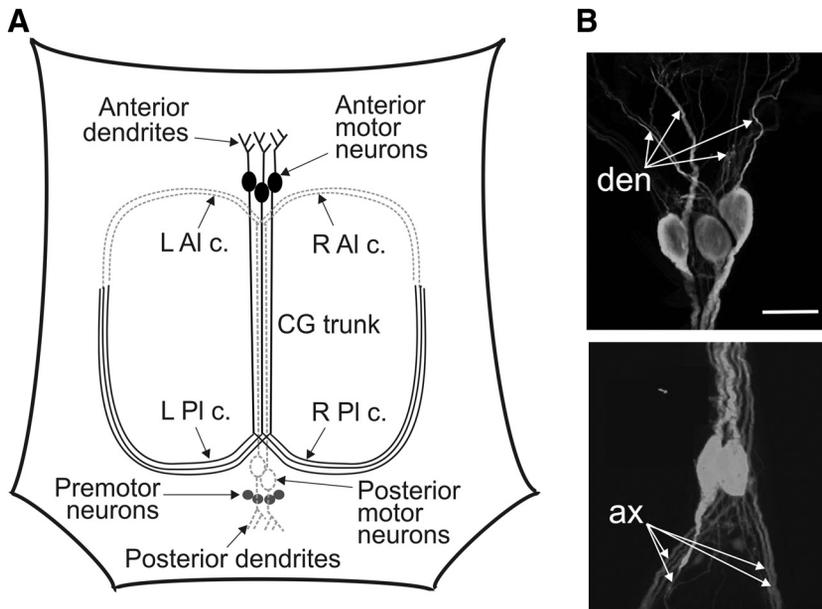


FIG. 1. Anatomy of the *Callinectes* cardiac system. A: sketch of the major structures (not drawn to scale). The cardiac ganglion (CG) lies on the inner dorsal wall of the single-chamber myocardium (black surrounding outline). Three large motor neurons at the anterior end of the CG project their axons (solid black lines) through the CG trunk to the posterior end, where they bifurcate and project to both the left posterolateral connective (L PI c.) and the right posterolateral connective (R PI c.). Two large motor neurons at the posterior end of the CG project their axons (dashed gray lines) through the CG trunk to the anterior end, where they bifurcate and project to both the left anterolateral connective (L AI c.) and the right anterolateral connective (R AI c.). Both the anterior and the posterior motor neurons project dendrites into the adjacent myocardial muscle. At the posterior end of the CG are also four smaller local premotor interneurons. B: images of Neurobiotin-filled motor neurons at the anterior (top) and posterior (bottom) end of the CG (the intervening CG trunk is omitted) showing their well-segregated dendrites (den) and axons (ax). Calibration bar = 80  $\mu$ m.

burst frequency, for instance, was higher in the isolated CG: the heartbeat was slowed by the presence of the myocardium. Furthermore, modulators had different, sometimes diametrically opposite, effects in the two cases. *Callinectes* FMRF-amide-like peptide, for example, increased the burst frequency and decreased the number of spikes per burst and the burst duration in the isolated CG but did the converse in the CG embedded in the myocardium (Fort et al. 2007a), and similar observations were made with dopamine and crustacean cardioactive peptide (CCAP) (Fort et al. 2004, 2007b). These observations implied that the CG was receiving information about the presence, and so perhaps the mechanical state, of the myocardium. There was, indeed, an obvious candidate for the pathway through which this information could flow. Alexandrowicz (1932) described dendrites of the CG neurons ramifying on the muscle fibers adjacent to the ganglion (Fig. 1, A and B, shows these dendrites in the *Callinectes* heart). He noted differences in form between these local dendritic arborizations and the more distant terminals of the motor neuron axons and proposed that the dendrites are mechanosensitive. In support of their mechanosensory role, several investigators reported that a quiescent heart could contract in response to focal mechanical stimuli in the region of the dendrites (Alexandrowicz 1932; Hartline 1967; see Cooke 2002).

In other crustacean cardiac systems, two other key studies have been performed in recent years that partly motivate our investigation here. In the isopod *Ligia pallasii*, Sakurai and Wilkens (2003) demonstrated that both passive stretch and active contractions of the myocardium do indeed dynamically modify the CG motor pattern in that system. However, although they assumed it, they did not demonstrate that the feedback was through the mechanosensitive dendrites. In the lobster *Homarus americanus*, on the other hand, Mahadevan et al. (2004) did describe a mechanism of feedback, but a second, quite different mechanism, whereby the myocardium produces a gaseous messenger, nitric oxide (NO), that diffuses back to the CG and slows its rhythm (see further DISCUSSION). Thus, although proposed >75 yr ago, the mechanosensory role of the dendrites has hitherto remained unresolved.

In this paper, we study the peripheral feedback in the *Callinectes* cardiac system. We perform lesion studies to interrupt the putative CG-myocardial loop either in its efferent limb carried by the motor neuron axons or its afferent limb carried by the dendrites, probing the effect on the CG motor pattern with both active contractions and passive stretches of the myocardium. Our aim is, first, to confirm that the mechanical state of the myocardium modifies the CG motor pattern, and second, to test the hypothesis that the mechanosensitive dendrites mediate this feedback.

## METHODS

### Animals and solutions

Specimens of *C. sapidus* (male and female) were captured in the San José Lagoon in the Hato Rey district of San Juan, Puerto Rico. They were housed under ambient light and temperature conditions in synthetic sea water (Instant Ocean) adjusted to a salinity of 18–20 ppt. To reduce fat deposits within the heart, the crabs were not fed. They were typically used within 1 wk of capture. All experiments used crab saline with composition based on Pantin's saline for *Cancer pagarus*, which contained (in mM) 487 NaCl, 13.6 KCl, 13.4 CaCl<sub>2</sub>, 13.6 MgCl<sub>2</sub>, 1.4 Na<sub>2</sub>SO<sub>4</sub>, and 3 HEPES, adjusted to pH 7.4 with NaOH. All experiments were performed at room temperature.

### Semi-intact working heart (SIWH)

This preparation was as described previously (Fort et al. 2004). The heart was pinned in a silicone elastomer (Sylgard)-lined petri dish in an arrangement as similar as possible to that in the intact crab. A small incision was made in the ventral wall of the heart to expose part of the nerve ring through which the axons of the CG motor neurons innervate the myocardium (see Fig. 1A). The exposed ring was cut, usually across one of the posterolateral connectives, and the severed end proximal to the ganglion was drawn into an extracellular suction electrode to record the CG motor neuron spike pattern. The recording was made with a differential AC amplifier (Model 1700, A-M Systems, Carlsborg, WA) and digitized with a PowerLab (AD Instruments, Colorado Springs, CO) data-acquisition system (total sampling rate: 100 kHz). To record the contractions of the heart, the myocardium was attached with a hook and nylon thread to a Grass FT03

isometric force transducer (Grass Astro-Med, West Warwick, RI) or an Aurora 300C dual-mode muscle lever system (Aurora Scientific, Aurora, Ontario, Canada) and placed under a resting tension of  $\sim 0.5$  g, unless specified otherwise (see following text). The preparation was continuously perfused internally with saline at a constant rate (2 ml/min) and pressure.

### Isolated cardiac ganglion (ICG)

This preparation was as described previously (Fort et al. 2004) or somewhat modified. The heart was pinned ventral side up in a Sylgard-lined petri dish. A cut was made in the ventral musculature to expose the CG. In de-efferentation experiments, the myocardium was dissected away, but the CG connectives were not initially cut and the entire nerve ring remained attached to the CG. In de-afferentation experiments, the standard ICG preparation was used in which all four CG connectives were cut and the myocardium including the nerve ring was removed. In either case, a small noncontracting remnant of muscle containing the dendritic endings of the CG neurons was retained at either end of the ganglion. Dissection was achieved principally by teasing away the adhering muscles. In the de-afferentation experiments, extracellular suction electrode recordings were obtained as in the SIWH preparation from at least one of the four cut connective roots. In all experiments, intracellular recordings were obtained from anterior and/or posterior motor neurons using standard glass microelectrodes filled with 2 M KCl (10–30 M $\Omega$ ). The preparation was continuously superfused with saline (2 ml/min).

### De-efferentation and de-afferentation procedures

In portunid crabs such as *Callinectes*, the dendritic endings of the CG neurons are mainly confined to two well-localized regions between the connective roots at the anterior and posterior ends of the ganglion (see Fig. 1, A and B) (see also Fort et al. 2004). The topographic separation between the axonal and dendritic projections from the CG enabled us to perform selective de-efferentation experiments by transecting the connective roots with fine mini-vannas scissors (No. 15000-10, Fine Science Tools, Foster City, CA). In selective de-afferentation experiments, conversely, the dendritic projections were transected with the same scissors or ligated with a fine hair. In the ligation experiments, the hair was looped loosely around the dendrites prior to mounting the preparation in the recording chamber; after collecting control data, the ligature was tightened by pulling the ends of the hair with fine tweezers. The de-efferentation and de-afferentation experiments were performed in the SIWH or the ICG preparation, or in the modified ICG preparation in which the entire nerve ring was initially left attached to the CG before being transected at the connective roots, as described in RESULTS. To evaluate the effect of the de-efferentation or de-afferentation, the parameters of the CG motor neuron spike pattern were averaged typically over a 1-min interval of the recording before the de-efferentation or de-afferentation procedure and then over another 1-min interval after the parameters had stabilized after the procedure (see RESULTS). When the experiment was completed, Neurobiotin injection (see following text) was performed to confirm the efficacy of the procedure.

