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Dr. Philip Brownell

Neurons in the abdominal ganglion of the marine mollusk, Aplysia californica , appear to respond directly to changes in blood flow and oxygen tension within the ganglion. When circulation through the ganglion is reduced, the spontaneous activity of some identifiable neurons are excited while others are inhibited. Many of the neurons which respond most strongly are cells involved in regulating the activity of the heart and vasculature. Most notably, the heart excitor interneuron, L10, is excited for prolonged periods when ganglionic circulation is reduced, and this response is reversed when flow resumes. The neurosecretory white cells (R3-R14), which innervate major arteries and the branchial vein, are also excited during periods of reduced ganglionic circulation.

Injection of ink-gelatin mixtures into the vasculature of the abdominal ganglion shows that blood enters the ganglion through a stereotypical pattern of arterial branches. The cell bodies of larger

neurons in the ganglion are surrounded by vascular spaces while the neuropil is not vascularized. The cell body of the heart excitor interneuron, L10, is positioned near the caudal artery where it enters the ganglion. This close morphological relationship between ganglionic vasculature and motor circuits controlling the heart suggests a basis for feedback regulation of cardiovascular functions.

In dissected preparations of the respiratory organs and the heart with intact innervation from the abdominal ganglion, reduced circulation to the ganglion stimulated two physiological responses that increased cardiac output. The initial response was contraction of the gill, stimulated by a burst of activity in the respiratory pumping circuit and Interneuron II. The second response involved long-term excitation of the heart which was correlated with the excitation of the heart excitor interneuron, L10. The physiological importance of this direct interaction between ganglionic circulation and central neurons controlling circulation is discussed.

GANGLIONIC CIRCULATION AND REGULATION OF CARDIOVASCULAR ACTIVITY IN *APLYSIA CALIFORNICA*

by

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Chapter I

GENERAL INTRODUCTION

Circulation is an example of a visceromotor behavior that is regulated by the central nervous system in response to disturbances in the internal and external environments of an animal. Circulation can be adjusted through a complex system of neuronal reflexes and endocrine actions that act on the heart or other circulatory pumps, and the vessels which route blood to various parts of the body. In mammalian systems the nervous system plays a particularly important role in regulating cardiovascular functions in accordance with circulatory demand. Pressure receptors and chemoreceptors embedded in the walls of vessels and the heart constantly monitor hydrostatic pressure and the concentrations of O_2, CO_2, and hydrogen ions in the blood. Input from these receptors is integrated centrally in the brain stem, and this information is used to adjust cardiovascular functioning through visceromotor pathways. Through such negative feedback regulation, cardiac output and circulation are stabilized at set-points, or levels of operation suitable for the momentary requirements of the body (Bard, '68). The mammalian brain is a complex integrative system however, so the extent to which these mechanisms of regulation can be defined and experimentally manipulated is limited.

In this regard invertebrates offer the important advantages of large, readily accessible cells and ease in experimental manipulation. One of the most studied models is the marine mollusk, Aplysia californica . The Aplysia heart, like those of vertebrates, has a myogenic pacemaker which is regulated by excitatory and inhibitory innervation from the abdominal ganglion (Kandel, '76, '79). Several investigators (Koester et al., '73, '74; Mayeri et al., '74; Liebeswar et al., '75; Sawada et al., '81), have now identified excitatory and inhibitory cardiac motorneurons, and at least seven vasomotor neurons which regulate the three major arteries exiting the heart. All of these motorneurons are under the control of the heart excitor interneuron, L10, and a group of functionally coupled interneurons, called Interneuron II (Int II), which act to inhibit the heart and coordinate the fixed action of respiratory pumping (Kupfermann and Kandel, '69; Kupfermann et al., '71; Mayeri et al., '71; Kupfermann et al., '74; Byrne, '83). The somata of many of the neurons are large (200μ-1000μ in diameter), and they lie on the outer surface of the ganglion where they are accessible to penetration by intracellular electrodes. In dissected preparations of Aplysia , they can be identified individually by their position, visual appearance, and synaptic interactions with other neurons in the ganglion. This renders the individual cells identifiable from animal to animal, so the role each cell plays in regulating the heart and vasculature can be studied in some detail.

Recent studies in Aplysia have shown that a variety of

conditions, including temperature change, noxious stimulation, moderate hypoxia, and presentation of food, all act to modulate the spontaneous activity of the heart (Dieringer et al., '78; Feinstein et al., '77; Koch and Koester, '82). There remains however, a surprising lack of information concerning the sensory mechanisms involved in the regulation of cardiovascular activity. Indeed, in contrast to the case in vertebrates where the sensory elements involved in regulation are well described, we still do not understand how cardiovascular functioning is monitored in Aplysia , and how this information impinges on motor circuits controlling circulation.

Anatomically, the abdominal ganglion appears to be in an appropriate location for monitoring cardiovascular functions. Blood enters the ganglion through a branch off the anterior aorta, the largest of the three major vessels exiting the heart (Fig. 8). This morphology raises the possibility that cardiac output might be sensed directly by cardiac control neurons in the abdominal ganglion. Changes in circulatory output (eg. pressure and chemical composition of blood) might alter the impulse activity of regulatory neurons in a manner that would modulate activity of the heart in accordance with circulatory demand.

In chapter II of this thesis, I have described the morphological and physiological relationship between circulation in the abdominal ganglion and neuronal cell bodies contained in the ganglion. I found that the heart excitor interneuron, L10, and several other neurons presumed to be involved in cardiovascular regulation, were located

adjacent to major branches of the ganglionic artery. Some of these neurons were excited by changes in ganglionic blood flow, while others were inhibited. The strongest effects were seen in the neuronal activities of Ll0 and Int II, the interneurons that most strongly influence cardiac output.

In chapter III, I have described a potential functional significance of these neuronal responses to manipulations of ganglionic blood flow. I monitored cardiac and respiratory activities in a dissected preparation and recorded from the heart excitor interneuron, Ll0, while experimentally manipulating circulation through the ganglion. The results of these studies suggests that cardiac and respiratory interneurons sense changes in ganglionic circulation directly, and that these responses contribute negative feedback regulation of cardiac and respiratory activities.

Chapter II

EFFECTS OF GANGLIONIC CIRCULATION ON NEURONS CONTROLLING CARDIOVASCULAR FUNCTIONS IN *APLYSIA*

ABSTRACT

The abdominal ganglion of *Aplysia californica* receives most of its blood supply through a small caudal artery that branches off the anterior aorta near its exit from the heart. By injecting an ink-gelatin mixture into the caudal artery, we observed a stereotypic pattern of arterial branching within the ganglion, and a general proximity of major branches to neurons controlling circulatory functions. This was particularly evident for the heart excitor interneuron, L10, which lies next to the caudal artery where it enters the ganglion. In electrophysiological experiments, L10 was reversibly excited by decreasing blood flow or oxygen tension within the ganglion. This effect was expressed as an increase in frequency and pattern of L10 firing. Some L10 follower cells (RB and LD cells) were directly affected by changes in L10 activity, while others (L3 and RD cells) appeared to respond independently of L10's synaptic influence. Neurosecretory cells that innervate major vessels in the region of the heart were also excited in the absence of ganglionic circulation. These results indicate that central neurons can be affected directly by circulatory status within the nervous system. The manner in which neurons are affected in the abdominal ganglion of

Aplysia suggests that ganglionic circulation may contribute to negative feedback regulation of cardiac output in this animal.

