Modulation of an Integrated Central Pattern Generator-Effector System: Dopaminergic Regulation of Cardiac Activity in the Blue Crab *Callinectes sapidus*

Timothy J. Fort, Vladimir Brezina, and Mark W. Miller*

1 Institute of Neurobiology and Department of Anatomy
University of Puerto Rico Medical Sciences Campus
201 Blvd del Valle, San Juan, Puerto Rico 00901

2 Department of Physiology & Biophysics
Mount Sinai School of Medicine
1 Gustave Levy Place, New York, NY 10029

Address for correspondence:
Dr. Mark W. Miller
Institute of Neurobiology
University of Puerto Rico
201 Blvd del Valle
San Juan, Puerto Rico 00901

Tel: (787) 724-1237
Fax: (787) 725-3804
email: mmiller@neurobio.upr.clu.edu

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Abstract

Theoretical studies have suggested that the output of a central pattern generator (CPG) must be matched to the properties of its peripheral effector system to ensure production of functional behavior. One way that such matching could be achieved is through coordinated central and peripheral modulation. In this study, morphological and physiological methods were used to examine the sources and actions of dopaminergic modulation in the cardiac system of the blue crab, Callinectes sapidus. Immunohistochemical localization of tyrosine hydroxylase (TH) revealed a prominent neuron in the commissural ganglion, the L-cell, that projected a large-diameter axon to the pericardial organ (PO) via an indirect and circuitous route. Within the PO, the L-cell axon gave rise to fine varicose fibers, suggesting that it releases dopamine in a neurohormonal fashion onto the heart musculature. In addition, one branch of the axon continued beyond the PO to the heart, where it innervated the anterior motor neurons and the posterior pacemaker region of the cardiac ganglion (CG). In physiological experiments, exogenous dopamine produced multiple effects on contraction and motor neuron burst parameters that corresponded to the dual central-peripheral modulation suggested by the L-cell morphology. Interestingly, parameters of the ganglionic motor output were modulated differently in the isolated CG and in a novel semi-intact system where the CG remained embedded within the heart musculature. These observations suggest a critical role of feedback from the periphery to the CG and underscore the requirement for integration of peripheral (neurohormonal) actions and direct ganglionic modulation in the regulation of this exceptionally simple system.
Introduction

The pivotal role of neuromodulation in the regulation and optimization of motor behavior is now firmly established (Kupfermann, 1979; Pearson, 1993; Katz, 1999). Compelling evidence has come from studies of many model systems (e.g., Weiss et al., 1992; Hultborn and Kiehn, 1992; Sillar et al., 2002) including, notably, the decapod crustaceans. In crustacean motor systems, modulation occurs at every level from sensory-motor integration (Glanzman and Krasne, 1983; Edwards et al., 2002) through central pattern generation (Miller and Sullivan, 1981; Harris-Warrick et al., 1988; Harris-Warrick and Marder, 1991) to neuromuscular transmission (Florey and Rathmayer, 1978; Kravitz et al., 1980; Breen and Atwood, 1983).

In neuromodulatory architectures, two broad themes have been distinguished: in the terminology of Cropper (1987) and Katz and Frost (1996), ‘intrinsic’ and ‘extrinsic’ modulation. Intrinsic modulation is locally integrated into the structure of the ‘mediating’ network. It is automatically set in motion when the network is active to provide local adjustments, although these adjustments may have global consequences in a well-connected, dynamically sensitive network such as a central pattern generator. The functional roles of intrinsic modulation have been relatively well studied (see, e.g., Katz and Frost, 1996; Brezina and Weiss, 1997; Katz, 1999; Marder and Thirumalai, 2002).

Extrinsic modulation, by contrast, arrives from an external source such as a modulatory neuron that is not itself part of the mediating circuitry. Such extrinsic modulatory systems of crustaceans have been described as overall ‘gain setters’ (Kravitz, 1988; Ma et al., 1992). Because extrinsic modulation is not obligatorily coupled to the activity of any particular part of the mediating system, it is free to exert widespread actions. In crustaceans, dopamine is of particular interest in this regard because it appears to regulate multiple motor systems (Berlind, 1977; Miller et al., 1984, 1985; Meyrand and Moulins, 1986; Barthe et al., 1989; Rajashekhar and Wilkens, 1992; Wood, 1995; Harris-Warrick et al, 1998). In those species in which it has been localized, dopamine has been found to be present in a relatively small number of neurons with extensive projections (Cournil et al., 1994, 1995, Wood and Derby, 1996; Tierney et al., 2003). Based on observations of this kind, it has been conjectured that the role of extrinsic
modulation in motor systems is to modulate multiple parts of motor circuits, and multiple motor
circuits, so as to integrate their activities into a global, coordinated whole. Whether external
modulation really plays this role, and what functional consequences might flow from such
actions, has, however, not been rigorously established. In part this has been because of the lack
of a suitable simple experimental preparation. Our first aim in this paper is to identify and
characterize such a preparation.

The cardiac system of decapod crustaceans is an exceptionally simple system which is
known to receive substantial modulatory regulation. The heartbeat in marine species is driven by
a simple (usually 9 neurons) central pattern generating circuit, the cardiac ganglion (CG),
positioned within the dorsal wall of the heart (Cooke, 1988; 2002). The CG is directly controlled
by a small number of modulatory fibers that originate in the central nervous system (Maynard,
1960; Field and Larimer, 1975; Yazawa and Kuwasawa, 1994; Delgado et al., 2000). The
heartbeat is also regulated by modulators that originate from the pericardial organs (POs),
neurohaemal structures that flank the heart and release bioactive products into the general
circulation (Alexandrowicz and Carlisle, 1953; Cooke and Sullivan, 1982). Recognition of the
crustacean CG as “an autonomously active, rhythmic, pattern-forming, neural system integrating
its own spontaneity with sensory and neurohumoral influences” (Cooke, 1988), led early
investigators to emphasize its utility as a very simple model for more complex nervous systems

Perhaps the most important aspect of global coordination is that between center and
periphery. Experimental studies suggest that motor systems are, as theoretical studies suggest
they must be, coordinately modulated both in the center and in the periphery to ensure efficient,
adaptive behavior (Calabrese, 1989; Meyrand and Marder, 1991; Chiel and Beer, 1997; Brezina
et al., 2000b). The present study was intended to establish the crustacean cardiac system, seen as
a simple model of a central pattern generator complete with its effector system, as a suitable
preparation in which to study the coordination of central and peripheral modulation. Anatomical
and physiological methods were used to show that a single central dopaminergic neuron is likely
to exert actions both on the periphery (neurohormonal modulation of contractions of the heart
muscle) and on the central pattern generator (direct innervation of the CG) of the cardiac system.
of the blue crab *Callinectes sapidus*. These inferences were examined with experiments in which dopamine was applied to intact hearts, isolated cardiac ganglia, and a novel semi-intact working heart preparation in which central and peripheral effects could be compared directly.

Materials and Methods

Specimens of *Callinectes 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 water that was obtained from collection sites. To reduce fat deposits within the heart, crabs were not fed. They were typically used within three weeks of capture.

Histology

Tyrosine hydroxylase (TH) immunohistochemistry. Specimens were covered in ice (30 min) to achieve immobilization. Tissues were dissected, secured to Sylgard-lined Petri dishes with minuten pins, and fixed for one hour in freshly prepared 4% paraformaldehyde. Standard wholemount immunohistochemical protocols were followed (see Miller et al., 1991 for detailed buffer composition, incubation and wash procedures). Ganglia were washed (5X, room temperature with agitation) in PTA (0.1 M phosphate buffer containing 2% Triton X-100 and 0.1% sodium azide). Following preincubation with normal goat serum (0.8%), tissues were immersed (48 hrs, room temperature) in a 1:200 dilution of the primary TH antibody (mouse monoclonal, Immunostar, Stillwater MN). Following repeated PTA washes (5X, at least 30 min each, room temperature), ganglia were incubated in secondary antibodies conjugated to a fluorescent marker (Alexa 488 goat anti-mouse IgG (H+L) conjugate; Molecular Probes, Eugene, OR: A-11029). The secondary antibody dilutions ranged from 1:1,000 to 1:3,000. The Alexa 488 was viewed with the G-2A filter block of the 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 Corel Draw 9 for addition of labels, cropping and organization of panels.