### Myocardial stretch

Myocardial stretch experiments were performed in the SIWH preparation. In early experiments in which the Grass force transducer was used, the transducer was simply moved so as to stretch or relax the myocardium (although only the concomitant changes in tension were actually recorded by the transducer). In experiments with the Aurora muscle lever system, the myocardium was stretched or relaxed by length commands to the Aurora system (both length and tension changes were recorded in this case). In many experiments, the myocardium was simply stretched or relaxed manually, within a relatively

brief interval of time, from one maintained length to another (see Figs. 8 and 9).

With the Aurora system, a second series of more controlled experiments was performed in which the length commands took the form of programmed random waveforms (see Fig. 10). These waveforms were designed to have, within limits, Gaussian white-noise properties. Each waveform was constructed in advance of the experiment in *Mathematica* (Wolfram Research, Champaign, IL) as a sequence of amplitude values drawn from a Gaussian distribution with zero mean and arbitrary SD. During the experiment, a custom-built random waveform stimulator, a computer incorporating a D/A board (DT331, Data Translation, Marlboro, MA) controlled by a C++ program running under Microsoft Windows, was used to output a voltage waveform defined by the sequence of amplitude values, switching from one value to the next every 40 ms (i.e., at 25 Hz). The resulting square waveform was smoothed with a low-pass filter (Model 900 8-pole Bessel filter, Frequency Devices, Ottawa, IL) at 0.3–6 Hz (3 Hz in most experiments reported here) and scaled in amplitude so as to be appropriate to the size of the myocardium in that particular preparation before being fed into the length command input of the Aurora system. Each random stretch waveform was 1 or 3 min long, and several waveforms were repeated and alternated, separated by rest intervals of 1–3 min. The baseline length of the myocardium, on which the random stretch waveforms were superimposed, was maintained at a preset value, generally somewhat stretched, throughout. The same or comparable series of random stretch waveforms was then repeated after transection of the dendrites.

### Neurobiotin injection

The methods of Delgado et al. (2000) were used with modifications. Microelectrode tips were filled with 4% Neurobiotin (Vector Laboratories, Burlingame, CA) dissolved in 0.5 M KCl, 50 mM Tris (pH 7.6). The electrode shafts were filled with 2 M KCl, resulting in resistances of 10–30 M $\Omega$ . Depolarizing current pulses (1–2 nA for 0.5 s repeated every 1 s for 30–120 min) were used to eject the Neurobiotin into the cell body of the impaled motor neuron. This procedure did not appear to affect the resting potential or spontaneous electrical activity of the neuron. The preparation was then usually left at room temperature for 2–3 h to allow the Neurobiotin to diffuse from the injection site to distant processes and other dye-coupled neurons. The preparation was repinned if necessary, fixed in 4% paraformaldehyde (24 h), transferred to a microcentrifuge tube, washed five times (30 min each) with PTA (0.1 M phosphate buffer containing 2% Triton X-100 and 0.1% sodium azide), and then incubated in Alexa Fluor 488-conjugated streptavidin (Molecular Probes, Eugene, OR) diluted (1:3,000 to 1:5,000) in PTA (24–48 h, room temperature). The Alexa Fluor 488 was viewed with the G-2A filter block of a Nikon Optiphot or using the preconfigured FITC channel of a Zeiss Pascal LSM5 laser scanning confocal microscope. Standard images were captured using the ACT1 (Nikon) software package. Confocal images were reconstructed (AIM Software) from sequential images captured in the *z*-axis plane of the tissue. Images were transported as TIFF files to Adobe Photoshop (Version 6) for adjusting overall contrast and brightness. Finally, they were imported to CorelDraw 9 for addition of labels, cropping, and organization of panels.

## RESULTS

### De-efferentation modifies the CG motor pattern

In decapod crustaceans, the CG motor neurons project their axons to the myocardium through four connectives—the left and right anterolateral and posterolateral connectives—that then form a nerve ring within the myocardium (reviewed by Cooke 2002). Transection experiments in lobsters showed that the motor innervation of the entire lobster myocardium could

be accounted for by the motor neuron projections through the four connectives (Kuramoto and Kuwasawa 1980), and the same appears to be true in portunid crabs including *Callinectes* (Fort et al. 2004; Tazaki and Cooke 1979), as sketched in Fig. 1A. Based on this anatomy, we hypothesized that sequential transection of the four connectives—"de-efferentation" of the myocardium—would decrease the amplitude of the myocardial contractions and then eliminate them altogether, and thereby, if there is indeed feedback by which the contractions modify the CG motor pattern, reveal that feedback.

The transection of the connectives might of course have direct effects itself, reflecting either the acute injury to the neurons whose axons were cut or the neurons' reaction or adaptation to the injury [although, where studied in invertebrate neurons, effects of the latter type have been found to develop slowly, over hours and days, relative to the effects sought here (see, e.g., Kuwada and Wine 1981; Walters et al. 1991)]. To rule out such effects, we first performed control experiments in which we transected the connectives in a modified ICG preparation in which the myocardium had been removed but the entire nerve ring was initially retained attached to the CG (Fig. 2A). We recorded the activity of the CG motor neurons with an intracellular microelectrode inserted into one of the motor neuron cell bodies. In *Callinectes*, all five motor neurons are electrically coupled and generally fire in synchrony (Fort et al. 2004; see also Tazaki and Cooke 1979), so that recording from just one of them was sufficient. A control recording was first obtained (a representative example is shown in Fig. 2B, top). The microelectrode was then removed and all four connectives were cut within 3 mm of the CG (at the locations marked in Fig. 2A, X). The motor neuron was then re-impaled, usually within 5 min of the transection, and another recording was obtained (Fig. 2B, bottom). The

posttransection activity was then monitored for 1–4 h; typically it exhibited no major changes over that time.

As Fig. 2B suggests, in these control experiments the connective transection did not change the CG motor neuron activity in any obvious way. In group data from four experiments (Fig. 2C), there was no statistically significant change in any of the three standard parameters of the CG motor neuron spike pattern that we quantified in this work: the spike burst frequency (C1), number of spikes per burst (C2), or burst duration (C3) (for statistical analyses, see figure legend). Moreover, there was no change in the motor neuron membrane potential, measured at its most hyperpolarized interburst level [control:  $-51.1 \pm 3.1$  (mean  $\pm$  SD) mV, connectives cut  $-52.0 \pm 2.8$  mV;  $t = -0.238$ ,  $P > 0.05$  by 2-tailed Student's  $t$ -test;  $n = 4$ ]. Thus the connective transection per se had no effect on the CG motor neuron activity within the time frame of our experiments.

We then performed analogous experiments in the SIWH preparation in which the CG remained embedded in the contracting myocardium. In this preparation, we recorded the CG motor neuron spikes with an extracellular suction electrode and the contractions of the myocardium with a force transducer. To record the spikes, we had to cut one of the connectives, in these experiments always the left posterolateral connective, already under "control" conditions. Again, because all five motor neurons fire in synchrony, the spikes recorded from that connective were representative of the spikes being transmitted simultaneously through all four connectives (see Fort et al. 2004). After obtaining a control recording, the remaining three connectives were then cut in the following sequence (see Fig. 2A): right posterolateral connective (R Pl c., 1st cut), right anterolateral connective (R Al c., 2nd cut), and left anterolateral connective (L Al c., 3rd cut). Immediately after each cut,