INTRODUCTION

The abdominal ganglion of Aplysia californica contains a neuronal circuit that controls circulation in this animal (Koester et al., '73). The circuit involves identifiable excitatory and inhibitory interneurons (Koester et al., '73) and several cardiac and vasomotor neurons that regulate activity of the heart and three major aortae which receive blood from the heart (Mayeri et al., '75; Sawada et al., '81). Although many of these neurons in this circuit are spontaneously active and presumably influence on-going activity of the cardiovascular system, there is currently no information concerning the mechanisms that regulate cardiac activity of these cells in accordance with circulatory demand. Because of the prevalence of negative feedback mechanisms for regulating cardiovascular activity in vertebrates, it is reasonable to expect that blood pressure or other circulatory parameters (eg. concentrations of O_2, CO_2, or hydrogen ions in blood) are monitored directly and that this information is used to influence the activity of neurons controlling circulation. It is notable that the abdominal ganglion, which contains the heart command circuit, is located near the origin of arterial blood flow. The ganglion receives its blood through a short branch from the largest artery exiting the heart. The cell bodies of L10 and other circulation-related neurons lie adjacent to this artery and its branches within the ganglion. This morphological arrangement thus provides a basis for direct

monitoring of circulation by neurons that control circulation. In this and the following paper we examine the physiological relationships between abdominal ganglion neurons and the circulatory system serving the ganglion.

MATERIALS AND METHODS

Animal Maintenance and Preparation . Mature Aplysia californica weighing 200 - 500 g were obtained from Sea Life Supply (Sand City, Ca.) or Pacific Biomarine, (Venice, Ca.). They were maintained in a natural seawater aquarium at 12-15°C under a natural photoperiod, and fed daily with lettuce.

The abdominal ganglion was removed from unanesthetized animals and pinned to a Sylgard (Dow Chemical) substrate, taking care not to puncture the small caudal artery that mediates blood flow from the anterior aorta to the ganglion. The ganglionic artery was cannulated with PE 20 tubing which had been drawn to a fine tip over heat. The tapered end of the cannula was inserted into the artery and tied in place with suture thread (5/0), and the other end was attached to a Teflon sample injection valve (Rheodyne no. 5020). The preparation was superfused with buffered artificial seawater (10 mM Tris, pH 7.8 with 0.2 % glucose added), or 1:1 mixtures of this solution with blood taken from the hemocoel during dissection. The same fluid was infused into the artery of the ganglion through the sample injection valve (Fig. 1). In all of the experiments a peristaltic pump (Rainin, Rabbit SS) was used to infuse blood or seawater at a constant rate of 2.5 uL/min. When called for, circulation to the ganglion was stopped by momentarily reversing the pump until the artery collapsed. Vascular infusion of fluids of low oxygen tension (Fig. 4) was accomplished under constant flow conditions by loading the sample

loop of the injection valve with deoxygenated (nitrogenated) blood or seawater and switching flow streams at the appropriate time. All experiments were conducted at room temperature (19-22 °C).

Intracellular Recording . Individual neurons were identified by their relative position in the ganglion, pigmentation, and synaptic interactions with other neurons, according to the criteria of Frazier et al., ('67). Cells were penetrated by glass microelectrodes (2M KAc) directly through the ganglionic sheath overlying the somata. Generally, electrical recordings made in this manner were stable for hours and unaffected by the mechanical disturbance associated with changing the rate of blood flow through the ganglion. Electrical activities of 2 to 4 neurons were monitored simultaneously and recorded on chart and tape recorders. Action potential ("spike") frequencies were analyzed and displayed in real time with the aid of a microcomputer. Injury discharges due to mechanical disturbance of the penetrated cells were indicated by an immediate increase in spike rate and a decline in spike amplitude. Cells affected in this manner by the onset and termination of ganglionic circulation were not included in our analysis.

Vascular Anatomy and Histology . The morphological relationship between ganglionic vasculature and neurons was examined by injecting ink (Speedball, water soluble), or a 1:2 mixture of ink and 10 % gelatin (Baker Chem) into the caudal ganglionic artery through a

cannula. The gelatin was liquified by warming to 37°C before use. Histological sections of the ink-infused ganglion were prepared by mixing (vortex; 5 sec) 10-20 µl of 25% glutaraldehyde into the ink-gelatin mixture immediately before vascular injection. The ganglion was then fixed in Bouin's fluid, dehydrated, embedded in paraffin, sectioned at 10 µm, and stained with chrome-hematoxylin (Humason, after Pearse, '79).

RESULTS

Vascular Morphology of the Abdominal Ganglion

Blood flows into the abdominal ganglion mainly through a single artery that branches off the anterior aorta near its exit from the heart and enters the caudal end of the ganglion. Occasionally, the right bag cell cluster and right connective of the ganglion receives a separate, small arterial branch from the dorsal artery.

We injected mixtures of ink and gelatin into the caudal artery of the abdominal ganglion to examine the structural relationships between vasculature and neurons in the ganglion. Although the fine structure of the vascular arborization was quite variable, the major branches of the artery showed a consistent pattern. As shown in Figure 2, the caudal artery typically bifurcates as it enters the ganglion, forming right and left branches. The branch to the left hemiganglion (shown on the right side of Fig. 2 A,B,C) arborizes profusely in the lower left quadrant, frequently enveloping the cell body of neuron L10 (arrow in A) and other neurons in this region. The left branch also gives rise to discrete vessels that travel several millimeters down peripheral nerves (arrow in C) and connectives in close aposition to axonal bundles. The right branch of the caudal artery arborizes throughout the right hemiganglion, especially in the region of the white cells, and along the proximal branchial nerve.

In histological sections of ganglia infused with the ink-gelatin-glutaraldehyde mixture (Fig. 2 D), we observed that most

of the larger neuronal cell bodies were surrounded by vascular spaces while the neuropil region was avascular. Vascular spaces tended to isolate the somata into discrete clumps but individual cells were largely in direct contact with the blood space. The soma of cell L10 was usually adjacent to the left branch of the caudal ganglionic artery and surrounded by vascular sinuses that branched off of the artery.

Effects of Ganglionic Circulation on Neuronal Activity

Most of the ganglionic neurons we surveyed appeared to be sensitive to the infusion of fluids into the ganglionic artery. They appeared insensitive, however, to the type of fluid or the rate of arterial infusion, as long as it was kept within a physiological range. Generally, oxygenated artificial seawater gave greater long term stability of neuronal activity so this medium was used in most experiments. A standard infusion rate of 2.5 µL/min was chosen because it distended the caudal artery to the same diameter observed in freshly dissected preparations where the heart was still functioning.