**Nerve backfills.** The biotin-avidin protocol followed the methods of Xin et al. (1999) with modifications based upon Díaz-Ríos et al. (1999). The tissue of interest was pinned out near a small vaseline well that was formed on the Sylgard surface. The nerve being examined was cut and drawn into the well. Care was taken to avoid contact between the end of the nerve and the vaseline. The tip of the nerve was cut one more time and then the crab saline inside the well was withdrawn and replaced with a saturated aqueous solution (1.6 mg / 30 µl) of biocytin (Sigma Chemical, St. Louis MO). The walls of the well were then built up with successive layers of vaseline, forming an ‘igloo’ that effectively isolated the biocytin pool from the saline surrounding the ganglion. The preparation was covered and incubated overnight at 14° C. The well was then removed, and ganglia were washed 3 - 5 times, repinned, and fixed in paraformaldehyde as described above. The fixed ganglia were transferred to micro-centrifuge tubes, washed 5 times (30 min each) with PTA solution and incubated overnight (room temperature, with shaking) in Rhodamine

**Neurobiotin injection.** Methods for intracellular staining were modified from the methods of Delgado et al. (2000). Microelectrode tips were filled with 4% Neurobiotin (Vector Laboratories) dissolved in 0.5 M KCl, 50 mM Tris (pH 7.6). The electrode shafts were filled with 2 M KCl, resulting in resistances ranging from 10 to 30 MΩ. Depolarizing current pulses (1 - 2 nA; 0.5 sec; 1 Hz; 10 - 60 minutes) were used to eject the Neurobiotin. This procedure did not appear to affect the resting potential or spontaneous electrical activity of the injected neuron. The preparations were usually left at room temperature for 2 - 3 hours in order to allow material
to diffuse from the injection site (cell body) to distant processes and dye-coupled cells. They were then repinned if necessary, and fixed in paraformaldehyde as described above. The fixed ganglia were transferred to microcentrifuge tubes, washed 5 times (30 min each) with PTA solution and incubated in Rhodamine600 Avidin D diluted (1:3,000 to 1:5,000) in PTA (24 - 48 hrs, room temperature). Tissues were then washed 5 times with PTA, examined and processed for THli as described above.

**Physiology**

*Working Heart* (Fig. 1A). Hearts were removed intact, the sternal artery was cannulated with a modified syringe needle and mounted in a 20 ml organ bath. The heart was suspended using a fine monofilament nylon thread attached to the force plates of a Grass FT .03 isometric force transducer and placed under a resting load (approx. 0.5 g). Perfusion with saline was maintained at a constant rate (2 ml / min) and pressure. The crab saline composition was based upon Pantin’s saline for *Cancer pagurus*: 487 mM NaCl, 13.6 mM KCl, 13.4 mM CaCl$_2$, 13.6 mM MgCl$_2$, 1.4 mM sodium sulfate, 3 mM HEPES (N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]), adjusted to pH 7.4 with sodium hydroxide. Perfusion rate and pressure were maintained when dopamine trials were performed.

*Semi-Intact Working Heart* (Fig. 1B). Hearts were dissected and pinned in Sylgard-lined petri dishes, 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 containing the motor neuron axons. The ring was cut and the severed end proximal to the ganglion was drawn into an extracellular suction electrode. The heart was then connected to the force plates of a Grass FT .03 isometric force transducer with a hook and nylon thread and placed under a resting load (approx. 0.5 g). The preparation was continually internally perfused with saline at a constant rate (2 ml/min) and pressure. Perfusion rate and pressure were maintained when dopamine trials were performed.

*Isolated Cardiac Ganglion* (Fig. 1C). Hearts were pinned ventral side up in Sylgard-lined petri dishes. A cut was made in the ventral musculature exposing the cardiac ganglion. Dissection was achieved principally by teasing away the adhering muscles. Previous
investigators (Tazaki and Cooke, 1979a) noted that the region within the confluence of the motor roots at each end of the ganglion contains the dendritic endings of the ganglionic neurons (see also Fig. 2B). A small non-contracting remnant was therefore retained at either end of the ganglion. Extracellular suction electrode recordings were obtained from at least one of the 4 cut ganglionic roots. Membrane potentials were recorded from anterior and/or posterior motor neurons using 2 M KCl filled or Neurobiotin tipped microelectrodes (10-30 MΩ). Preparations were continuously superfused with saline (2 ml/min).

In all physiological preparations, effects of dopamine were evaluated by comparing parameter values averaged over a one-minute period during the strongest response to the applied dopamine (typically about five minutes after the perfusion switch) to control values averaged over a one-minute period before the dopamine application. The preparation was washed (at least 20 min) between the application of different dopamine concentrations.

Results

Functional topography of the *Callinectes* cardiac system

We first sought to characterize features of the *Callinectes* cardiac system relevant to its suitability for examining the coordination of central and peripheral modulation. Injection of Neurobiotin into any of the large neurons within the cardiac ganglion revealed, through dye coupling, its full complement of five motor neurons. The cell bodies of three of the motor neurons were located at the anterior end of the ganglion (Fig. 2A, asterisks). These cells projected large caliber axons into the ganglionic trunk in the posterior direction. Two large posterior motor neurons (Fig. 2B, asterisks) projected axons in the anterior direction. The five axons came into close apposition within the anterior half of the trunk. The axons originating from the anterior cells bifurcated upon reaching the posterior end of the ganglion and projected branches into each of the posterior connectives (Fig. 2B). Similarly, the axons of the posterior cells bifurcated at the anterior end of the ganglion and projected branches into each of the anterior connectives. A single dendritic process emerged from the distal end of each motor neuron (Fig 2B). These processes branched within or close to the ganglion and terminated as
fine projections in the cardiac muscle adjacent to the ganglion within the confluence of the major connectives (see also Tazaki and Cooke, 1979a, 1983a,b). When large quantities of the tracer were injected, small neurons could be discerned in the most posterior region of the ganglionic trunk (Fig. 2B, *dashed box*). Although the dye coupling between the large cells and the small cells was relatively weak, four small cells were observed in the best instances.

Intracellular recording from the large motor neurons combined with extracellular recording from the major connectives was used to examine the electrical properties and functional organization of the cardiac ganglion (Fig. 2C,D). In the isolated CG, all motor neurons produced simultaneous spontaneous rhythmic burst activity (Fig. 2C). In other decapods, electrical coupling among CG motor neurons and common synaptic input is thought to underlie such coordinated bursting and hence simultaneous contraction of the entire myocardium (Hagiwara, 1961; Tazaki and Cooke, 1979a; Mirolli et al., 1987). Indeed, as in other decapods (Hartline, 1979; Friesen, 1975a,b; Tazaki and Cooke, 1979a, 1983a), each of the large motor neurons in *Callinectes* received synchronous EPSPs in the initial and late phases of each burst (Fig. 2D, *arrows*). Impulses corresponding to these EPSPs were not detected in the motor trunks, indicating that they originate from the small interneurons, the projections of which are confined to the CG (Tazaki and Cooke, 1979a, 1983a,b; Mirolli et al., 1987).