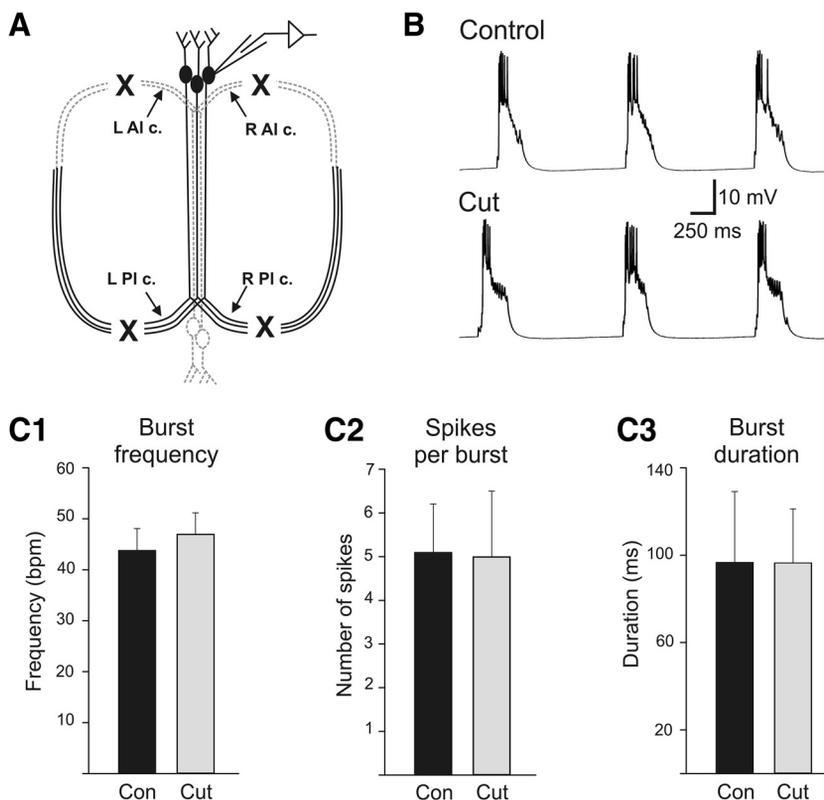


FIG. 2. Control de-efferentation experiments in the modified isolated CG (ICG) preparation reveal no direct effect of de-efferentation on the CG motor neuron spike pattern. *A*: in the de-efferentation procedure, the four CG connectives were cut at the locations marked (X). *B*: representative intracellular recording from an anterior motor neuron before (top) and after (bottom) de-efferentation. *C*: quantification of the standard parameters of the CG motor neuron spike pattern: group data from 4 experiments. Means  $\pm$  SD are plotted. There was no statistically significant effect of the de-efferentation on the burst frequency [C1: control  $43.8 \pm 4.3$  bursts per min (bpm), connectives cut  $47.0 \pm 4.2$  bpm;  $t = -0.997$ ,  $P > 0.05$  by 2-tailed Student's  $t$ -test], number of spikes per burst (C2: control  $5.1 \pm 1.1$ , cut  $5.0 \pm 1.5$ ;  $t = 0.106$ ,  $P > 0.05$ ), or burst duration (C3: control  $96.8 \pm 32.4$  ms, cut  $96.5 \pm 24.7$  ms;  $t = 0.123$ ,  $P > 0.05$ ).

the motor neuron spikes and contractions were considerably disturbed, but they stabilized within 2–3 min. Another recording was obtained, and the activity was monitored for 30–45 min before the next cut.

Figure 3A shows excerpts from the recordings under control conditions and after each cut from a representative experiment, and B expands and compares single contractions and motor neuron spike bursts from these recordings. Figure 4 shows the group data from all experiments. Clearly, in these experiments with the myocardium left in place, the connective transection did have substantial effects, not only on the contractions, as was to be expected, but also on the motor neuron spike pattern.

After each successive cut, the amplitude of the contractions was progressively reduced (Figs. 3, A and B, and 4A). When all four connectives were cut, the contractions were often eliminated (as in Fig. 3), although in some preparations very small and rapid rhythmic contractions could be detected in the muscle fibers adjacent to the CG. Statistical analyses of the group data in Fig. 4A showed that the reduction in contraction amplitude was highly significant (see figure legend).

Concomitantly with this decrease in contraction amplitude, there was a progressive increase in the frequency of the motor neuron spike bursts (Figs. 3, A and B, and 4B). Because each burst produced one contraction, the frequency of the contractions also necessarily increased. As the bursts increased in frequency, they at the same time became weaker, decreasing progressively in both the number of spikes per burst and the burst duration (Figs. 3, A and B, and 4, C and D). When all four connectives were cut, the bursts were often reduced to single spikes, and in some preparations spikes of different amplitudes appeared in the extracellular record (e.g., in Fig. 3, A and B, 3rd cut), likely signs of the partial desynchronization of the CG motor neurons that is sometimes observed when the system is unphysiologically stressed (see, e.g., Fort et al. 2007a). Statistical analyses of the group data in Fig. 4, B–D, showed that the changes in burst and contraction frequency, number of spikes per burst, and burst duration were all highly significant (see figure legend).

### De-afferentation modifies the CG motor pattern

In the experiments just described, the de-efferentation had a direct effect, presumably, just on the myocardial contractions in the cardiac periphery. Yet the motor neuron spike patterns generated centrally by the CG were also modified, presumably by feedback from the periphery. To determine whether this feedback was mediated by the dendrites of the CG neurons that project into the myocardial muscle, we performed a complementary set of “de-afferentation” experiments in which we disrupted the dendrites. Selective de-afferentation was made possible by the fact that in portunid crabs such as *Callinectes* the dendritic endings of the CG neurons are mainly confined to two well-localized regions between the connective roots at the anterior and posterior ends of the ganglion (Fort et al. 2004; Tazaki and Cooke 1979) (see Fig. 1, A and B), where they can be disrupted without injury to the motor neuron axons projecting through the connectives. We disrupted the dendrites in two ways, by cutting the dendritic region with sharp scissors or by ligating it with a fine hair.

First, as in the case of de-efferentation, we performed control de-afferentation experiments in the ICG preparation from which the myocardium had been removed. This was all the more necessary as previous work has suggested that the generation of the CG rhythm may be highly sensitive to dendritic injury (see review by Cooke 2002). In these experiments, a control intracellular recording was obtained from one of the CG motor neurons (a representative example is shown in Fig. 5B, top). The microelectrode was then removed, both the anterior and the posterior dendrites were either cut or ligated (at the locations marked in Fig. 5A, ×), and the motor neuron was re-impaled, usually within 5 min. Although the transection or ligation procedure did result in some injury to the motor neurons, as evidenced by transient increases in burst frequency, these effects were completely reversed within 10 min (Fig. 5B, bottom). Group data obtained 30 min after the transection or ligation (Fig. 5C) showed that neither procedure had any significant effect on the motor neuron burst frequency, number of spikes per burst, or burst duration (for statistical analyses,

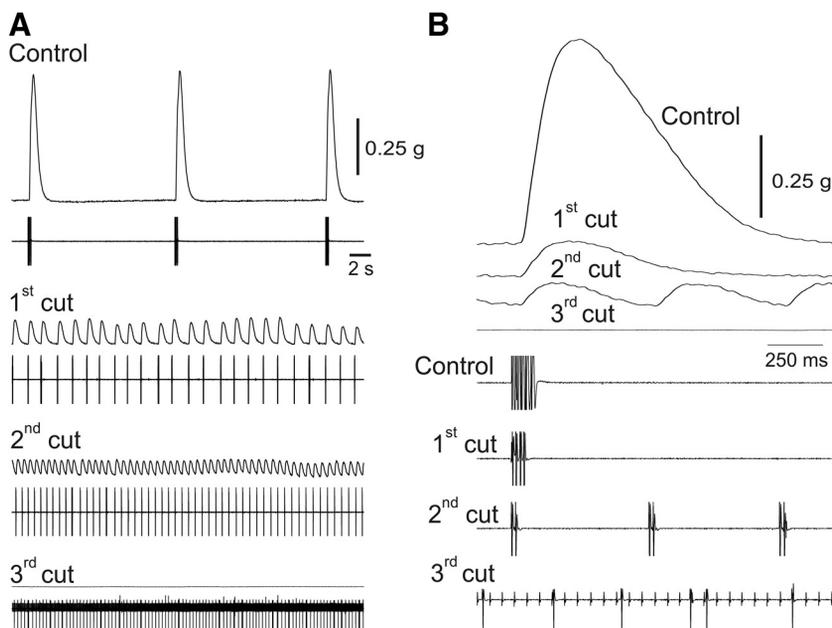


FIG. 3. In the semi-intact working heart (SIWH) preparation, de-efferentation modifies not only contractions but also the CG motor neuron spike pattern: representative records. A: myocardial contractions recorded with a force transducer (top of each pair of records) and the CG motor neuron spike bursts recorded with an extracellular suction electrode from the left posterolateral connective (bottom of each pair of records), with only the left posterolateral connective cut (control) and then after further successive cuts of the right posterolateral connective (1st cut), the right anterolateral connective (2nd cut), and the left anterolateral connective (3rd cut). B: expanded segments of the records in A aligned for comparison.