L10 Response. Of the 20 identified neurons we surveyed, the heart excitor interneuron, L10, was among those most strongly affected by reduction of ganglionic circulation. Previous studies (Mayeri et al., '79) reported that L10 showed a particularly unstable pattern of

activity when the ganglion was isolated from the animal. In our experiments, L10 was usually silent when the ganglion was first isolated from the animal, but its rate of impulse discharge slowly increased until it fired in strong bursts of spikes. As shown in Figure 3 A, infusion of oxygenated blood or seawater into the ganglion artery reversed this tendency. The pattern of inhibitory synaptic input to L10 changed somewhat with arterial infusion, but this was variable and did not account for the decrease in L10 activity. This would suggest that the long-term effect of circulation on L10 was direct and not mediated synaptically. However, when ganglionic circulation was turned off, L10 was usually inhibited for a brief period by synaptic input from Interneuron II, the complex of interneurons that coordinate respiratory pumping of the branchial organs. The inhibitory effect occurred within the first 4 minutes (mean = 1.94 +/- 1.81 min) of terminating arterial infusion.

In six experiments where circulation to the ganglion was maintained for 15 minutes and then shut off for 25 or 35 minutes (Fig. 3 B), the rate of L10 firing gradually increased until circulation was turned on again. The duration and extent of L10 excitation was dependent on the period of decreased circulation, and in each case the response was reversible.

L10 was also excited by reductions in oxygen tension of fluids circulating through the ganglion. As shown in Fig. 4, the average spike rate of L10 increased 20 to 50% above baseline levels when arterial infusion of oxygenated seawater was switched to deoxygenated

(pO_2 < 10 mmHg) seawater without interrupting the rate of flow. The response to reduced oxygen tension was not always reversible, however, and generally did not excite L10 as much as stopping arterial infusion altogether. This would suggest that variation in arterial flow plays a more important role than blood oxygen tension in controlling the firing rate of L10 in the isolated ganglion.

Response of L10 Follower Cells. L10 affects changes in heart activity through its synaptic actions on motorneurons (follower cells) that innervate the heart and vasculature. To determine how the effects of ganglionic circulation on L10 are expressed at the level of these motorneurons, we recorded from clusters of cells (RB and LD) to which they belong. The most important motorneurons innervating the heart are the excitatory cell RB_{HE}, a serotonergic neuron in the RB cluster, and two heart inhibitor neurons, LD_{HI1} and LD_{HI2}. RB_{HE}, and other RB cells, receive excitatory synaptic input from L10 while the LD cells are inhibited by L10.

As shown in Figure 5, RB and LD follower cells responded to changes in ganglionic circulation in a manner predicted by the effect of this manipulation on L10. With arterial infusion turned off, RB and LD cells discharged at relatively high and low rates, respectively, reflecting the elevated activity of L10 during this time (Fig. 5 C). When ganglionic circulation was restored, these follower cells returned to baseline levels of activity, again reflecting the response (decrease in activity) of L10.

Some cells, however, did not show the response predicted by the effects of ganglionic circulation on L10. RD neurons are inhibited by synaptic input from L10, yet their activity decreased rather than increased when ganglionic circulation was on, even though inhibitory synaptic input from L10 was reduced during this time. This suggests that some L10 follower cells are capable of responding directly to changes in ganglionic circulation independently of L10 synaptic input. Figure 6 shows the results of a similar experiment in which the responses of two L10 follower cells, an RB cell and identified cell L3, were recorded. The RB neuron, which is excited by L10, showed the predicted increase in activity when ganglionic circulation was turned off. However, cell L3, which is inhibited by L10, also showed an excitatory response. The time course of the L3 response was similar to the excitatory response of neurosecretory white cells (R4 in this example), which are not innervated by L10. This suggests that the L3 neuron, like the RD cluster cells, responds independently of L10, possibly by a direct influence of ganglionic circulation.

White Cell Responses. The neurosecretory white cells (R3-R13) of the right upper quadrant of the ganglion, and white cell R14 showed excitatory responses to reduced ganglionic circulation. The patterns of their responses varied, however, with some cells responding phasically (see R11 and R12 in Fig. 7), and others responding tonically to reduced circulation. Like other responsive neurons we examined, there was no change in synaptic input to white cells that

could account for the patterns of their responses.

DISCUSSION

L10 is the excitatory command interneuron which codes for increased cardiac output (Koester et al., '73, '74; Mayeri et al., '74). As previously noted by others (Mayeri et al., '79), we found that L10 activity steadily increased when the ganglion was isolated from the animal. In the absence of circulatory feedback, L10 activity gradually moves through four modes: silent, tonic firing, low-frequency bursting discharge, and high-frequency bursting. Studies by Koester et al. ('74); (see also Kandel, '76), concluded that these modes of firing resulted from the intrinsic pacemaker capability of the cell and was not generated by intraganglionic synaptic input that could be recorded in the cell body. The factors which influence this transformation in L10 activity have not been investigated until now.

Our results suggest that L10 activity is dependent upon the flow and oxygen tension of fluids circulating through the ganglionic vasculature. Furthermore, the manner in which L10 is affected appears to provide a negative feedback mechanism for regulating heart activities in accordance with circulatory and metabolic demands. In the presence of ganglionic circulation, L10 fired at low rates, and occasionally it was silent. The excitatory effect of L10 on the heart under these conditions would be minimal. In the absence of ganglionic infusion, L10 becomes excited, frequently to the point that it begins to fire strong bursts of spikes. Such strong effects on L10 should

excite the heart and increase cardiac output.

The excitatory response of L10 was preceded by a transient period of inhibition. This was caused by burst activity in Interneuron II (Int II), a group of coupled interneurons that coordinate respiratory pumping of the gill (Kupfermann et al., '69, '70, '74; Mayeri et al., '71; Byrne, '83). Int II driven gill contractions should force blood into and through the heart, thus contributing to increased cardiac output.

L10 exerts its excitatory effect on the heart primarily through the heart excitor motorneuron RB_{HE} (Mayeri et al., '74). RB_{HE} is a unique cell found in the RB cluster near the entry point of the caudal artery of the abdominal ganglion. Without neural innervation of the heart, we were unable to positively identify RB_{HE} in this study, but we did observe responses of other unidentified RB cells. Cells in the RB cluster share common electrophysiological properties (Frazier et al., '67; Kandel et al., '67; Winlow and Kandel, '76), so responses of these cells to ganglionic circulation are likely to be similar. The excitatory responses of the RB cells we observed followed the same time course as the L10 response to decreased arterial infusion (Fig. 3), suggesting that L10's input was responsible (Fig. 5, 6).

L10 also acts to inhibit motorneurons (LD_{HI1}, LD_{HI2}) that inhibit the heart. We were unable to identify the LD_{HI} cells in the isolated ganglion, but we did record from LD cluster cells. LD neurons were excited during periods of increased ganglionic infusion,

which further supports the hypothesis that the L10 response has a concerted effect on cardiac motorneurons. However, other L10 follower cells (RD, L3), and several neurosecretory white cells (R3-R13) that do not receive synaptic input from L10, also responded to changes in ganglionic infusion, indicating a direct action of ganglionic circulation that is independent of L10's influence.

Some of the neurons that responded to changes in ganglionic circulation have circulatory functions that are less defined. Neurosecretory cells R3-R13 and white cell R14, for example, send axons to major arteries and the branchial vein (Price and McAdoo, '79). It has been suggested that R3-R14 effect muscles surrounding these vessels (Sawada et al., '81). Our results show that these cells are excited when ganglionic circulation is reduced, but the physiological significance of the action remains to be determined.