In the crab cardiac ganglia that have been investigated to date, the spatiotemporal properties of the electrical coupling and synaptic signaling result in precise synchrony of the motor neuron firing (Tazaki, 1972; Tazaki and Cooke, 1979a,b; 1983a,b; Berlind, 1982; Mirolli et al., 1987). Simultaneous recording from an anterior motor neuron, a posterior motor neuron, a branch of the anterior connective, and a branch of the posterior connective revealed that such synchrony of firing is also a property of the *Callinectes* CG (Fig. 2C,D). Intracellular recordings from any of the anterior motor neurons (Fig. 2C,D, *Ant. MN*) revealed, in each burst, five to ten non-overshooting impulses (10 – 15 mV) superimposed upon a slow potential (500 – 900 msec in duration). The motor neuron slow potential has been shown to reflect the combined actions of an endogenous regenerative driver potential and the chemically mediated EPSPs originating from the small interneurons (Tazaki and Cooke, 1979a; 1983a). In somatic recordings of posterior motor neurons (Fig. 2C,D, *Post. MN*), the amplitudes of the impulses (3 – 5 mV) and the EPSPs
(0.5 – 2 mV) were considerably smaller than in the anterior motor neurons (10 – 15 mV and 2 –
4 mV), probably reflecting their origin in the anterior half of the ganglion (see Tazaki and
Cooke, 1983a). Examination of a single burst with an expanded time axis revealed that the
impulses of the posterior neurons occurred in precise synchrony with those of the anterior motor
neurons (Fig. 2D, two upper records). Moreover, recordings from each of the major connectives
(Fig. 2D, two lower records) exhibited identical impulse trains that corresponded to the
synchronous firing of the two sets of motor neurons.

The semi-intact working heart preparation

The observed synchrony of motor neuron firing suggested that recording from any of the four
connectives would reflect the motor output of the ganglion and hence the patterned input to the
entire cardiac musculature. This reasoning prompted us to develop a preparation, termed the
semi-intact working heart (S-IWH; see Materials and Methods), in which we could record the
motor output of the ganglion in a minimally dissected contracting heart (Fig. 1B). As this
preparation required some removal of cardiac muscle and severing one connective, we
performed an initial comparison of the parameters of heartbeat activity in the S-IWH with those
in the fully intact working heart (WH) preparation (Fig. 1A). Contraction amplitudes were
substantially reduced in the S-IWH (compare Fig. 1A2 and 1B2), due presumably to the reduced
mass of heart muscle and the reduced motor drive resulting from cutting the connective.
However, the heartbeat frequency of the S-IWH (15.8 ± 1.8 beats per minute, mean ± SE, n = 12)
did not differ significantly from that of the intact WH (16.5 ± 1.2 beats per minute, n = 20; two-
tailed Student’s t-test, \( p = 0.74 \); Fig. 3A1). Moreover, the time to peak contraction (WH: 336.6 ±
29.7 msec, S-IWH: 364.9 ± 30.5 msec; \( p = 0.53 \)) and the contraction duration (WH: 594.0 ±
104.7 msec, SIWH: 612.0 ± 112.6 msec; \( p = 0.91 \)) did not differ between the two preparations
(Fig. 3A2,3).

We further compared the motor patterns recorded in the S-IWH with those recorded in the
isolated cardiac ganglion (ICG) preparation (Fig. 1C). The burst frequency of the ICG (24.0 ±
3.2 bursts per minute, n = 9) was significantly greater than that of the S-IWH (16.5 ± 1.2 bursts
per minute, n =12; \( p < 0.05 \); Fig. 3B1), as was the number of impulses per burst (CG: 15.4 ± 2.6,
Together, these observations demonstrated that the semi-intact working heart preparation retained the essential properties of the intact heart. However, there were significant differences between the motor patterns produced by the S-IWH and the isolated CG, underscoring the importance of examining cardiac modulation in the context of the entire, integrated CPG-effector system.

**Dopaminergic regulation of the cardiac system: Anatomical substrates**

In a series of immunohistochemical experiments, we used a monoclonal antibody generated against tyrosine hydroxylase (TH) to identify possible sources and modes of dopaminergic modulation of the *Callinectes* cardiac system. In a previous mapping of the catecholaminergic system of *Callinectes* by Wood and Derby (1996), the staining pattern of TH-like immunoreactivity (THli) was found to be virtually identical to that observed with an antibody against dopamine (see also Cournil et al., 1994). In view of this, and the reported inability to detect norepinephrine and epinephrine in crustacean nervous tissue using chromatographic techniques (Sullivan et al., 1977; Barker et al., 1979), THli is commonly equated with the localization of dopamine in these species.

We observed THli in a limited number of neurons in the brain and ventral nerve cord (Figs. 4 and 5). In agreement with the previous description (Wood and Derby, 1996) the largest central THli neuron was observed in the commissural ganglion (Fig. 4A, *arrow*). This cell corresponds to a dopaminergic neuron, termed the ‘L-cell’ (Selverston et al., 1976), that has been described in a number of crustacean species, including the crab *Carcinus maenas* (Cooke and Goldstone, 1970), the lobsters *Panulirus interruptus* (Kushner and Maynard, 1977), *Homarus gammarus* (Cournil et al., 1984, 1994), *Homarus americanus* (Siwicki et al., 1987; Pulver et al., 2003) and the crayfish *Oronectes rusticus* (Tierney et al., 2003). The L-cell axon gave rise to multiple neurites within the commissural ganglion prior to its entry into the circumesophageal connective (C conn.). Four additional smaller THli neurons were observed in the commissural ganglion (Fig. 4A, *arrowheads*). A slender fiber projected into the superior esophageal nerve (*son*; Fig.
This fiber did not appear to be a collateral of the L-cell axon, but it was also not possible to associate it definitively with any of the small neurons. Branches of the small neurons appeared to contribute to the central neuropil network, but their individual projections could not be distinguished from each other or from that of the L-cell.

Upon entering the C conn., the large axon originating from each L-cell projected anteriorly toward the brain (Fig. 4B). However, slightly posterior to the tritocerebrum, the L-cell axon reversed direction and coursed posteriorly again in the medial portion of the C conn., bypassing the commissural ganglion and continuing without branching toward the subesophageal ganglion (Fig. 4B, arrowheads; see also Wood and Derby, 1996). Near the point at which the L-cell axon reversed its direction at the junction of the C conn. and the brain, it gave rise to a small branch that contributed to the THli innervation of the tritocerebrum (Fig. 4C,D, see also Tierney et al., 2003). Upon reaching the subesophageal ganglion, the L-cell axon coursed slightly past the origin of the first segmental nerve (SN1; nomenclature of Maynard, 1961a,b) and then turned back to enter the nerve at a characteristic acute angle (Fig. 5A,B).

As projections from the CNS to the heart and pericardial organs are typically associated with the subesophageal segmental nerves (see Maynard, 1961b; Cooke and Sullivan, 1982), the projection of the L-cell axon into SN1 provided a possible anatomical substrate for dopaminergic regulation of the heart. We performed double-labeling experiments to confirm that the L-cell axon indeed projected into SN1. Biocytin backfills of the nerve revealed a bundle of fibers that curved in the anterior direction upon entering the subesophageal ganglion. The position and trajectory of this fiber bundle suggested that it originated from the C-cell cluster in the anterior thoracic ganglia (Maynard, 1961b; Wood et al., 1996). Toward the posterior edge of the nerve, a single large diameter fiber (Fig. 5B1, arrow) could be followed into the ganglion, where it turned sharply to enter the C Conn. Only this fiber was observed to label for THli (Fig. 5B2, arrow), indicating that the sole dopaminergic fiber in SN1 originates from the L-cell. Occasionally, a smaller THli fiber also entered SN1, but it always terminated close to the ganglion and was never backfilled.
The large L-cell axon in SN1 could be followed to the pericardial organ in the lateral region of the thorax. Upon reaching the PO, it ramified repeatedly, projecting branches into each of the major bars and longitudinal trunks of the PO (Fig. 6; terminology of Alexandrowicz, 1953). Within the central core of each bar and trunk, multiple smooth THli fibers ran in parallel fashion (Fig. 6C,D). These fibers gave rise to finer, more irregular branches that reached the superficial cortex or secretory layer of the PO (Fig. 6E; see Maynard and Maynard, 1962). No immunoreactive cell bodies were observed in the PO. These observations are consistent with previous descriptions of the anatomical features of catecholaminergic projections to the pericardial organs of six other brachyuran species (Cooke and Goldstone, 1980) and the embryonic lobster *Homarus americanus* (Pulver and Marder, 2002).