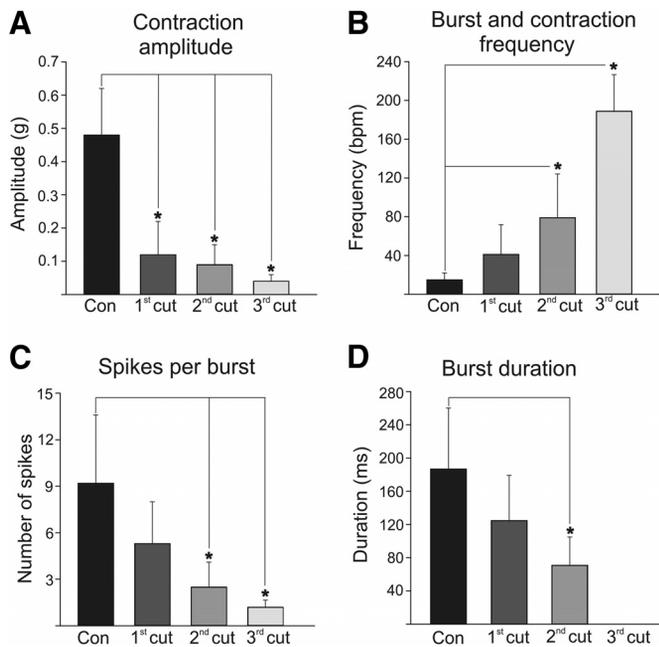


FIG. 4. In the SIWH preparation, de-efferentation modifies not only contractions but also the CG motor neuron spike pattern: group data from 6 experiments (not all measurements could be made in all experiments). Means  $\pm$  SD are plotted. Separately in each of A–D, statistical analysis was performed with 1-way ANOVA with the following overall results. A: contraction amplitude: control  $0.48 \pm 0.14$  g, 1st cut  $0.12 \pm 0.10$  g, 2nd cut  $0.09 \pm 0.06$  g, 3rd cut  $0.04 \pm 0.02$  g;  $F(3,15) = 18.75$ ,  $P < 0.001$ . B: burst and contraction frequency: control  $15.0 \pm 7.1$  bpm, 1st cut  $41.2 \pm 30.7$  bpm, 2nd cut  $79.1 \pm 45.0$  bpm, 3rd cut  $189.0 \pm 37.6$  bpm;  $F(3,18) = 24.88$ ;  $P < 0.001$ . C: number of spikes per burst: control  $9.2 \pm 4.4$  spikes, 1st cut  $5.3 \pm 2.7$  spikes, 2nd cut  $2.5 \pm 1.6$  spikes, 3rd cut  $1.2 \pm 0.5$  spikes;  $F(3,19) = 9.31$ ,  $P < 0.001$ . D: burst duration: control  $187.0 \pm 73.2$  ms, 1st cut  $124.8 \pm 54.4$  ms, 2nd cut  $71.0 \pm 34.0$  ms;  $F(2,13) = 4.80$ ,  $P < 0.03$ . In each of A–D, the ANOVA was then followed by pairwise comparisons using the Holm-Sidak method; all significant differences from control ( $P < 0.05$ ) are marked by the asterisks. In D, no meaningful burst duration could be quantified after the 3rd cut because only one spike per burst remained in most experiments.

see figure legend). Moreover, there was no significant change in the motor neuron membrane potential following the transection (control:  $-50.4 \pm 3.6$  mV, cut:  $-52.2 \pm 1.6$  mV;  $t = -1.006$ ,  $P > 0.05$  by 2-tailed Student's  $t$ -test;  $n = 5$ ) or ligation (control:  $-50.4 \pm 2.9$  mV, ligated:  $-49.8 \pm 2.7$  mV;  $t = 0.341$ ;  $P > 0.05$ ;  $n = 5$ ).

Having established that the de-afferentation procedures per se had no long-lasting effects, we performed analogous experiments in the SIWH preparation in which the CG remained embedded in the contracting myocardium. Figure 6A, then expanded in B, show records before and after both the anterior and the posterior dendrites were cut in a representative experiment. Figure 7 shows the group data from all transection and ligation experiments. Figure 6C shows a Neurobiotin fill of the motor neurons following one of the transection experiments, a procedure that we routinely performed to confirm that we had selectively eliminated the dendrites but spared the motor neuron axons.

In the SIWH preparation, in contrast to the ICG preparation, the transection or ligation of the dendrites had substantial effects. Both procedures had essentially identical effects, which were also very similar to those of de-efferentation: decreased contraction amplitude, increased burst and contraction frequency, and decreased number of spikes per burst and burst duration (Fig. 6, A

and B). Statistical analyses of the group data in Fig. 7 showed that the effects of both transection and ligation on all four parameters were statistically significant (see figure legend).

#### Myocardial stretch modifies the CG motor pattern

Information was thus apparently being conveyed from the myocardium to the CG through the dendrites. Presumably this was information about the mechanical state of the myocardium, sensed by the mechanosensitive dendrites. If so, simply stretching or relaxing the myocardium passively might also modify the CG motor neuron spike pattern in a dendrite-dependent manner.

To test this prediction, we performed experiments in the SIWH preparation in which we stretched or relaxed the myocardium by moving the attached force transducer or, in later experiments with an Aurora muscle lever system (a clamp system in which both length and force are measured and either one can be set as the controlled variable), by commanding a change in the length input to the system. All of the manipulations were thus in the first instance changes in myocardial length, although they were always necessarily accompanied by changes in myocardial tension. With the force transducer, we recorded only the latter changes in force, while with the Aurora system we were able to record both the length and force changes. At the same time, we recorded the CG motor neuron spikes with an extracellular suction electrode.

In an initial exploratory set of experiments, we simply stretched or relaxed the myocardium manually, within a relatively brief interval of time, from one maintained length to another. We observed clear changes in the motor neuron spike pattern in response to repeated stretches and relaxations of various amplitudes in 12 experiments. Representative excerpts from three experiments are shown in Fig. 8. The parameter of the spike pattern that was most obviously modified was the burst frequency, in this figure more conveniently plotted as its reciprocal, the cycle period of the CG rhythm.

The effects that we observed were complex. The predominant effect was a decrease in the cycle period, an acceleration of the CG rhythm, on stretch, or equivalently an increase in the cycle period, a slowing of the rhythm, on relaxation (Fig. 8, A and B; see also Fig. 9). The response to relaxation could be dramatic, sometimes almost entirely stopping the heart (Fig. 8A). In a number of cases, however, we observed the opposite effect, an increase in the cycle period on stretch and a decrease on relaxation (Fig. 8C). Sometimes there was a biphasic response to stretch or relaxation (Fig. 8B), suggesting a combination of opposing effects with different time courses. Furthermore, the effects were often transient (e.g., Fig. 8C) and there was strong history dependence. Thus over the course of an experiment, a particular value of the myocardial length or tension did not correspond to any single value of the cycle period, and, conversely, the same cycle period could occur with very different degrees of myocardial stretch, as can clearly be seen in Fig. 8C. We observed no obvious correlation between the type of effect and the baseline state, for example the baseline length or tension, of the myocardium, although this point will need more systematic examination in future work.

Although the cycle period was the parameter of the spike pattern that was most consistently modified, in some cases

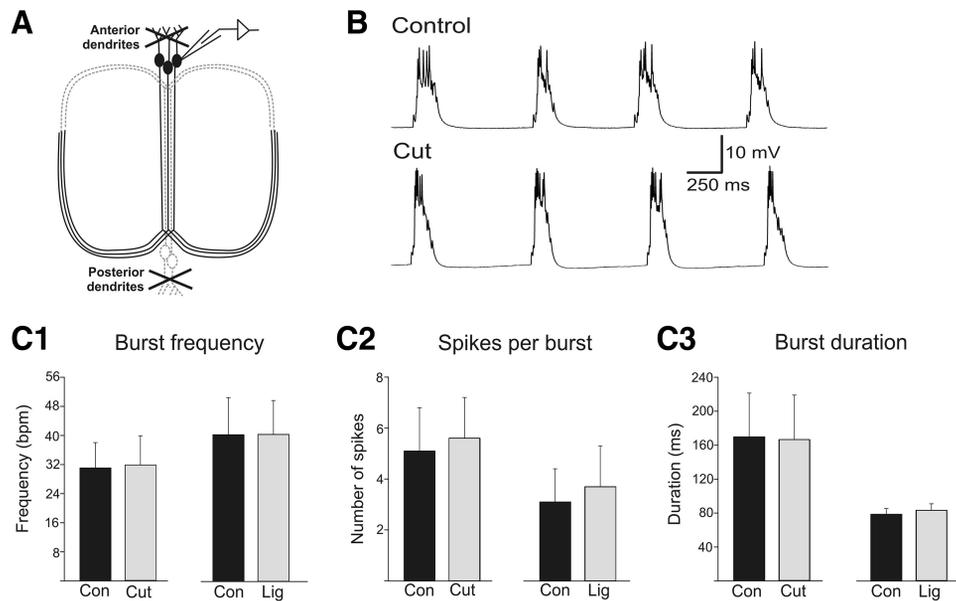


FIG. 5. Control de-afferentation experiments in the ICG preparation reveal no direct effects of the de-afferentation procedures on the CG motor neuron spike pattern. *A*: in the de-afferentation procedures, both the anterior and the posterior dendrites were either transected or ligated at the locations marked ( $\times$ ). *B*: representative intracellular recording from a motor neuron before (*top*) and after (*bottom*) transection of the dendrites. *C*: quantification of the standard parameters of the CG motor neuron spike pattern: group data from 8 experiments with dendrite transection and 6 experiments with dendrite ligation. Means  $\pm$  SD are plotted. There was no statistically significant effect of the dendrite transection on the burst frequency (*C1, left*: control  $31.1 \pm 7.0$  bpm, dendrites cut  $31.9 \pm 8.0$  bpm;  $t = -0.199$ ,  $P > 0.05$  by 2-tailed Student's *t*-test), number of spikes per burst (*C2, left*: control  $5.1 \pm 1.6$  spikes, cut  $5.6 \pm 1.6$  spikes;  $t = -0.674$ ,  $P > 0.05$ ), or burst duration (*C3, left*: control  $169.9 \pm 51.5$  ms, cut  $166.5 \pm 52.5$  ms;  $t = 0.130$ ,  $P > 0.05$ ). Likewise, there was no statistically significant effect of the dendrite ligation on the burst frequency (*C1, right*: control  $40.2 \pm 10.2$  bpm, dendrites ligated  $40.3 \pm 9.3$  bpm;  $t = -0.296$ ,  $P > 0.05$ ), number of spikes per burst (*C2, right*: control  $3.1 \pm 1.3$  spikes, ligated  $3.7 \pm 1.6$  spikes;  $t = -0.653$ ,  $P > 0.05$ ), or burst duration (*C3, right*: control  $78.7 \pm 6.7$  ms, cut  $83.4 \pm 7.5$  ms;  $t = -0.115$ ,  $P > 0.05$ ).

there were clear effects also on the number of spikes per burst and burst duration. Figure 9 shows an example in which these parameters were not only modified, but they as well as the cycle period apparently became more regular from one cycle to the next on relaxation and more irregular on stretch. Thus the myocardial stretch did not merely speed up or slow down the heart, but modified the CG motor pattern in a deeper manner.