Our results indicate that neuronal activity can be influenced directly by circulation in the central nervous system. In Aplysia this may be a mechanism of feedback regulation of cardiovascular activity. In chapter III, we examined this possibility in a dissected preparation where activity of cardiac and respiratory organs can be monitored and related to the changes in ganglionic blood flow.

Fig. 1. Experimental set up for examining the effects of ganglionic circulation on activity of central neurons. Spontaneous impulse activity was recorded through intracellular electrodes. A sample injection valve was used to infuse deoxygenated saline without interrupting arterial flow.

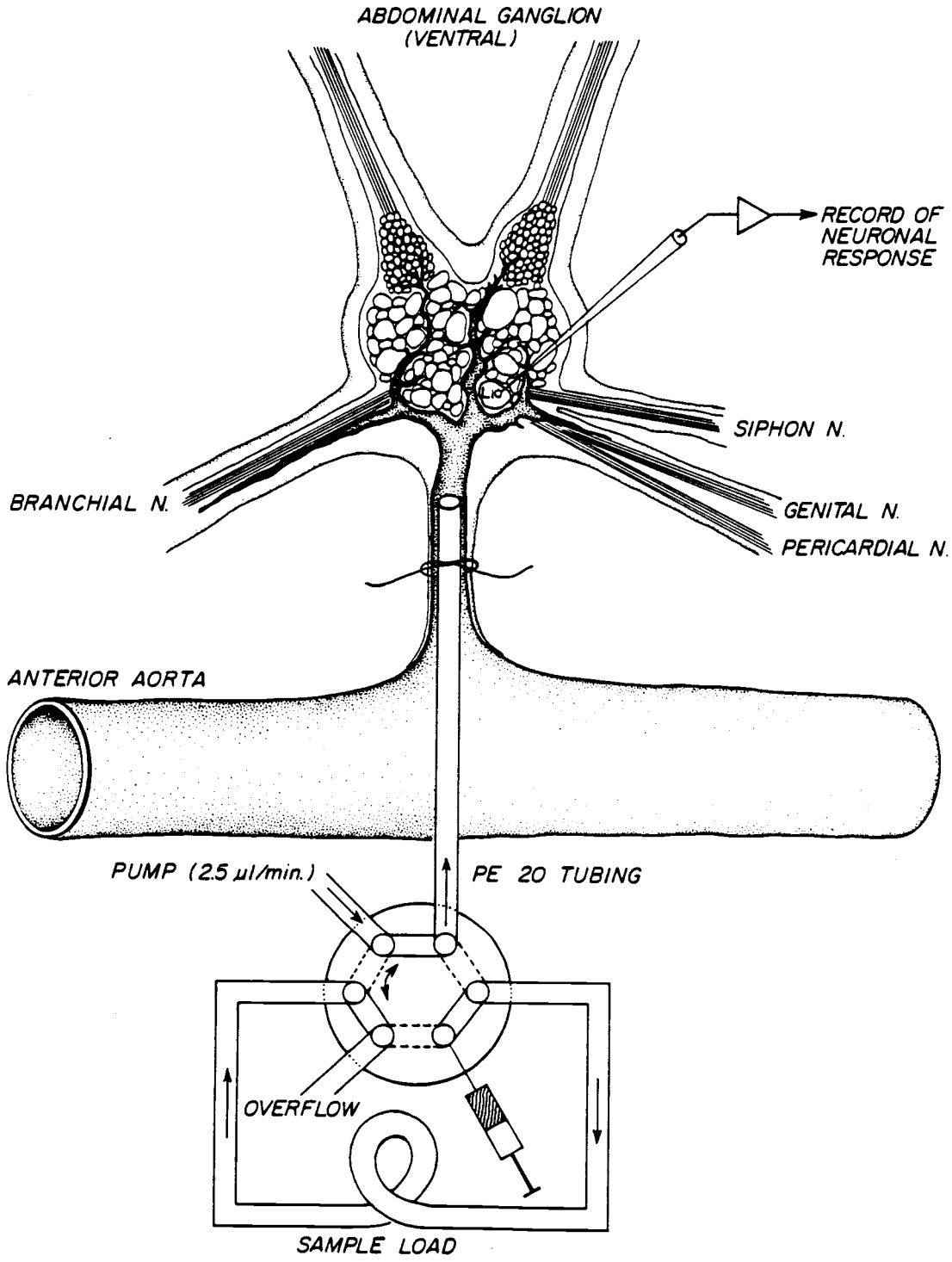
ABDOMINAL GANGLION
(VENTRAL)
RECORD OF
NEURONAL
RESPONSE
SIPHON N.
BRANCHIAL N.
GENITAL N.
PERICARDIAL N.
ANTERIOR AORTA
PUMP (2.5 µl/min.)
PE 20 TUBING
OVERFLOW
SAMPLE LOAD

Figure 1

Fig. 2. Vascular arborization in the abdominal ganglion. A, B, and C show three stages during injection of ink into the caudal ganglionic artery. Note the position of the heart command interneuron, L10, (arrow in A), relative to major arterial branches. Arrow in C indicates arterial branches in peripheral nerves. D: Histological section of ink-filled ganglion showing the distribution of vascular spaces (ink) relative to neuronal cell bodies.