Unexpectedly, however, a single branch of the L-cell axon was observed to depart from the anterior bar region of the PO in a side twig that gave rise to the dorsal nerve projecting to the heart (Fig. 6B, arrow). Within the heart, the THli fiber extended, without branching, to the cardiac ganglion. No THli innervation of the myocardium was observed. Upon reaching the CG, each L-cell fiber produced collaterals that formed a distributed varicose innervation surrounding the three large anterior motor neurons (Fig. 7A). The fibers then coursed to the posterior region of the CG where they branched locally and terminated abruptly in the vicinity of the small interneurons (Fig. 7B, arrowhead). No THli innervation was observed around the cell bodies of the two posterior motor neurons. Moreover, no THli fibers were observed to leave the CG in any of its motor roots or dendritic processes.

Together, the distribution of THli material in *Callinectes* suggested that a single central dopaminergic neuron, the L-cell, might regulate cardiac activity in complementary ways. Dopamine released from its terminals in the pericardial organs is likely to act in a neurohormonal fashion on the entire cardiac system, while dopamine released from its terminals in the cardiac ganglion might act as a local modulator within specific regions of the ganglion.

### Cardioactive actions of dopamine

We tested the effects of exogenous dopamine on each of the three preparations: the fully intact working heart, the semi-intact working heart, and the isolated cardiac ganglion. In the
intact WH, DA produced increases in contraction frequency and amplitude (Fig. 8). For both parameters, the DA dose-response relation revealed threshold responses in the nanomolar range, with gradual increases in the magnitude of the response up to micromolar range (Fig. 8A,C,D). Despite increases in contraction amplitude of 80 – 100% above control values in the presence of high DA concentrations, no obvious changes in the shape of the contractions (i.e., in their temporal characteristics such as rise time and decay time) were noted in the presence of DA (Fig. 8B, inset). Phase plots in which the rate of change of the force was plotted against the force of contraction (Fig. 8B) had similar shapes in the presence of DA as under control conditions, indicating that the rates of rise and decay both changed in direct proportion to the increase in the force of contraction.

Of the three preparations, we focused particularly on the S-IWH, as it allowed simultaneous recording of both the contractions and the underlying motor activity of the CG. The effects of DA on the contractions could then be compared to those in the WH, while the effects on the motor activity could be compared to those in the ICG. Furthermore, by showing that the contractions were always coupled one-for-one with the bursts of the underlying motor pattern (Figs. 1B2, 9A), the S-IWH provided one parameter, namely the frequency of the contractions or bursts, that could be compared across all three preparations.

As already described, with the exception of absolute contraction amplitudes, the basal properties of the contractions in the S-IWH were similar to those in the WH (Fig. 3A). The effects of DA on the contractions were also similar: both contraction frequency and amplitude were increased (Fig. 9A-C). Apparent thresholds were in the nanomolar range, and by fitting the DA dose-response values with standard sigmoidal functions (smooth curves in Fig. 9B-E; see figure legend), we obtained estimates of the half-maximally effective DA concentration, EC50, of $2.4 \times 10^{-7}$ M for frequency and $1.7 \times 10^{-7}$ M for amplitude (vertical dashed lines in Fig. 9B and 9C, respectively). Apart from frequency, DA had much less potent effects on the parameters of the neural motor patterns. Burst duration was increased, but only by 20 – 40%, and only with a much higher apparent threshold ($\sim 3 \times 10^{-7}$ M) and EC50 of $6.8 \times 10^{-7}$ M (Fig. 9D). The number of impulses per burst was not significantly changed by DA at any of the concentrations tested (Fig. 9E).
In the ICG, application of DA increased burst frequency, burst duration, as well as the number of impulses per burst. It had a relatively small effect on the burst frequency (maximal increase approximately 20%), but with a low EC$_{50}$, $1.4 \times 10^{-8}$ M (Fig 10A,B). On the other hand, the effects of DA on the burst duration and the number of impulses were much larger (increases of 200% and 60% respectively), but had much higher EC$_{50}$ values, $1.2 \times 10^{-6}$ M and $2.0 \times 10^{-6}$ M respectively (Fig. 10C,D).

In Fig. 11 we have superimposed all of the DA dose-response curves from Figs. 8-10 for comparison. Detailed consideration of Fig. 11 is deferred until the Discussion, but clearly the data could not be explained by a single unified effect of DA. Rather, there appeared to be several effects, with different magnitudes and different values of EC$_{50}$. Furthermore, remarkably, the same parameters were modulated by DA differently in the S-IWH and ICG preparations (e.g., in Fig. 11C,D; see statistical analysis in Fig. 11 legend). How this might happen is considered in the Discussion.

**Discussion**

**Functional topography of the *Callinectes* cardiac system**

An important aim of the present work was to establish the *Callinectes* cardiac system as a suitable experimental preparation for the study of extrinsic modulation and its functional consequences. For this, it was necessary at the outset to confirm the basic functional topography of the *Callinectes* cardiac system, even though this could be expected to be in many ways similar to that studied in other crab species. Indeed, the general morphological and physiological features of the *Callinectes* CG that we have described here (see also Hawkins and House, 1978) are in agreement with descriptions in other crabs, including *Eriocher japonicus* (Tazaki, 1972), *Podophthalmus vigil* (Berlind, 1982), and *Carcinus maenas* (Saver et al, 1999). In particular, our characterization enables us to conclude that the number (five), position (three anterior and two posterior), and physiological properties of the motor neurons in the *Callinectes* system
correspond to the previous detailed description of crab cardiac functional anatomy in *Portunus sanguinolentus* (Tazaki and Cooke, 1979a,b;1983a).

Of particular importance for our subsequent investigation, the five motor neurons were found to fire in precise synchrony. In other crab species, the synchrony is thought to reflect highly effective electrical coupling close to the region of synaptic integration and impulse initiation of all five motor neurons. This critical integrative area is located within the anterior portion of the CG (Tazaki and Cooke, 1979a, 1983a; Mirolli et al., 1987). The synchrony appears to be unique to crab cardiac systems. In lobsters, the motor neurons of the CG also fire in coordinated bursts, but, because the individual neurons have unique, often multiple sites of impulse initiation, not in a precisely synchronized fashion (Hartline, 1967; Friesen 1975a). The synchrony of motor neuron firing in the crab CG is experimentally advantageous because it permits monitoring of the motor output from any of the connectives projecting from the CG to the heart musculature. Recordings from muscle fibers demonstrate that excitatory junctional potentials corresponding to this motor pattern occur throughout the myocardium (Benson, 1981; our observations). No evidence for synaptic drive to the muscle from any other source is observed.

**Catecholaminergic innervation of the cardiac system**

In this work we have used THli as a marker of catecholaminergic localization. For the reasons already presented in Results, the catecholamine that the THli reflects in *Callinectes*, and probably other crustaceans, is very likely to be dopamine.