To quantitatively understand these complex effects, a more powerful analysis will be needed. To gather the data for such an analysis, we performed another set of experiments in which we used the Aurora system to stretch and relax the myocardium

with a systematic series of random waveforms programmed to have Gaussian white-noise properties (for details see METHODS). The intent of such waveforms is to elicit at the same time all of the effects that the myocardial stretch may have, no matter (within limits) what their direction, magnitude, and time course may be. A representative example of one such waveform and its effect on the motor neuron spike pattern is shown in Fig. 10A. The cycle period is plotted, and it is clear that during the interval of random stretch the cycle period became more variable than during the preceding or following baseline intervals. This increased variability reflected, although presumably

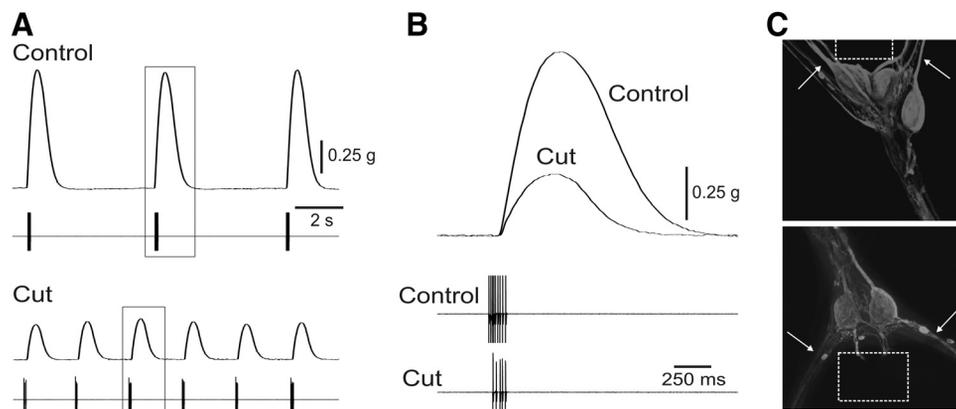


FIG. 6. In the SIWH preparation, de-afferentation modifies not only contractions but also the CG motor neuron spike pattern: representative records. *A*: myocardial contractions recorded with a force transducer (*top* of each pair of records) and the CG motor neuron spike bursts recorded with an extracellular suction electrode from the left posterolateral connective (*bottom* of each pair of records), before and after both the anterior and posterior dendrites were cut. *B*: the boxed segments of the records in *A* expanded and aligned for comparison. *C*: Neurobiotin fills of the motor neurons after a de-afferentation experiment. The images confirm that both the anterior and the posterior dendrites that would have been in the regions outlined by the dashed rectangles in the *top* and *bottom* images, respectively (compare Fig. 1B), were successfully cut while the motor neuron axons within the connectives (arrows) remained intact.

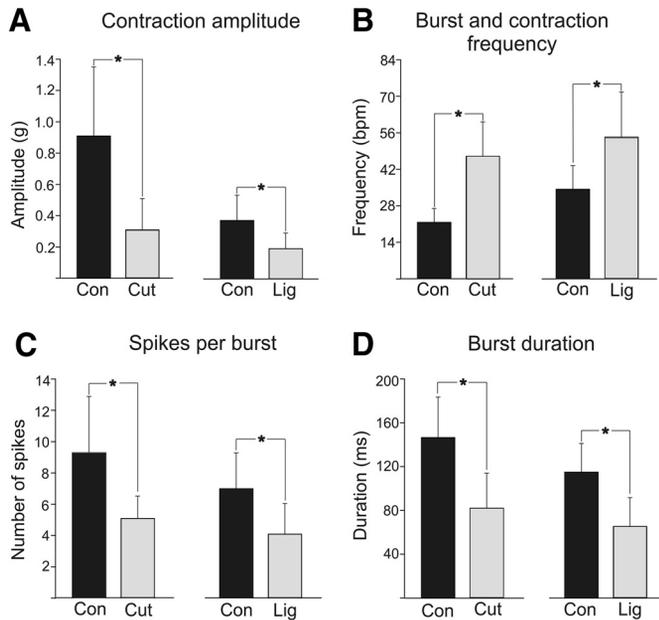


FIG. 7. In the SIWH preparation, de-afferentation modifies not only contractions but also the CG motor neuron spike pattern: group data from 7 experiments with dendrite transection and 6 experiments with dendrite ligation. Means  $\pm$  SD are plotted. Statistical analyses were performed with 2-tailed Student's *t*-tests. There were statistically significant effects (\*) of the dendrite transection on contraction amplitude (A, left: control  $0.91 \pm 0.44$  g, cut  $0.31 \pm 0.20$  g;  $t = 2.507$ ,  $P < 0.05$ ), burst and contraction frequency (B, left: control  $21.7 \pm 5.4$  bpm, cut  $47.0 \pm 13.1$  bpm;  $t = -4.642$ ,  $P < 0.001$ ), number of spikes per burst (C, left: control  $9.3 \pm 3.6$  spikes, cut  $5.1 \pm 1.4$  spikes;  $t = 2.916$ ,  $P < 0.05$ ), and burst duration (D, left: control  $146.6 \pm 36.8$  ms, cut  $81.9 \pm 32.1$  ms;  $t = 3.503$ ,  $P < 0.05$ ), and of the dendrite ligation on contraction amplitude (A, right: control  $0.37 \pm 0.16$  g, ligated  $0.19 \pm 0.10$  g;  $t = 2.359$ ,  $P < 0.05$ ), burst and contraction frequency (B, right: control  $34.2 \pm 9.0$  bpm, ligated  $54.2 \pm 17.3$  bpm;  $t = -2.716$ ,  $P < 0.02$ ), number of spikes per burst (C, right: control  $7.0 \pm 2.3$  spikes, ligated  $4.1 \pm 1.9$  spikes;  $t = 2.318$ ,  $P < 0.05$ ), and burst duration (D, right: control  $115.0 \pm 26.1$  ms, ligated  $65.5 \pm 26.2$  ms;  $t = 3.280$ ,  $P < 0.05$ ).

in some complex manner, the variability of the imposed myocardial stretch. When the dendrites were cut, the increase in variability did not occur (Fig. 10B).

A full mathematical analysis of this dataset, to extract kernels quantitatively representing the effects of the myocardial stretch, is beyond the scope of this paper and will be presented elsewhere (see DISCUSSION) (see also Stern et al. 2009a). For the purposes of this paper, however, we performed a basic analysis of the data, simply in terms of the increased overall variability, to demonstrate in a statistically rigorous manner that the cycle period of the CG rhythm was indeed modified by these random myocardial stretch patterns and that this modification was dependent on the integrity of the dendrites. Figure 10C compares the distributions of the cycle period values during one representative random stretch pattern (thick outline) and the adjacent baseline intervals (thin outline), before and after transection of the dendrites. During the random stretch pattern, the distribution became noticeably broader when the dendrites were intact but not when they were cut. To quantify the dispersion of the values in such distributions, we computed the coefficient of variation (CV; the SD of the distribution divided by its mean) and normalized the CV during each random pattern by the CV during the adjacent baseline intervals. Figure 10D shows the group data from four experiments. Statistical analysis (see figure legend) confirmed that

the CV of the cycle period was indeed significantly increased by the random stretches when the dendrites were intact (black bar), but not when they were cut (gray bar).

## DISCUSSION

In his initial description of the dendritic projections from the crustacean CG, Alexandrowicz (1932) wrote: "The fact that the dendrites of the cells end in the muscles suggests that the rhythmical discharges in the nerve-cells are under the influence of the rhythmical action of the musculature." Our results confirm that, indeed, the rhythmic motor pattern generated by the *Callinectes* CG is modified by feedback from the contractions of the myocardial musculature that are produced by that motor pattern, and that this feedback is mediated at least in part by the dendrites.