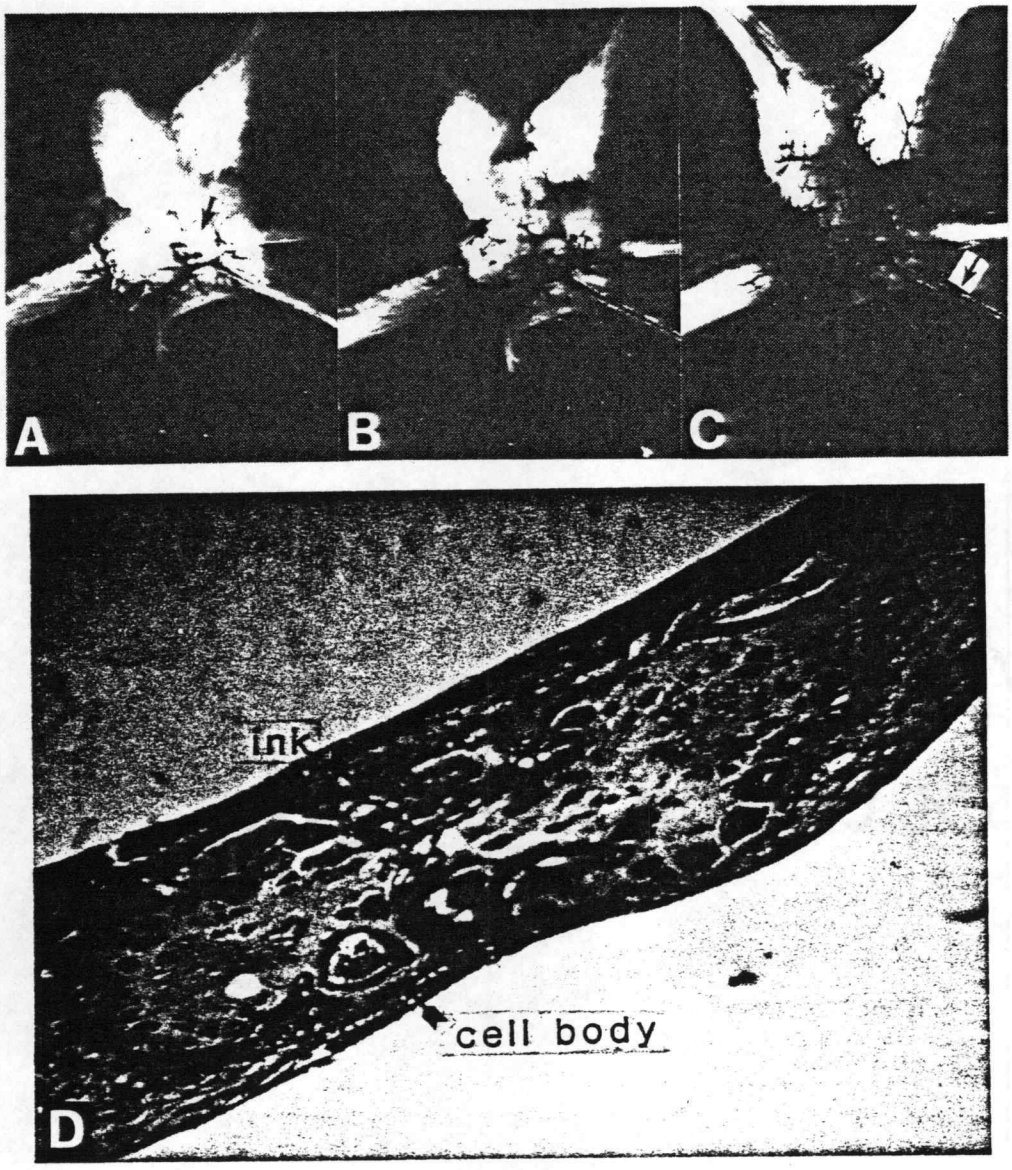

Figure 2

Fig. 3. Effects of ganglionic circulation on the impulse activity of the heart excitor interneuron, L10. A: Infusion of oxygenated seawater into the caudal artery of the abdominal ganglion reversibly inhibited L10 spike activity. Arrows indicate the beginning (on: 2.5 µL/min) and end (off: 0 µL/min) of arterial infusion. B: L10 spike rate gradually increased over several minutes when arterial infusion was turned off, and returned to baseline levels with the same time course when flow resumed. The period and extent of L10's response depended on the period of reduced circulation. C: Three examples showing short-term effects of decreased ganglionic circulation on L10 activity. During the first 1 to 4 minutes of response, L10 was briefly but strongly inhibited (*) by synaptic input from Interneuron II. (open symbols - arterial infusion on; closed symbols - arterial infusion off)

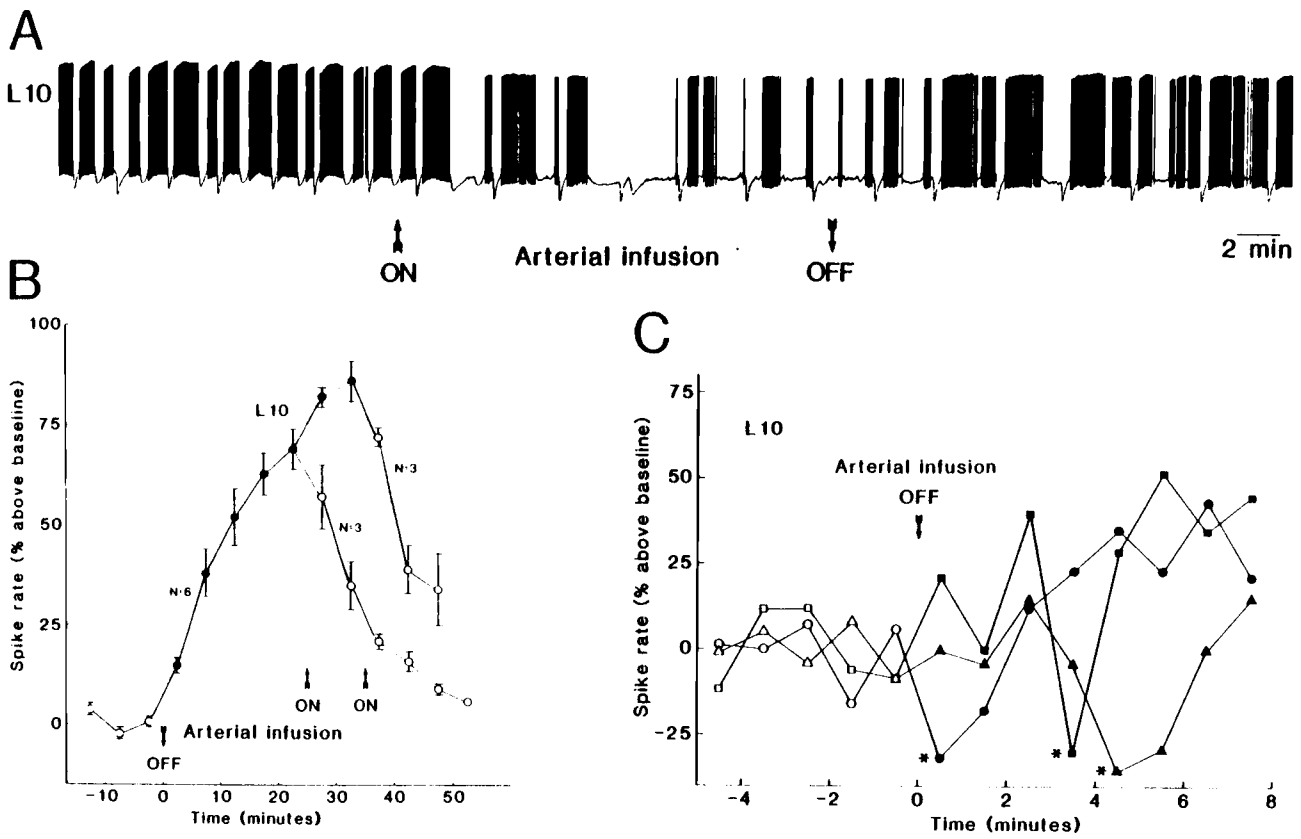
A
L10
ON
Arterial infusion
OFF
2 min
B
Spike rate (% above baseline)
100
75
50
25
0
L10
N=6
N=3
N=3
ON
ON
Arterial infusion
OFF
-10
0
10
20
30
40
50
Time (minutes)
C
Spike rate (% above baseline)
75
50
25
0
-25
L10
Arterial infusion
OFF
-4
-2
0
2
4
6
8
Time (minutes)

Figure 3

Fig. 4. Effect of oxygen tension on spontaneous activity of L10. In three separate experiments, spontaneous activity of L10 increased when a constant flow (2.5 μL/min) of oxygenated seawater (pO_2 = 150 mmHg) was switched to deoxygenated seawater (pO_2 < 10 mmHg). In two of the preparations, the excitatory effect on L10 was reversed when the ganglion was again infused with oxygenated seawater.