The distribution of THli in *Callinectes* (see also Wood and Derby, 1996) indicates that the catecholaminergic innervation of the cardiac system originates from a single CNS neuron, the L-cell. The convoluted course of the L-cell axon that we have observed in the CNS is very characteristic. Maynard (1961) reported that the largest fiber in the crab segmental nerve 1, which he designated the ‘a’ fiber, followed a unique course upon entering the subesophageal ganglion, turning ‘sharply’ toward the circumesophageal connective. Using histofluorescent methods in *Carcinus maenas*, Cooke and Goldstone (1970) then determined that the ‘a’ fiber originated from a large catecholaminergic cell in the comissural ganglion. They were able to trace the large axon of this cell to the brain, back to the subesophageal ganglion, and into a
segmental nerve projecting to the pericardial organ. In *Callinectes* itself, Wood and Derby previously identified the L-cell and followed its axon to the brain and back past the commissural ganglion. These investigators likewise postulated that the *Callinectes* L-cell projected to the PO. Here we have confirmed this projection. In addition, we have made the novel finding, critical for understanding the regulation of the cardiac system, that a branch of the L-cell axon then continues beyond the PO, to the heart. Within the heart, the innervation of the cardiac ganglion by this fiber is consistent with previous descriptions of cardioaccelerator fibers in several crustacean species (Maynard, 1960; Field and Larimer, 1975; Yazawa and Kuwasawa, 1994; Sakurai and Yamagishi, 1998).

In studies of the stomatogastric system of lobsters, the L-cell was found to receive depolarizing input corresponding to the esophageal motor rhythm of the foregut (Selverston et al., 1976). Subsequently, the firing pattern of the L-cell of *Homarus* was shown to be influenced by four distinct foregut rhythms (Robertson and Moulins, 1981). Given the previously demonstrated excitatory effects of dopamine on the pyloric central pattern generator in the stomatogastric ganglion (Anderson and Barker, 1981), the L-cell was postulated to regulate the activity of the gut through a positive feedback loop via release of dopamine from its terminals in the PO (Robertson and Moulins, 1981). The projections of the L-cell that we have documented here suggest that the L-cell may play an even broader integrative role that includes cardiac responses to increased metabolic or behavioral demands (see Guirguis and Wilkens, 1995). The demonstration of long lasting (16 – 18 hr) increases in heart rate associated with food detection and consumption in *Callinectes* (McGaw and Reiber, 2000) is consistent with such a broader role.

Neurons that correspond to the L-cell in a range of decapods all appear to exhibit a catecholaminergic phenotype: *Carcinus maenas* (Cooke and Goldstone, 1970); *Panulirus interruptus*: (Barker et al., 1979; Kushner and Barker, 1983), *Homarus gammarus* (Cournil et al., 1984, 1994); *Homarus americanus* (Siwicki et al., 1987), *Cancer irroratus* and *borealis* (Marder, 1987), *Callinectes sapidus* (Wood and Derby, 1996, and this study), and *Macrobrachium rosenbergii* (Sosa et al., 2002). However, there appears to be substantial variability in the cotransmitter content of L-cells. In *Homarus gammarus* (Cournil et al., 1984) and
Macrobrachium rosenbergii (Sosa et al., 2002), the L-cell contains serotonin immunoreactivity. Proctolin-like immunoreactivity is present in the L-cell of Homarus americanus (Siwicki et al., 1987), Cancer irroratus and C. borealis (Marder et al., 1986), but not in Procambarus clarkii (Siwicki and Bishop, 1986), Panulirus interruptus (Siwicki and Bishop, 1986), or Callinectes, the subject of the present study (Wood et al., 1996; Wood and Derby, 1996). In view of such cotransmitter diversity, it will be interesting to examine the generality of the L-cell projection to the CG that we have identified. It is possible that the cotransmitter diversity reflects somewhat different modes of use of the L-cell in different species. Some species may use the L-cell mostly for hormonal release through the PO, others for direct modulation of the CG, and still others in the dual mode of modulation that, we propose, occurs in Callinectes.

The limited distribution of THli within the CG appears to arise exclusively from the single pair of L-cell fibers that enter the heart via the dorsal nerves (Fig. 7; see also Yazawa and Kuwasawa, 1994). The presence of dopamine within these fibers may account for earlier biochemical measurements of catecholamines in the lobster cardiac ganglion (Ocorr and Berlind, 1983). The localization of THli does not support a role for dopamine as the neurotransmitter of the motor neurons, a function for which L-glutamate is currently a leading candidate (Cooke, 1966; Benson, 1981; Yazawa et al., 1998; Delgado et al., 2000). The absence of THli innervation in the region of the cell bodies of the posterior motor neurons (Fig. 7B) suggests that the innervation of the five motor neurons may not be uniform. Direct modulation of the posterior motor neurons cannot be excluded, however, as their synaptic input and impulse initiation occur in the anterior portion of the ganglion, in a region that receives substantial THli innervation (Fig. 7A; see Tazaki and Cooke, 1983a; Mirolli et al., 1987; Cooke, 2002). It is also notable that the THli projections are confined to the ganglion itself. In this regard, they differ from the “System II” cardioregulatory fibers described by Alexandrowicz (1932) and the catecholaminergic innervation of the hermit crab, where the regulatory axons innervate myocardial cells (Yazawa and Kuwasawa, 1994). Finally, the localization of THli terminals to the neuropil and somatic regions of the ganglion indicates that the distal dendritic processes projecting into adjacent muscle fibers (Fig. 2B) are also not targets of this regulation. This contrasts to GABAergic inhibitory regulation for which extensive dendritic innervation has been observed (“System I” fiber of Alexandrowicz, 1932; Delgado et al., 2000).
In sum, we propose that the pattern of L-cell innervation that we have found indicates a dual mode of action of the L-cell neurotransmitter, dopamine, on the cardiac system of Callinectes. Dopamine released from the L-cell terminals in the pericardial organs is likely to act in a neurohormonal fashion on the entire cardiac system, including its periphery, the cardiac musculature. At the same time, dopamine released from the terminals in the cardiac ganglion acts as a local modulator of the central motor pattern generated by the ganglion. We propose that this dual innervation provides an anatomical substrate for the physiological actions of dopamine that we have found, as discussed next.

**Cardioactive actions of dopamine**

The cardioactive effects observed here are in many ways similar to those reported in other arthropods where dopamine typically produces increases in contraction frequency and amplitude (Berlind et al., 1970; Florey and Rathmayer, 1978; Cooke and Sullivan, 1982; Augustine et al., 1982). As dopamine also produces increases in burst frequency, burst duration, and the number of impulses per burst in isolated cardiac ganglia (Miller et al., 1984; Berlind, 1998), it is easy to assume that all of the effects observed in the cardiac system are a simple consequence of its central actions. However, when the actions of dopamine are quantitatively compared on the CG with and without the peripheral cardiac musculature, as we have done here, it becomes clear that matters are likely to be considerably more complex.

The similarity between the basal contraction parameters of the S-IWH and the fully intact WH (Fig. 3A1-3), coupled with the comparable dose-dependence of the effect of dopamine on contraction amplitude (Fig. 11A), support the conclusion that (except for the absolute magnitude of the contraction) the properties of the S-IWH faithfully reflect those of the intact system. In the S-IWH, and so presumably in the WH, there is essentially no effect of dopamine on the burst parameters of the underlying motor pattern, such as burst duration (Fig. 11C) or the number of impulses per burst (Fig. 11D). Yet there is a large increase in contraction amplitude (Fig. 11A). This increase could be a secondary consequence of the dopamine-induced increase in burst and contraction frequency (Fig. 11B; see Mahadevan et al., 2004). Alternatively or in addition, however, it could also be produced by a direct action of dopamine on the peripheral cardiac musculature.
Peripheral effects of modulators, including dopamine, acting both presynaptically at neuromuscular junctions and postsynaptically on the myocytes themselves, are common in other muscles of decapods (e.g., Florey and Rathmayer, 1978; Kravitz et al., 1980; Lingle, 1981; Breen and Atwood, 1983; Fischer and Florey, 1983; Jorge-Rivera et al., 1998; Djokaj et al., 2001), other arthropods, and invertebrates generally (Evans and Myers, 1986; Calabrese, 1989; Worden, 1998). A peripheral dopaminergic action would presumably be due to dopamine released neurohormonally from the pericardial organs (dashed arrows in the summary diagram presented in Fig. 12A and in more detail in Fig. 12B), since the cardiac musculature is not directly innervated by the dopaminergic L-cell.