Beyond this, however, the detailed interpretation of our results is not as straightforward as it might appear because there are evidently multiple effects (as our stretch experiments show) and furthermore because any primary effect has multiple secondary consequences that ramify through the interconnected network structure of even this "simple" system. Before attempting an interpretation, it is helpful in this regard to compare and contrast our results with the relevant previous results.

### Comparison with previous work

First, in the early work in lobster hearts, focal mechanical stimulation of the dendrites was found to contract the heart (Alexandrowicz 1932; Hartline 1967; see Cooke 2002). Furthermore, inflating or otherwise stretching the heart increased the heartbeat frequency and sometimes appeared to be required for the heart to beat at all (Hartline 1967; Kuramoto and Ebara 1984, 1985), leading to the idea that the normal inflation of the heart with blood maintains the CG rhythm. Wilkens (1993), however, has argued that the faster heartbeat in such experiments is not due to the mechanical stimulus but rather to increased delivery of oxygen to the tissue. In our experiments here, passive stretches of the myocardium did often increase the frequency of the CG rhythm—the experiment in Fig. 8A suggests, indeed, almost an absolute requirement for stretch—but sometimes decreased it, and furthermore both our de-afferentation and de-afferentation results suggest that larger active contractions are associated with a slower, rather than faster, rhythm.

Second, in our previous work in *Callinectes*, we proposed that the differences that we observed in the parameters of the CG motor pattern between the isolated CG and the CG embedded in the myocardium, and in the effects of modulators in the two cases (see INTRODUCTION), reflected feedback from the myocardium that was removed when the CG was isolated (Fort et al. 2004, 2007a,b). For example, the CG rhythm became faster when the CG was isolated, implying that feedback from the myocardium normally slows the rhythm. Our de-afferentation and de-afferentation results generally support this explanation (see further in the following text).

Third, in the lobster heart, Mahadevan et al. (2004) likewise proposed that feedback from the myocardium slows the CG rhythm. As a mechanism for this, however, they provided convincing evidence that NO, produced in the myocardial

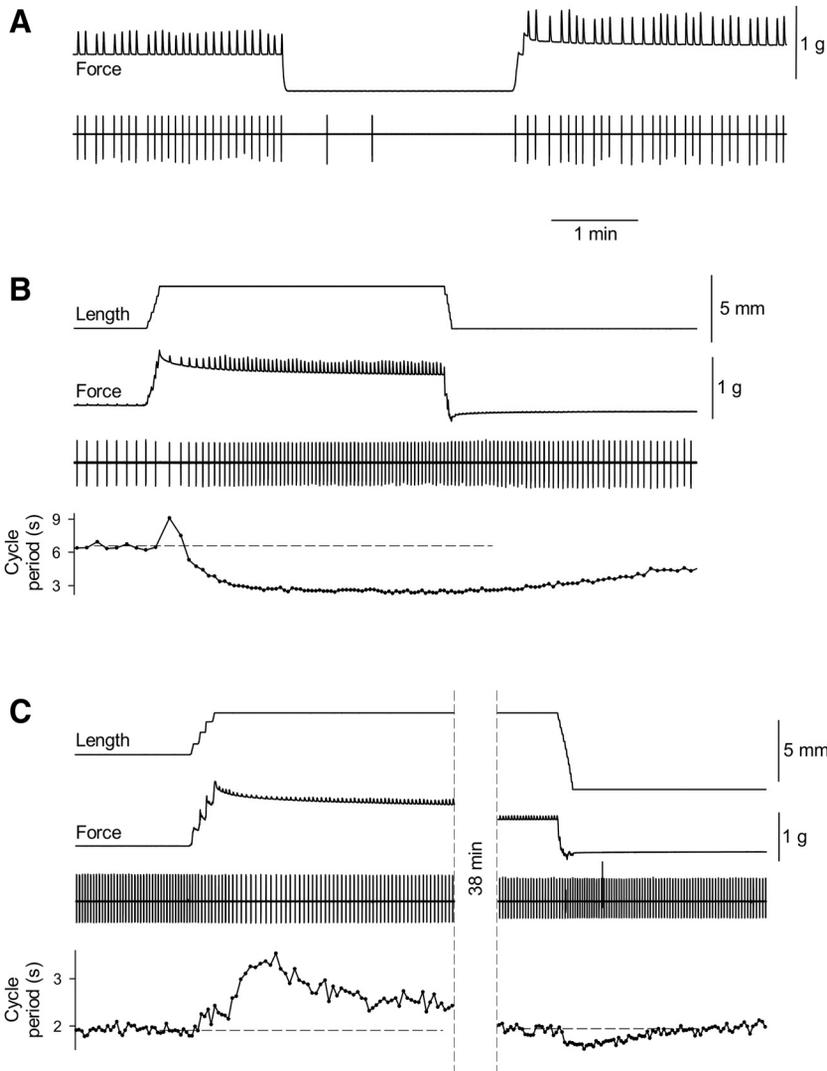


FIG. 8. Stretch of the myocardium modifies the cycle period of the CG rhythm. Representative excerpts from three experiments in SIWH preparations. *A*: myocardial force (tension) recorded with a force transducer (*top*) and CG motor neuron spike bursts recorded with an extracellular suction electrode from a CG connective (*bottom*) while the myocardium was relaxed and then re-stretched by moving the force transducer. *B* and *C*: myocardial length and force recorded with the Aurora muscle lever system, motor neuron spike bursts, and the cycle period computed from them (*top* to *bottom* in each of *B* and *C*). The myocardium was stretched and then relaxed by length commands to the Aurora system. The time scale bar under excerpt *A* applies to all three excerpts.

muscle by a nitric oxide synthase (NOS) that may be  $Ca^{2+}$ -dependent and so perhaps increase its activity in parallel with contractile activity, diffuses back to the CG and slows its

rhythm (see also Goy 2005). Because NOS is found also in crab myocardium (Scholz et al. 2002), this mechanism may well operate also in the *Callinectes* heart.

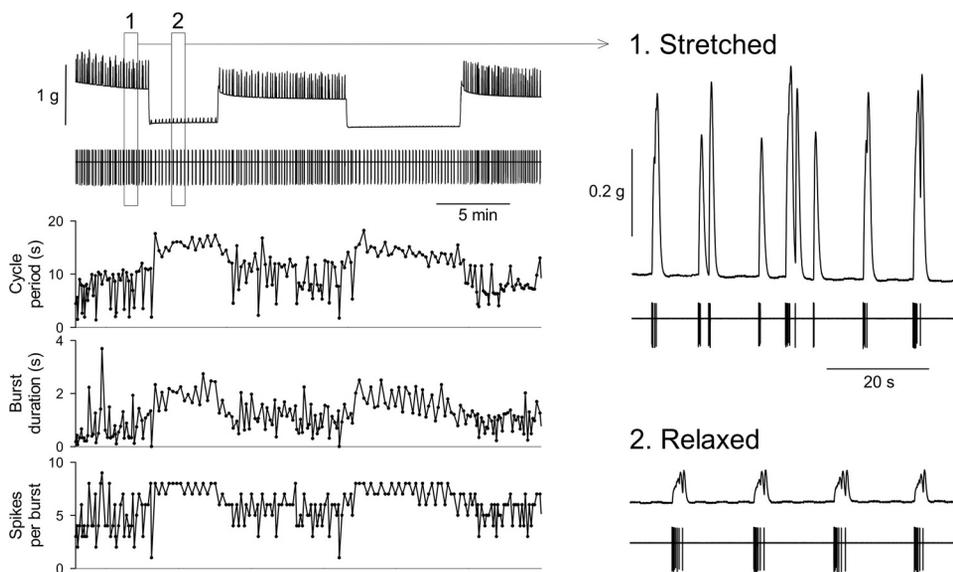


FIG. 9. Stretch of the myocardium can modify the detailed pattern of the CG motor neuron spikes. Representative excerpt from an experiment in a SIWH preparation, like that in Fig. 8A except that in this case parameters such as the spike burst duration and the number of spikes per burst were also clearly modified and are plotted. The indicated segments of the recording, showing the contractions of the myocardium and the spike bursts under the stretched and relaxed conditions, are expanded on the right.