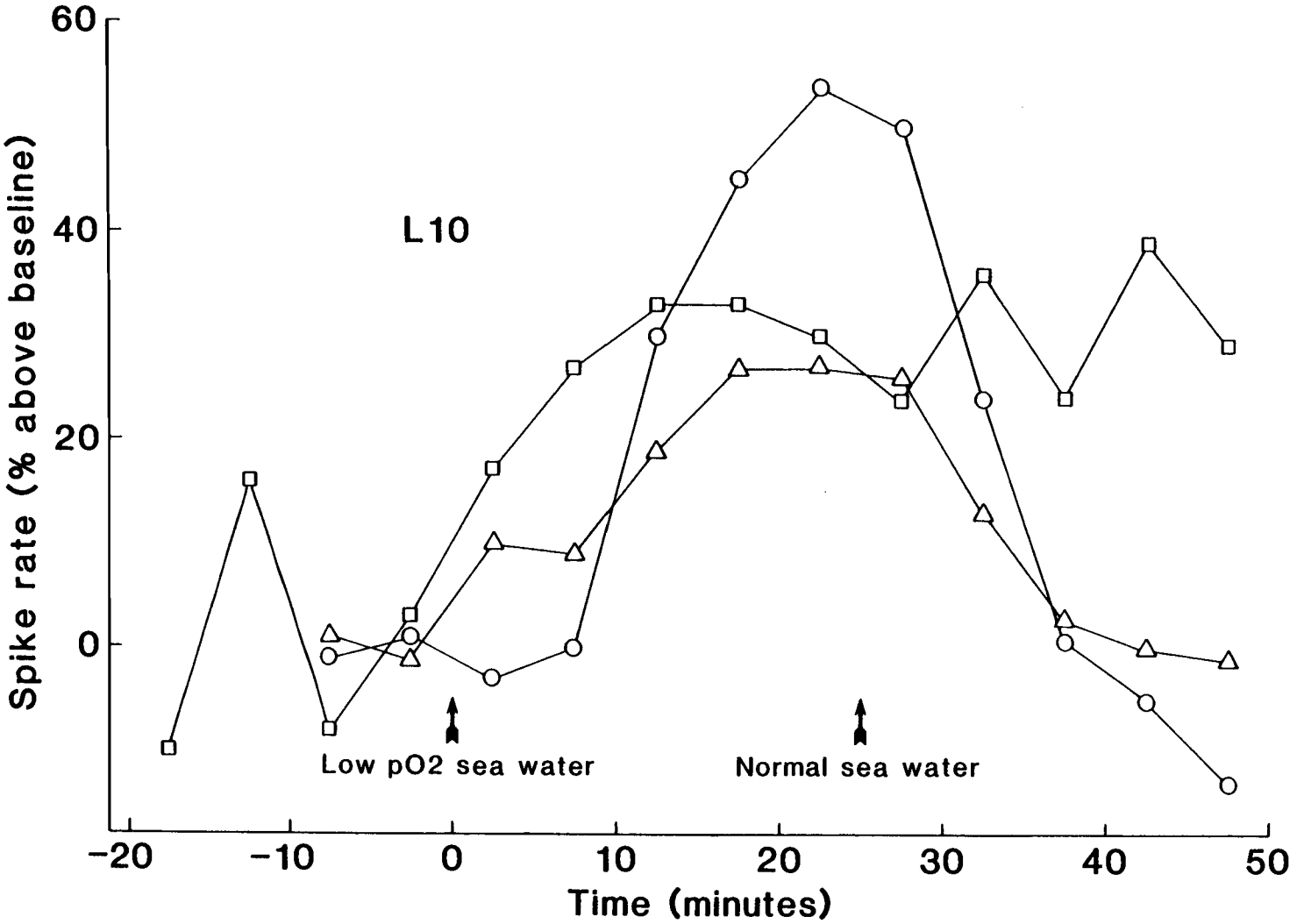
60
40
20
0
Spike rate (% above baseline)
L10
Low pO2 sea water
Normal sea water
-20
-10
0
10
20
30
40
50
Time (minutes)

Figure 4

Fig. 5. Responses of L10 follower cells to changes in ganglionic circulation. A: Follower cells that are excited synaptically by L10 (RB neuron in this example) showed decreased spike activity during periods of arterial infusion. Of the follower cells that are inhibited by L10, (LD and RD in this example), one (LD) was excited by infusion of oxygenated sea water while the other (RD) was inhibited. Neuronal traces at C1 and C2 are expanded in C. B: The effects of decreased ganglionic circulation on L10 follower cells developed slowly and were fully reversible. C: Expanded traces from A (arrows C1, C2) showing changes in synaptic input to L10 follower cells. L10 synapses excite the RB neuron and simultaneously inhibit LD and RD cells (dotted lines). Note that the frequency of L10 synaptic input was decreased during the period of arterial infusion.

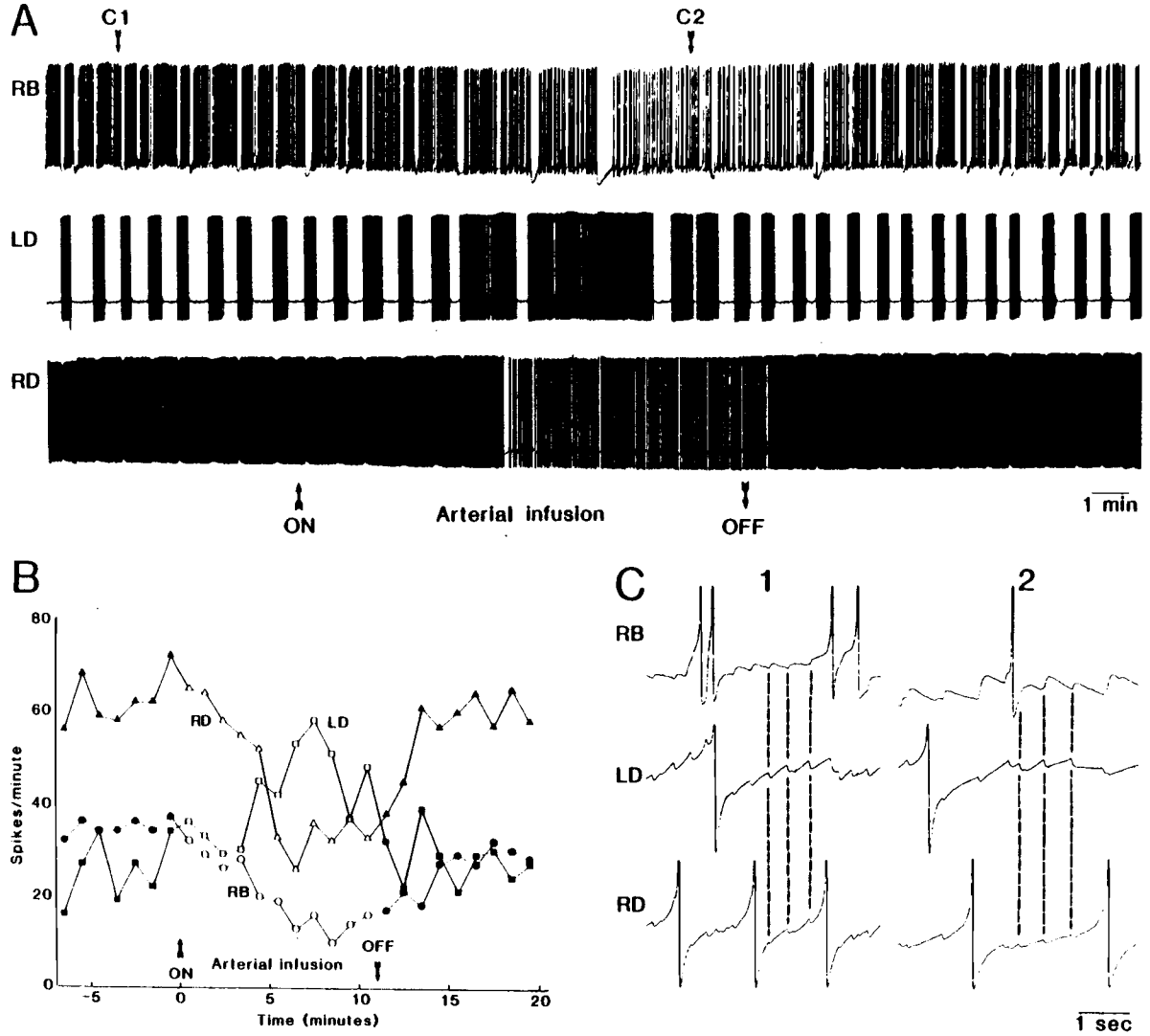
A
C1
C2
RB
LD
RD
ON
Arterial infusion
OFF
1 min
B
Spikes/minute
80
60
40
20
0
RD
LD
RB
ON
Arterial infusion
OFF
-5
0
5
10
15
20
Time (minutes)
C
1
2
RB
LD
RD
1 sec