All of the other effects of dopamine are likely to be initiated centrally, through the direct innervation by the L-cell of the CG. The data are consistent with a single central effect of dopamine on the burst frequency of the motor pattern and consequently, since contractions follow the bursts one-for-one (Fig. 1B2), on contraction frequency (Fig. 11B). The effects of dopamine on burst or contraction frequency are statistically indistinguishable in the WH and S-IWH (see Fig. 11 legend). In the ICG, the effect is significantly smaller, but in a characteristic way. Because the absolute burst frequency is higher in the ICG under control conditions [Fig. 3B1, and ‘Control’ inset of Fig. 11B], the smaller increase brings the frequency at high dopamine concentrations to almost exactly the same absolute value in the ICG as in the S-IWH [‘10^-6 M DA’ inset of Fig. 11B] and WH. This maximal frequency, in the range of 20-25 bursts or beats per minute, may thus constitute a ceiling above which the cardiac ganglion cannot accelerate. A single central effect of dopamine on frequency appears, therefore, to be the most parsimonious explanation. In previous studies using ligatures and vaseline wells, dopamine was found to affect ganglionic burst frequency most strongly when applied to the posterior region of the CG (Miller et al., 1984; see also Berlind, 1998, 2001a). We propose therefore that the effect on frequency is achieved primarily by dopamine release from the projections of the L-cell that terminate in the area of the small interneurons (thin solid arrows in Fig. 12A,B), which act as pacemakers of the crab cardiac system (Tazaki and Cooke, 1979a, 1983a,b).

There is yet a third effect of dopamine, which is seen in the ICG and is thus presumably a direct central effect: the large increase in burst duration and the number of impulses per burst (Fig. 11C,D). This effect is clearly different from that on frequency, since it occurs at much
higher dopamine concentrations. Interestingly, dopaminergic actions on the large neurons of the crabs *Portunus* and *Podophthalmus*, manifested in a somewhat comparable manner as a lowered threshold for eliciting repetitive driver potentials, only occurred with high dopamine concentrations (10 to 100 times higher than the actions on the small interneurons; Miller et al., 1984). We propose therefore that the third effect of dopamine may be achieved by release of dopamine from the L-cell terminals in the anterior motor neuron region of the CG (*thick solid arrows* in Fig. 12A,B). The ‘high’ DA concentrations required to produce these effects are still only of the order of $10^{-6}$ M, and thus quite possibly reached with endogenous release of dopamine. However, the physiological significance of these observations remains to be established.

The most intriguing feature of this third effect of dopamine is that it is seen in the isolated CG but not when the CG remains embedded within the cardiac musculature. Furthermore, as with frequency, even the basal values of these parameters, the burst duration and the number of impulses per burst, are different in the ICG and S-IWH (Fig. 3B2,3). We conclude that, in the intact system, feedback from the cardiac musculature regulates the parameters of the motor pattern produced by the CG as well as the modulation of these parameters. Several feedback mechanisms that could help explain our data are already known to exist in crustacean cardiac systems. First, the CG burst duration is typically found to be reciprocally related to the burst frequency. This relation is attributable, at least in part, to the properties of the motor neuron driver potentials, which are smaller at higher frequencies (Tazaki and Cooke, 1990). As dopamine increases burst frequency by acting on the small pacemaker interneurons, this relation, opposing the simultaneous increases in burst duration and the number of impulses per burst produced by the action of dopamine directly on the motor neurons, could serve to maintain these parameters approximately constant (Fig. 12B), as we have observed is the case in the intact system. However, at least in its simplest form, this mechanism would provide purely central feedback, operating within the CG itself. More interestingly, Sakurai and Wilkens (2003) have recently reported that increasing tension of the cardiac musculature—i.e., contraction amplitude (Fig. 12B)—exerts negative feedback effects on parameters of the motor pattern produced by the CG. Mechanistically, this kind of negative feedback from the periphery to the center could be implemented by direct mechanosensitive hyperpolarization of the motor neuron dendrites that
extend into the muscle surrounding the CG (Fig. 2B; Sakurai and Wilkens, 2003; see Cooke, 2002) or by a retrograde diffusible messenger (e.g., NO: see Mahadevan et al., 2004).

Central-peripheral integration through extrinsic modulation

The relation between the CG motor pattern and contractions of the myocardium is not well understood and is likely to be complex. Development of tension in crustacean muscle fibers is thought to be directly related to the degree to which the muscle membrane is depolarized (Orkand, 1962). For example, Anderson and Cooke (1971) showed that each burst of impulses from a lobster CG produced a complex synaptic depolarization that preceded and accompanied the early phase of cardiac contraction (see also Benson, 1981). Contraction was graded and could be elicited with depolarizations as small as 5 mV. Regenerative membrane responses were not detected in the lobster myocardium, but they have been observed in other crustacean hearts (compiled in Anderson and Cooke, 1971). Precisely to bridge such complexities of contraction-excitation coupling, a general analytical approach, termed the neuromuscular transform, was recently developed (Brezina et al., 2000a,b; 2003a,b). This approach simply considers the input-output relation between the motor neuron firing pattern and the muscle contraction. In the S-IWH preparation that we have developed here, we can record and, preliminary work (Fort et al., 2004) shows, also experimentally manipulate, simultaneously both the motor neuron firing pattern and the contractions. This ability promises to make the Callinectes cardiac system highly suitable to the neuromuscular-transform approach. Indeed, the work presented here has already produced a body of data that can begin to be analyzed in this way.

Work with the neuromuscular transform in Aplysia (Brezina et al., 2000b), as well as more general computational and neuroethological considerations (Chiel and Beer, 1997), suggest that central motor commands and the properties of the peripheral musculature must be matched and coordinated if functional behavior is to be produced. This coordination must be maintained throughout all plastic changes in the behavior. Such coordination can ideally be implemented by extrinsic modulation, originating from a single source but ramifying to both the center and the periphery. In this work, we have documented the likely existence of such a mechanism in the Callinectes cardiac system. The anatomy of the L-cell suggests that it releases dopamine both as a neurohormone onto the entire heart and as a local neurotransmitter within the CG, and we have
found multiple physiological effects of dopamine that probably correspond. Furthermore, by comparing the three preparations in which the cardiac musculature was removed from the CG to different degrees, we have found evidence for feedback coupling from the periphery to the center. Such feedback—in more complex systems, implemented by dedicated sensory neurons—is common in neuromuscular systems, and its presence immediately raises important dynamical issues, for example of stability, that the mechanisms of central-peripheral coordination and modulation must deal with. These issues were not addressed in the original work with the neuromuscular transform in *Aplysia*, largely because of a lack of suitable experimental preparation. In its exceptional simplicity and experimental tractability, yet already with multiple sites of modulation and feedback, we believe that the *Callinectes* cardiac system will prove to be a suitable preparation for the study of these questions.
Figure Legends