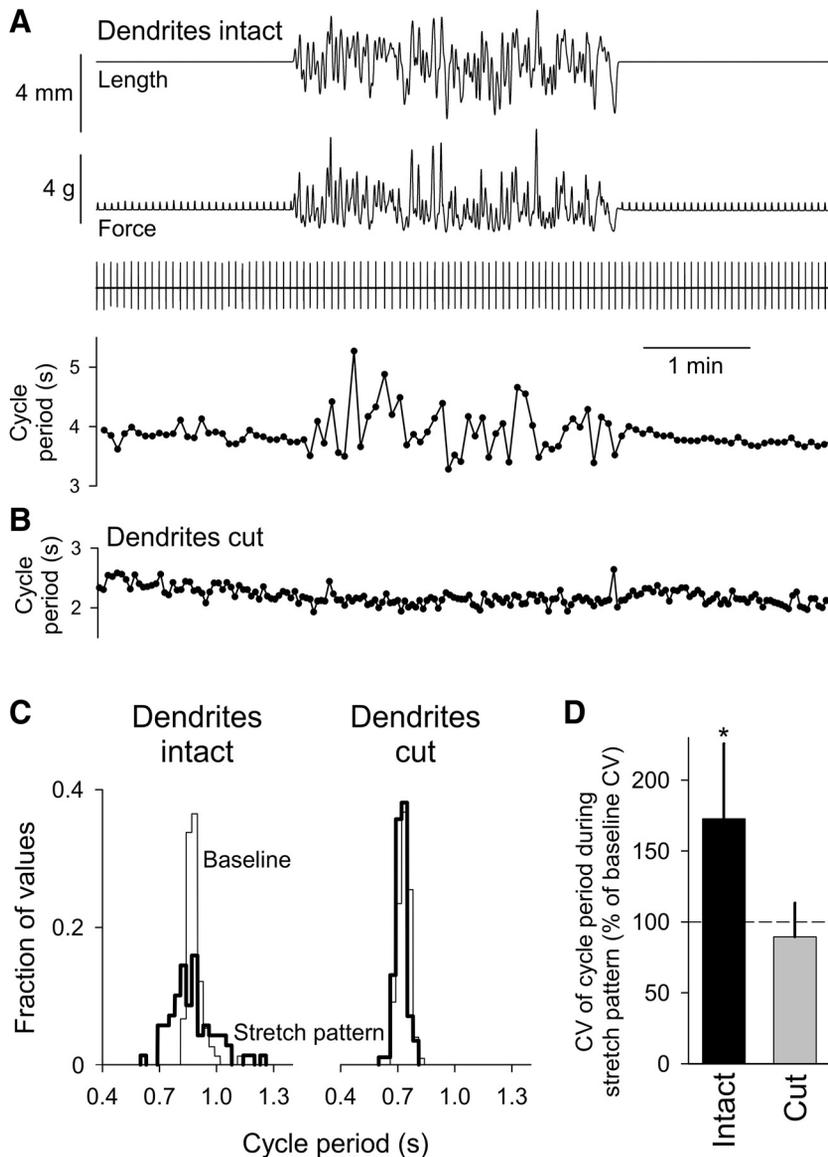


FIG. 10. Dendrite-mediated modification of the CG motor neuron spike pattern by myocardial stretch probed with random stretch patterns: basic statistical analysis of the modification of the cycle period of the CG rhythm. *A*: representative excerpt from an experiment in a SIWH preparation showing one of a series of 3-min random stretch patterns applied as length commands to the Aurora muscle lever system separated by steady baseline stretch (see METHODS). Plotted is the myocardial length and force recorded with the Aurora system, the CG motor neuron spike bursts recorded extracellularly from a CG connective, and the cycle period computed from them (top to bottom). *B*: the cycle period during the same random stretch pattern, in the same experiment, but with the dendrites cut. The plot is aligned so that the stretch pattern was applied during the same section of the plot as in *A*. *C*: typical histograms of the cycle period values computed (in a different experiment from that in *A* and *B*) during one random stretch pattern (thick histogram outline) and during a comparable interval of the adjacent baseline stretch (thin histogram outline), first with the dendrites intact (left) and then, in the same experiment, with the dendrites cut (right). *D*: basic statistical analysis of the increased variability of the cycle period induced by the random stretch. The coefficient of variation (CV) of the cycle period values during each random stretch pattern was expressed as a percentage of the CV of the cycle period values during the comparable interval of adjacent baseline stretch. Plotted are the means  $\pm$  SD of the resulting values pooled from 11 random stretch patterns applied with the dendrites intact and 10 comparable patterns applied with the dendrites cut, from 4 experiments. Testing of statistical significance with 1-way ANOVA [dendrites intact  $1.73 \pm 0.53$ , dendrites cut  $0.89 \pm 0.24$ ;  $F(3,38) = 18.07$ ,  $P < 0.001$ ] followed by pairwise comparisons using the Holm-Sidak method showed that the random stretch CV was significantly different from 100%, i.e., from the baseline CV, when the dendrites were intact ( $P < 0.001$ , \*) but not significantly different when the dendrites were cut ( $P > 0.2$ ).

Fourth, in the heart of *Ligia*, Sakurai and Wilkens (2003) made a number of especially relevant findings. The *Ligia* CG is even simpler, containing only six neurons that act simultaneously as pacemaker neurons, motor neurons, and, Sakurai and Wilkens proposed, also sensory neurons that, through their dendrites, sense feedback from the myocardium. Recording intracellularly the membrane voltage of the neurons—something that remains to be done in the *Callinectes* CG—Sakurai and Wilkens found that the neurons were hyperpolarized by both passive stretch and active contractions of the myocardium. The time course of the voltage response conformed closely to that of the mechanical stimulus, allowing cycle-to-cycle and even within-cycle feedback and suggesting mediation of the feedback by a fast process—Sakurai and Wilkens suggested mechanosensitive ion channels in the membrane of the dendrites—rather than a slower process such as NO diffusion. With maintained passive stretch of the myocardium, the net result of the feedback was a slowing of the CG rhythm, as in some, but not all, of our stretch experiments here. With phasic active contractions, however, there was, as each contraction relaxed and the hyperpolarization diminished, a re-

bound phase advance of the next spike burst. The net result of the feedback in the normally beating heart was thus a faster rhythm, the opposite of the effect of the NO-mediated feedback described by Mahadevan et al. (2004) and of our de-efferentiation and de-afferentation results.

#### Interpretation of the results in the interconnected system

We will focus particularly on the de-efferentiation and de-afferentation results. The general rule with such lesion experiments is that the observed difference in the activity of the system before and after the lesion does not represent simply the contribution of the lesioned process, but rather is the difference between the states of the entire system with and without the lesioned process (see, e.g., Kitano 2004). We will assume that, as in all of the experiments reported here, the system is observed only after it has equilibrated to a dynamical steady state, which in these experiments was always a limit cycle of period 1, that is, with all successive heartbeats essentially identical [although higher-order cycles spanning multiple heartbeats and other more complex dynamical behaviors can

and do occur in the *Callinectes* cardiac system (Fort et al. 2007a,b; Hokkanen 2000)]. When the system is an interconnected network containing feedback loops, the state to which it equilibrates can be surprising. Thus although the fact that our dendrite lesions, for example, had a significant effect indicates that the dendrites transmitted significant information, the magnitude of the effect does not directly show the magnitude, or potentially even the sign, of the information that they transmitted. Nevertheless, if we correlate the effects of the de-afferentation, de-efferentation, and stretch, we will be able to reach some reasonably firm conclusions.

Suppose, specifically, that the *Callinectes* CG is connected to the myocardium by the motor neuron axons, and the myocardium is connected back to the CG not only by the mechanosensitive dendrites but also by NO-mediated feedback like that described by Mahadevan et al. (2004). The CG itself very likely contains yet another feedback mechanism (see following text) that, as in other crustacean hearts (Benson 1980; Mayeri 1973; Tazaki and Cooke 1979, 1990), functionally connects the frequency of the CG spike bursts to their strength (number of spikes per burst and burst duration) in an inverse fashion, so that a slow CG rhythm has strong bursts and a faster rhythm weaker bursts.

De-afferentation is perhaps the easier intervention to interpret because it removes one connection, the dendritic feedback, from the network. This removal has an intrinsic effect on the CG motor pattern—if it did not then the behavior of the system would not change at all—that can most parsimoniously be assumed to be in the direction of the final effect that is observed, that is, toward a faster CG rhythm with weaker bursts. To simplify this discussion, we will suppose that the effect is primarily to speed up the rhythm, which then secondarily weakens the bursts by the feedback within the CG. This constitutes the intrinsic effect of the de-afferentation, but it will not be observed in isolation. Through the efferent motor neuron axons, the modified CG motor pattern produces modified myocardial contractions. The neuromuscular transform, the mapping from the motor neuron spike pattern to the contraction waveform (Brezina et al. 2000a), is not yet known in detail in the *Callinectes* heart, but there is reason to suppose that it combines two main effects that will operate here: first, an increase in contraction amplitude as the CG rhythm speeds up, due to synaptic facilitation of the myocardial neuromuscular junctions (see, e.g., Anderson and Cooke 1971; Mahadevan et al. 2004; Fig. 8B of Fort et al. 2007a), but opposed by a second effect, a decrease in contraction amplitude as the bursts weaken (see Fig. 8D of Fort et al. 2007b). The second effect is likely to predominate here. Therefore the motor pattern with a faster rhythm but weaker bursts produces smaller contractions. The system will not simply stabilize there, however, because in the de-afferented preparation the CG has not been removed from the myocardium, so that the NO-mediated feedback still remains to complete a loop from the periphery to the CG. If this feedback operates as described by Mahadevan et al. (2004), the smaller contractions will produce reduced levels of NO and thus modify the CG motor pattern further in the same direction, toward an even faster rhythm and, through the feedback within the CG, even weaker bursts, and so in turn, through the neuromuscular transform, smaller contractions still. Driven by this positive feedback loop, the system will continue to evolve toward a faster rhythm, weaker bursts, and

smaller contractions until it reaches a state in which the positive feedback loop ceases to operate. If the NO-mediated feedback is, as might be expected, relatively slow, this evolution might progress over many heartbeats. The final state, however, will be the state that we will actually have observed in our de-afferentation experiments.