Figure 5

Fig. 6. Changes in patterns of neuronal activity induced by turning off ganglionic circulation. A: Abdominal ganglion neurons generally showed higher levels of impulse activity in the absence of circulation. In this example, a neuron that is excited by L10 (RB), one that is inhibited by L10 (L3), and one that does not receive L10 synaptic input (R4), were all reversibly excited while arterial infusion was turned off. Neuronal traces at C1 and C2 are expanded in C. B: During the period of decreased circulation, the average spike rate of the RB neuron increased gradually with the same time course as the L10 excitatory response (see Fig. 3). The changes in spike rate of L3 and white cell R4 were more abrupt. C: RB was excited indirectly by increased excitatory synaptic input from L10, while L3 and R4 appeared to respond directly and independently of the inhibitory synaptic influence from L10 (indicated by co-occurance of EPSP's in RB and IPSP's in L3, dotted lines) which increased while arterial infusion was off.

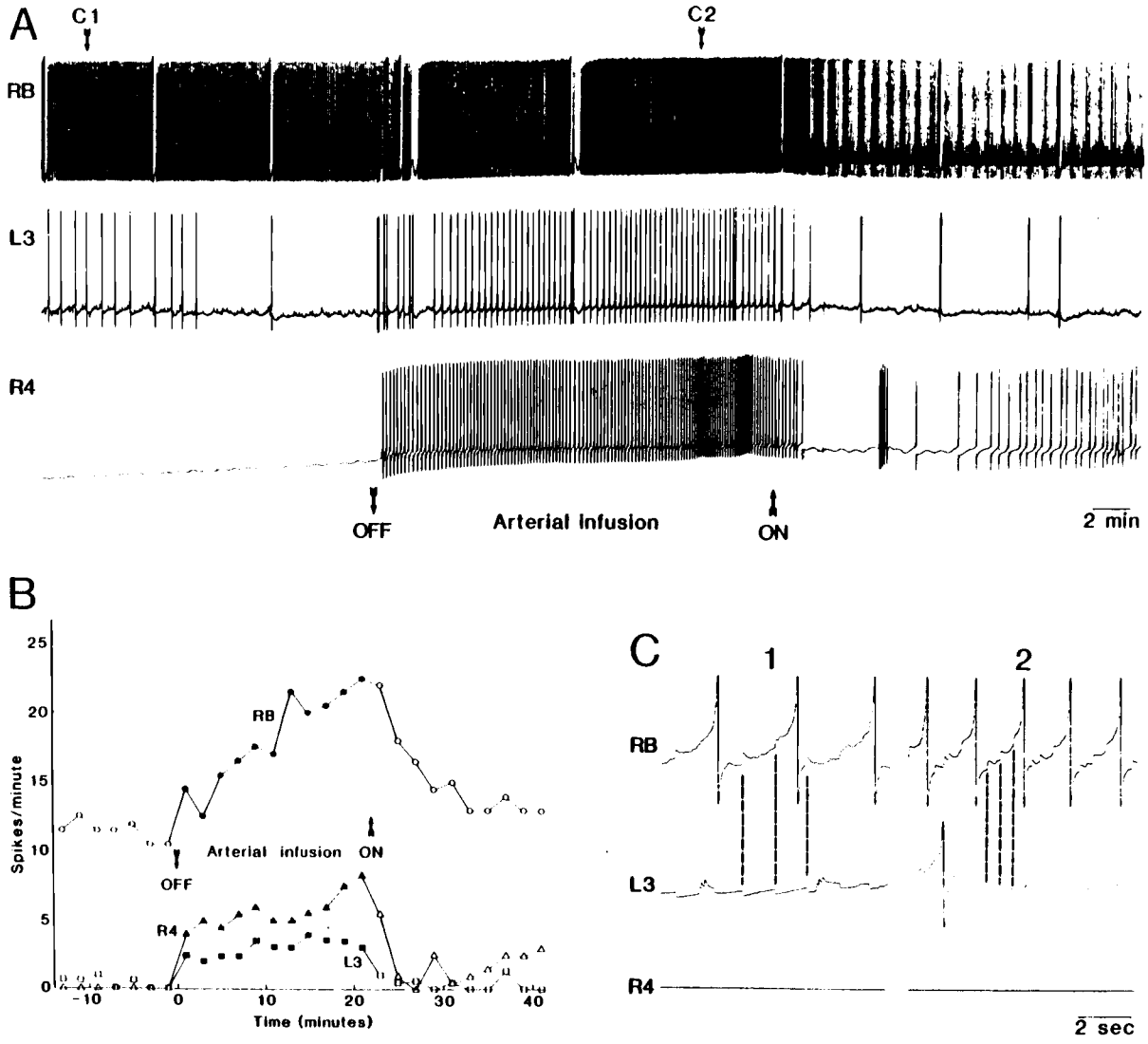
A
C1
C2
RB
L3
R4
OFF
Arterial Infusion
ON
2 min
B
Spikes/minute
25
20
15
10
5
0
RB
Arterial infusion
ON
OFF
R4
L3
-10
0
10
20
30
40
Time (minutes)
C
1
2
RB
L3
R4
2 sec

Figure 6

Fig. 7. Influence of ganglionic circulation on spontaneous activity of white cells. A: Simultaneous recordings from three white cells show both phasic and tonic responses to decreased arterial infusion. B: A separate experiment showing the reversible excitatory response of R14 to decreased arterial infusion.