Fig. 1. Comparison of three physiological preparations used in this study. All recordings shown here were obtained from a single specimen, enabling direct comparison. (A1) In the working heart (WH) preparation, the intact heart was suspended in a perfused organ bath. (A2) Repetitive contractions were recorded with an isometric force transducer. A single heartbeat is shown with an expanded time base to illustrate how the time to peak contraction and contraction duration were determined. The contraction duration was measured at the amplitude reached half-way through the time to peak contraction. (B1) In the semi-intact working heart (S-IWH) preparation, the heart was pinned out in a Sylgard dish and perfused. The CG, which is not exposed in this preparation, is illustrated in this schematic (dashed outlines; not to scale) to depict its approximate position and organization within the heart. A small incision was made in the posterior region of the ventral surface of the heart, one of the posterior roots was cut (dashed oval), and its proximal end was drawn into a suction electrode. (B2) In the S-IWH preparation, contractions were recorded with an isometric force transducer (upper trace) while simultaneously recording motor neuron impulse patterns from the suction electrode (lower trace). The expanded record of a single heartbeat shows that the onset of the contraction occurred 20–30 msec after the initial motor neuron impulse. Although the amplitude of the contraction was somewhat reduced in comparison with the WH (panel A), other contraction parameters, such as the frequency, time to peak, and duration did not differ appreciably between the two preparations. (C1) In the isolated cardiac ganglion (ICG) preparation, the CG was detached from the cardiac muscle, pinned to a Sylgard surface, and superfused. (C2) The somatic membrane potential of one or more motor neurons (upper record) was recorded with intracellular microelectrodes, and extracellular recordings of motor neuron impulse patterns (lower record) were made as in B. The expanded records show non-overshooting somatic motor neuron impulses superimposed on a slow depolarizing potential (upper record). The extracellular impulse pattern (lower record) corresponded precisely to that of the motor neuron. The motor pattern parameters of the isolated ganglion differed substantially from those of the S-IWH. The burst frequency, duration, and the number of impulses per burst were all increased (compare panels C2 and B2). Time calibrations shown in C apply to all panels.
Fig. 2. Functional topography of the *Callinectes* cardiac ganglion.  (*A*) Neurobiotin fill of the three large anterior motor neurons (*asterisks*). Each motor neuron gives rise to a large caliber axon that projects in the posterior direction into the ganglionic trunk (*arrows*). (*B*) The cell bodies of two large motor neurons located at the posterior end of the ganglion (*asterisks*). Same preparation as in *A*; the trunk connecting the two ends (approximately 5 mm in length) is not shown. The axons originating from the three anterior motor neurons bifurcate (one bifurcation is indicated by *arrow*) near the posterior end of the ganglion and project a branch into each of the posterior roots of the lateral connectives. The axons of the two posterior motor neurons project in the anterior direction. Finer dendritic processes extend from their posterior pole. These processes branch near the cell body (one dendritic branch point is indicated by *arrowhead*) and project into the muscle fibers adjacent to the ganglion within the confluence of the two posterolateral connectives. In the most posterior region of the ganglion (*dashed box*) the cell bodies of the small interneurons exhibited lower levels of dye coupling. *Calibration bar* in both *A* and *B* = 100 µm.  (*C*) Intracellular recording from an anterior motor neuron (*Ant. MN*) and a posterior motor neuron (*Post. MN*) together with simultaneous extracellular recording of the motor neuron impulse pattern from an anterior connective (*Ant. con.*) and a posterior connective (*Post. con./). Rhythmic (approximately 0.5 Hz) synchronous bursting is observed in the two motor neurons. In both cases, non-overshooting impulses are superimposed on a slow depolarization. The impulses and the slow depolarization are both larger in the anterior neuron than in the posterior neuron.  (*D*) A single burst (boxed in panel *C*) shown on an expanded time base. The inflections on the rising phase of the depolarization in the two motor neurons and the extended period of synaptic input that follows the motor neuron firing (*arrows*) reflect EPSPs originating from the small interneurons (see Tazaki and Cooke, 1979a). These EPSPs also are substantially larger in the anterior motor neuron. Only the motor neuron impulses are recorded by suction electrodes on the connectives (*two lower recordings*). Identical impulse trains, precisely synchronized with the intracellularly recorded impulses, are recorded from both connectives.

Fig. 3. Properties of the semi-intact working heart preparation.  (*A*) Comparison of the S-IWH and WH. Parameters of the heartbeat measured in the S-IWH, including (*A1*) the contraction frequency, (*A2*) the time to peak contraction, and (*A3*) the contraction duration (measured as in
Fig. 1A2), did not differ significantly from those measured in the WH. (B) Comparison of the S-IWH and ICG. Parameters of the motor pattern, including (B1) the burst frequency, (B2) the number of impulses per burst, and (B3) the burst duration, were all significantly greater in the ICG ($p < 0.05$; see main text). Means ± SE are plotted throughout, with the number of preparations $n$ indicated.

**Fig. 4.** Tyrosine hydroxylase-like immunoreactivity (THli) in the L-cells and their projections. (A) THli in the commissural ganglion (CoG). A diffuse system of fine THli fibers coursed throughout the central neuropil region of the CoG. THli was located in five neurons, all in the anterior portion of the ganglion. The cell body of the L-cell (arrow) was substantially larger than the others (arrowheads) and typically had an irregular shape (see also Cooke and Goldstone, 1970). Its stout axon coursed through the central neuropil region of the ganglion and then turned abruptly to enter the circumesophageal connective (C conn.). A second fine fiber present in the superior esophageal nerve (son) could not be associated definitively with any of the neurons in the CoG. (B) Each L-cell soma (arrows) in the paired commissural ganglia gave rise to a single large caliber axon that exited the ganglion and ascended toward the brain along the lateral edge of the C conn. Following its reversal of direction (panels C and D) the L-cell axon (arrowheads) coursed past the CoG along the medial edge of the C conn. (C) Within the brain, groups of THli somata were present within the anterior medial ventral protocerebrum (filled arrowhead; cluster 6 according to the nomenclature of Sandeman et al., 1992) and laterally in cluster 12 of the deutocerebrum (unfilled arrowhead). Prominent bundles of THli fibers, probably originating from neurons in the eyestalk ganglia (Wood and Derby, 1996), projected from the optic nerve (opt. n.) to ventral regions of the anterior medial protocerebrum, where their staining became impossible to follow. A more diffuse, but widespread THli innervation of the deutocerebrum and tritocerebrum originated, in part, from a limited number (4 to 6) fibers that ascended in each C conn. The largest such fiber, originating from the L-cell (panels A and B), approached the brain in the most lateral edge of each connective, but reversed its direction prior to reaching the posterior tritocerebrum (arrows). (D) Higher magnification of the junctional area between the C conn. and tritocerebrum from the same preparation as in panel C. Near the point at which the L-cell fiber reversed direction (arrow), it gave rise to a collateral
which in turn branched (arrowhead) to innervate medial and lateral regions of the tritocerebrum. 

Calibration bars = 100 µm in A and D, 200 µm in B and C.

**Fig. 5.** Projection of the L-cell axon into SN1. (A) Whole mount preparation of the ventral nerve cord. Several fine THli fibers descending in the C conn. contributed to longitudinal intersegmental tracts (arrowheads) projecting to the most posterior portions of the ganglionic mass. Within the abdominal ganglia, two large THli cell bodies were observed near the midline of the cord. These cells are likely to correspond to the anterior unpaired medial (aum) cells that were shown to provide a catecholaminergic innervation of the hindgut in crayfish (Mercier et al., 1991). Upon reaching the subesophageal ganglion, the L-cell axon descended in the lateral intersegmental (LIS) tract (Maynard, 1961), coursed slightly past the origin of the first segmental nerve (SN1) and then turned back and laterally to enter the nerve (arrows). sa, sternal artery. 