Interestingly, the final state in the de-afferentation experiments, in which the CG still remained in the myocardium, was not very different in its motor neuron burst frequency, number of spikes per burst, and burst duration from the state when the CG was completely removed from the myocardium in our isolated CG experiments in this paper (compare the cut and ligated parameter values in Fig. 7 with the control values in Figs. 5C and 2C). This bears out our previous proposal that the change in CG motor pattern that is observed when the CG is removed from the myocardium is explained by the loss of peripheral feedback. It implies, furthermore, that when the dendrites were lesioned in the de-afferentation experiments, the system evolved to a state where the NO-mediated feedback, and any other feedback from the myocardium that may exist, was minimized as well. This is also, of course, consistent with the possibility that in the *Callinectes* heart, in contrast to the lobster heart, the NO-mediated feedback is relatively weak compared with the dendritic feedback.

A similar interpretation can be offered for the de-efferentation results. In this case, no connection, at least until the last connective is cut, is removed entirely from the network; rather, the neuromuscular transform is modified so that a given motor neuron spike pattern produces smaller contractions than it did before. Essentially the same argument applies, however, as in the case of de-afferentation, and predicts the same evolution of the system—except driven in this case by both the NO-mediated and dendritic positive feedback loops—toward a faster rhythm, weaker bursts, and smaller contractions. This was indeed observed. In fact, the system apparently evolved even past the final state reached with de-afferentation or in the isolated CG, all the way to a state with an extremely fast rhythm, extremely weak bursts, and no contractions at all. The explanation for this is not entirely clear. One obvious difference between the de-efferented preparation and the de-afferented and isolated CG preparations is that the former still has intact dendrites that can sense the passive tension of the myocardium. An explanation in terms of passive tension would require that active and passive tension have opposite effects, the former slowing down and the latter speeding up the CG rhythm. In *Ligia*, Sakurai and Wilkens (2003) found that the two had effects in the same direction, presumably because they exerted similar tension on the dendrites. In our experiments here, however, we did find some indications of opposite effects of active contractions (tested alone in the de-efferentation experiments) and passive stretch (tested alone in the stretch experiments).

In the de-afferentation and de-efferentation scenarios in the preceding text, a factor essential for the continued operation of the positive feedback loops is the progressive change in the input to the peripheral feedback connections, that is, the progressive decrease in contraction amplitude. In our random stretch experiments in Fig. 10, however, we imposed the same stretch pattern, superimposed on the same baseline stretch, before and after the dendrite cuts, thus short-circuiting the

NO-mediated and any other peripheral feedback loops and revealing the effect of the dendritic feedback alone.

### *Premotor interneurons*

As our readout of the CG motor pattern, we focused throughout these experiments on the spike pattern of the motor neurons. This was partly for technical reasons—the large motor neurons are relatively easy to record from intracellularly and their spikes are easy to detect extracellularly in the CG connectives—but also for a more fundamental functional reason: because the motor neuron spikes are the only spikes that leave the CG for the myocardium, they are the only spikes that need be directly considered in analyzing the CG-myocardial interactions.

Nevertheless, the CG contains also the premotor interneurons (Fig. 1A). Neurobiotin fills of the motor neurons often enabled us to resolve the premotor neurons as well due to dye coupling between the two types of neurons (see Fort et al. 2004). In no instance, however, were we able to definitively ascribe specific dendrites to the premotor neurons. In contrast, the methylene blue stains of Alexandrowicz (1932) clearly demonstrated the presence of dendritic projections from the premotor neurons of *Cancer pagurus* and *Palinurus vulgaris* (see further Cooke 2002). The *Callinectes* premotor neurons thus very possibly have dendrites too, which we may have been cutting and ligating, together with those of the motor neurons, in our de-afferentation experiments.

For our purposes here, however, the importance of distinguishing which dendrites were cut is diminished by the fact that, although the premotor neurons do play a greater role in initiating and the motor neurons then in patterning the CG spike bursts, the two types of neurons are so extensively coupled to each other with electrical and chemical connections that, especially from the vantage point of the myocardium, they merge into a single network that simply elaborates the motor neuron spike pattern. It is the interactions within this CG network, notably the electrical coupling that implements feedback from the motor neurons to the premotor neurons (Berlind 1989; García-Crescioni et al. 2008), that are thought to give rise to such features of the spike pattern as the inverse relation between the frequency and the strength of the bursts (Benson 1980; Mayeri 1973; Tazaki and Cooke 1979, 1983a,b, 1990). Some of these features can indeed be observed in the isolated CG. However, the rapid and continuous modulation of the membrane voltage of the CG neurons by peripheral feedback found by Sakurai and Wilkens (2003) in *Ligia* raises the possibility that in the intact heart some of the relationships observed in the spike pattern may in fact be imposed on it by the peripheral feedback. This may be the case, for example, in our Fig. 9, where the complex patterning of the spikes and bursts when the myocardium is stretched may be generated entirely by the interactions within the CG network, but may alternatively be produced, or at least reinforced, by rapid feedback from the irregular contractions, which the irregular spike pattern then of course itself produces.

### *Self-regulation of the cardiac system for adaptive output*

The feedback from the myocardium back to the CG adds another layer to the network of interactions that regulates the

output of the cardiac system, furthermore a layer that reflects more directly the actual output and so enables the CG to “monitor and adjust its activity to the results of its actions” (Cooke 2002). For example, the inverse relationship between CG burst frequency and strength appears to have homeostatic significance, in that it produces an inverse relationship between the frequency and amplitude of the myocardial contractions (as seen in Figs. 3 and 6) that may keep the cardiac output approximately constant even as its temporal pattern changes. As already discussed, the inverse relationship between burst frequency and strength may originate entirely within the CG and control the contractions in a forward manner. If the relationship is adjusted by the feedback from the myocardium, however, then the homeostasis may be much more responsive to the actual cardiac output that is being produced.

Another example suggests itself when we note that neuromodulators that increase the cardiac output, typically by increasing both the burst and contraction frequency and the contraction amplitude, also tend to speed up the relaxation rate of the contractions, so that the contractions, although larger, are nevertheless briefer (see, e.g., Fig. 3A of Fort et al. 2007a). If, as described by Sakurai and Wilkens (2003) in *Ligia*, the feedback from the myocardium hyperpolarizes the CG neurons during the contraction but then, by a rebound excitation, initiates the next spike burst, then the feedback may ensure that each contraction is always initiated immediately after the relaxation of the previous contraction irrespective of the frequency and amplitude of the contractions, allowing the system to produce, in particular, fast, large, yet fully relaxing contractions. This would add a layer of feedback adjustment to the strictly forward mechanism that has been analyzed extensively in the feeding neuromuscular system of *Aplysia*, where the changes in contraction frequency, amplitude, and relaxation rate are coupled simply by virtue of being brought about simultaneously by the same modulator or set of coreleased modulators (Brezina et al. 1996, 2000b; Weiss et al. 1992).

The scenario just described would represent a form of entrainment, which was indeed demonstrated experimentally by Sakurai and Wilkens (2003) in the *Ligia* heart. As in other neuromechanical systems (Nishikawa et al. 2007; Pearson et al. 2006; Prochazka and Yakovenko 2007), phenomena such as entrainment and resonance (Hatsopoulos 1996; Iwasaki and Zheng 2006) may be expected to emerge from the dynamics of the coupled neuromechanical network and strengthen further its ability to self-tune its behavior so as to stabilize functionally adaptive output and, conversely, destabilize maladaptive output.

### *Modeling the complete cardiac system*

As our de-afferentation and de-efferentation scenarios showed, understanding the dynamical behavior of a network interconnected by multiple feedback loops, and its response to lesions and modulation, is by no means straightforward. It can best be done with the help of a mathematical model. We have constructed such a model, intended to represent the system completely at least on the level of the motor neuron spikes and contractions, of the *Callinectes* heart (Stern et al. 2006–2008, 2009b). We first model each of the connections in the cardiac network separately from experimental data obtained under open-loop conditions. Thus, to model the dendritic feedback

studied in this paper, we mathematically analyze the dataset of CG motor pattern responses to random stretches that here formed the basis of Fig. 10. Gaussian white-noise stretch waveforms were used in these experiments (see METHODS) to capture the effects of the feedback on (within limits) all time scales—inspection of Figs. 8–10 indeed appears to reveal both cycle-to-cycle and much slower effects—that can then be extracted mathematically as convolution kernels whose operation generates the effects (Stern et al. 2006, 2009a). These kernels, and similar kernels for all of the other connections of the network, are then embedded in a complete generative closed-loop model of the network (Stern et al. 2008). We are now running the model to predict and explain the behavior of the unmodulated network as well as the effects of the modulators that we observed in our previous work (Fort et al. 2004, 2007a,b), so as to provide further insight into the functional operation and regulation of this simple CPG-effector system.

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