Calibration bar = 500 µm. (B) Double-labeling experiments to confirm that the L-cell axon projects into SN1. (B1) Biocytin backfill of SN1 revealed a bundle of fibers (arrowhead) that curved in the anterior direction upon entering the subesophageal ganglion, likely corresponding to projections of the C-cell cluster in the anterior thoracic ganglia (Matsumoto, 1954; Maynard, 1961). Toward the posterior edge of the nerve, a single large diameter fiber (arrow) could be followed into the ganglion, where it turned sharply to enter the C conn. (B2) TH-like immunoreactivity; same preparation and field of view as in panel B1 shows labeling of the L-cell axon. Calibration bar = 200 µm.

**Fig. 6.** TH-like immunoreactivity in the pericardial organ (PO). (A) A single large-caliber fiber (arrow) originating in the CNS—i.e. the L-cell axon—entered the PO via SN1. (B) The large-caliber THli fiber gave rise to a smaller fiber (arrow) that projected, via the anterior bar (AB), into the twig that became the dorsal nerve (DN) en route to the heart. (C) Within the PO, the THli fiber divided to produce many smooth parallel fibers and varicosities of various sizes. (D) Higher magnification of PO shown in panel C. With the plane of focus on the core of the PO, multiple smooth fibers were observed. (E) Same magnification as in D; focusing on the surface or cortex of the PO revealed finer varicose fibers, indicative of release sites. Calibration bars in A - C = 200 µm; D and E =100 µm.
Fig. 7. TH-like immunoreactivity in the cardiac ganglion. (A) A single THli fiber reached the CG via each dorsal nerve (arrows). Upon entering the CG, these fibers gave rise to varicose branches that terminated in the dendritic and proximal axonal regions of the three large anterior motor neurons (asterisks, filled with Neurobiotin). (B) Very fine varicose THli branches (arrow) projected to the posterior end of the CG where they bypassed the two large motor neurons and terminated in the region of the four small interneurons (arrowhead). *Calibration bar* in both A and B = 100 µm.

Fig. 8. Actions of dopamine on the working heart preparation. (A) Contractions recorded from the WH (see Fig. 1A) shown with a compressed time base. Responses to three concentrations of dopamine (DA). Onset of DA perfusion is indicated by *upward arrows*. *Calibration bars*: vertical = 1 g, horizontal = 30 sec. (B) Preservation of shape of the modulated contraction. *Inset*: superimposed contractions from the WH under control conditions (Con) and in the presence of 1 × 10^{-6} M dopamine (DA). Although the contraction amplitude was increased by approximately 90%, the time to peak contraction and the decay time remained unchanged. *Main plot*: phase plot of the rate of change of the force (y axis) as a function of the force of contraction (x axis). Ten contractions are plotted under control conditions and in presence of 1 × 10^{-6} M DA. The similar shapes of the phase plots indicate that the rates of rise and decay of the contractions are both directly proportional to the amplitude of the contractions under the two conditions. (C, D) DA concentration dependence of contraction frequency (C) and amplitude (D). Means ± SE from 10 preparations. The *smooth curves* are best fits of the DA concentration dependence values with the equation % Change = \( a / \{1 + (EC_{50}/\log_{10}[DA])^b\} \), where [DA] is the DA concentration and \( a, b, \) and \( EC_{50} \) are parameters of the fit.

Fig. 9. Actions of dopamine on the semi-intact working heart preparation. (A1) *Upper record*: contraction force. *Lower record*: simultaneous extracellular recording from the posterolateral connective (see Fig. 1B). (A2) Peak response to perfusion of 1 × 10^{-6} M DA in the same preparation as in A1. (B-E) DA concentration dependence of contraction or burst frequency (B), contraction amplitude (C), burst duration (D) and the number of impulses per burst (E). Means ± SE from 6-17 preparations. The *vertical dashed lines* in B-D mark the
half-maximally effective concentrations, $EC_{50}$, obtained by fitting the equation $\% \text{ Change} = a/{1+(EC_{50}/\log_{10}[DA])^b}$ (smooth curves).

**Fig. 10.** Actions of dopamine on the isolated cardiac ganglion. *(A1)* Upper record: intracellular recording from a CG motor neuron. Lower record: simultaneous extracellular recording from the posterolateral connective (see Fig. 1C). *(A2)* Peak response to perfusion of $1 \times 10^{-6}$ M DA in the same preparation as in *A1*. *(B-D)* DA concentration dependence of burst frequency (*B*), burst duration (*C*), and the number of impulses per burst (*D*). Means ± SE from 6-10 preparations. The *vertical dashed lines* mark the half-maximally effective concentrations, $EC_{50}$, obtained by fitting the equation $\% \text{ Change} = a/{1+(EC_{50}/\log_{10}[DA])^b}$ (smooth curves).

**Fig. 11.** Comparisons of dopamine dose-response relations in the three preparations. Superimposed data and fitted curves from Figs. 8-10 for contraction amplitude (*A*), contraction or burst frequency (*B*), burst duration (*C*), and the number of impulses per burst (*D*). In *B*, the *insets* show the absolute frequency in beats or bursts per minute (BPM)—as opposed to the % change in response to DA shown in the main plot—in the the S-IWH and ICG under control conditions and in the presence of $1 \times 10^{-6}$ M DA. Means ± SE are plotted, with the number of preparations $n$ indicated. Statistical testing using 2-way ANOVA followed by Holm-Sidak multiple comparison tests showed significant differences ($p < 0.05$) between the WH and S-IWH in *A*; between the WH and ICG and between the S-IWH and ICG, but not between the WH and S-IWH, in *B*; between the S-IWH and ICG in *C*; and between the S-IWH and ICG in *D*. See Discussion.

**Fig. 12.** Schematic summary of dopaminergic regulation of the *Callinectes* cardiac system as suggested by this work. *(A)* Three modes of dopaminergic regulation originating from a single neuron, the L-cell, within the CNS. Neurohormonal release of DA (*dashed arrow*) from L-cell terminals in the pericardial organ (*PO*) reaches the entire heart, and, in particular, the cardiac musculature. In addition, the L-cell regulates the heart via its direct innervation of the cardiac ganglion (*CG*) (*solid arrows*). This innervation consists of a posterior projection to the posterior pacemaker interneurons (*thin solid arrow*) and an anterior
projection to the motor neurons (thick solid arrow).  (B) Details of the proposed integration of central and peripheral actions of DA in the cardiac system.

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Figure 2

A

B

C

D

Ant. MN

Post.MN

Ant.con.

Post.con.

5 sec

Ant. MN

Post.MN

Ant.con.

Post.con.

0.5 sec

10mV
Figure 3

A1

Contraction Frequency

![Bar chart showing contraction frequency for WH and S-IWH](chart1)

A2

Time to Peak Contraction

![Bar chart showing time to peak contraction for WH and S-IWH](chart2)

A3

Contraction Duration

![Bar chart showing contraction duration for WH and S-IWH](chart3)

B1

Burst Frequency

![Bar chart showing burst frequency for S-IWH and ICG](chart4)

B2

Impulses / Burst

![Bar chart showing impulses per burst for S-IWH and ICG](chart5)

B3

Burst Duration

![Bar chart showing burst duration for S-IWH and ICG](chart6)
Figure 4
Figure 5
Figure 6

A

B

C

D

E
Figure 7
Figure 10

A1

A2

B

C

D

Burst Frequency

% Change

[DA] (M)

Burst Duration

% Change

[DA] (M)

Impulses / Burst

% Change

[DA] (M)
Figure 11

A

Contraction Amplitude

- WH
- S-IWH

% Change

[DA] (M)

B

Frequency

- WH
- S-IWH
- ICG

% Change

[DA] (M)

C

Burst Duration

- S-IWH
- ICG

% Change

[DA] (M)

D

Impulses / Burst

- S-IWH
- ICG

% Change

[DA] (M)