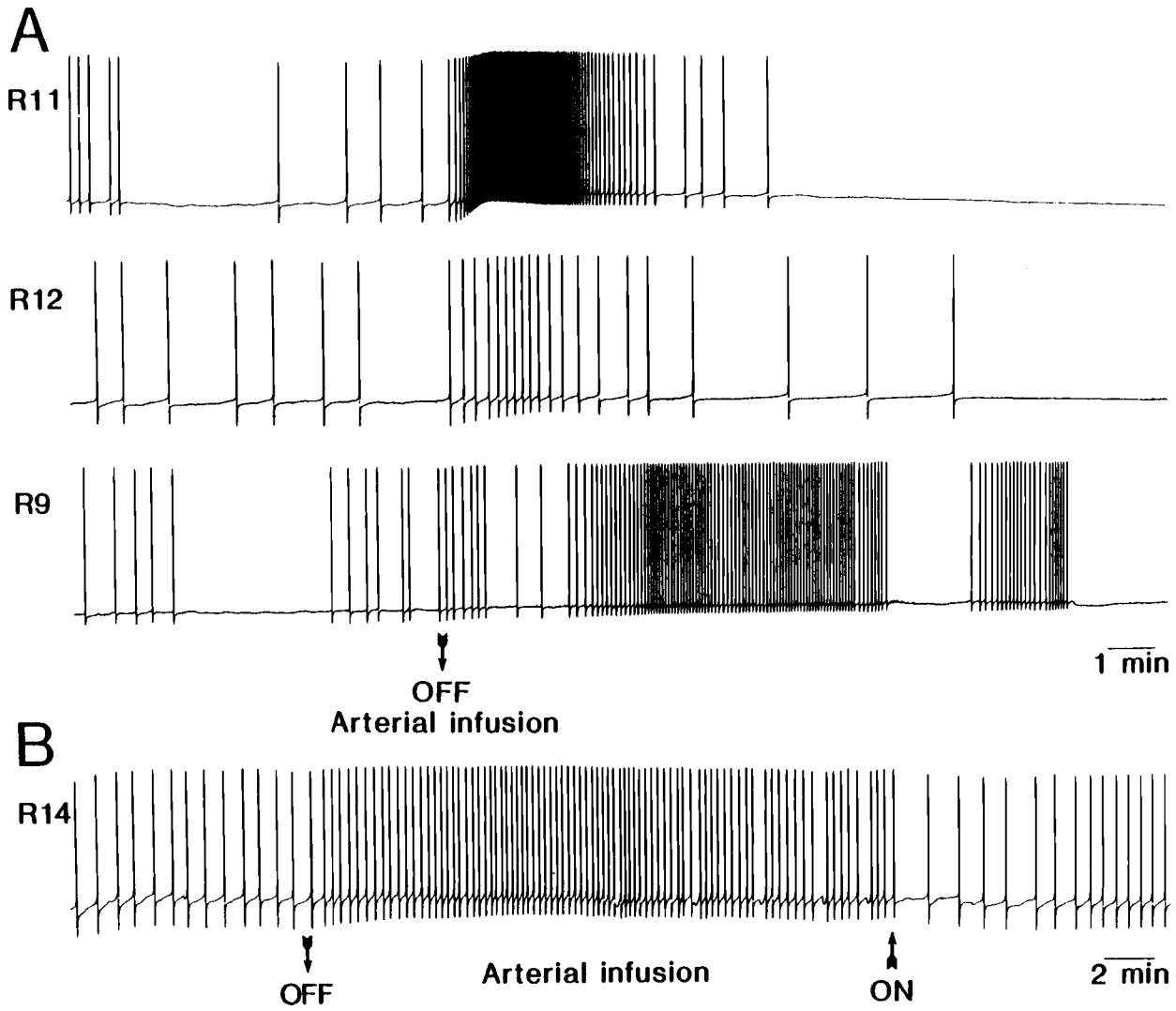
A
R11
R12
R9
OFF
Arterial infusion
1 min
B
R14
OFF
Arterial infusion
ON
2 min

Figure 7

CHAPTER III

GANGLIONIC CIRCULATION AND REGULATION OF GILL AND HEART ACTIVITIES IN *APLYSIA*

ABSTRACT

In isolated preparations of the abdominal ganglion of *Aplysia californica*, changes in the flow rate and oxygen tension of fluids circulating through the ganglionic vasculature influence the spontaneous activities of central neurons (chapter II). One of these cells, the heart excitor interneuron, L10, is excited by decreased ganglionic blood flow, suggesting a negative feedback interaction between circulation and the neural circuit regulating cardiac output. We examined this possibility in a dissected preparation of the heart and respiratory organs. Our results show that decreased circulation through the abdominal ganglion stimulates a transient increase in the rate and amplitude of respiratory pumping and pericardial contractions, and long-term excitation of the heart. Both of these responses increase cardiac output, and both appear to involve a direct action of ganglionic circulation on interneurons controlling the gill and heart. Our results suggest that a direct effect of ganglionic circulation on central neurons is part of the physiological mechanism that regulates cardiovascular homeostasis in this animal.

INTRODUCTION

In the preceding chapter we described the effects of ganglionic circulation on spontaneous activities of neurons with known cardiovascular functions in Aplysia . The effects of ganglionic circulation on one of these cells, the heart excitor interneuron, L10, was of particular interest because it suggests a novel mechanism for regulating cardiac output in this animal. L10, which is positioned adjacent to the major artery of the ganglion, is excited when the rate of blood flow through the ganglion is decreased. Excitation of L10 is known to increase cardiac output (Koester et al., '73, 74), which, in turn, should increase circulation to the ganglion and neutralize the excitation of L10. In vertebrate circulatory systems, circulatory performance is monitored by specialized sensory cells embedded in the walls of the major arteries and the heart (Bard, '68). Receptors of this type have not been found in molluscan systems however, suggesting that direct sensing of circulatory status by motor circuits controlling the heart may play a relatively important role.

In this paper we have investigated the importance of ganglionic circulation in controlling cardiac output in a dissected preparation of the heart and respiratory organs. Our results indicate that decreased circulation within the abdominal ganglion stimulates two physiological responses - contraction of the gill, and excitation of the heart - both of which act to increase cardiac output in a manner

that should sustain circulatory homeostasis.

MATERIALS AND METHODS

Animals . In all of these experiments we used mature Aplysia californica ranging in size from 200-500 g. The source of these animals and the conditions for maintaining them in the laboratory are described in chapter II.

Semi-intact preparation . Unanesthetized animals were pinned ventral surface up and hemocoel blood was removed through a longitudinal incision in the body wall. The three major arteries exiting the heart - the anterior, gastroesophageal, and abdominal aorta - were ligatured and cut, along with the abdominal ganglion connectives, leaving intact all other nerves from the ganglion to the peripheral organs (see Fig. 8 for anatomical relationship between heart, ganglion and vasculature). The spermalytic gland, opaline gland, ink gland and digestive tract were then ligated, where appropriate, and removed. The innervated branchial and pericardial organs were further isolated by cutting through the body wall in a circle around the pericardium.

After loosely pinning the isolated organs in a recording chamber, the efferent branchial vein was cannulated with polyethylene tubing (0.86 mm diameter) and infused with an oxygenated mixture of buffered artificial seawater (10 mM Tris, pH 7.8, with 0.2% glucose added) and blood taken from the hemocoel at dissection. The rate of infusion was adjusted by raising or lowering the height of the

infusion fluid reservoir to achieve a baseline heart rate of about 15 beats/minute (see Fig. 13). Blood pressure was monitored with a Narco pressure transducer (RP1500I) inserted into the gastroesophageal artery, and a 15 cm length of 0.86 mm diameter PE tubing was inserted into the abdominal aorta to act as a flow restrictor. In some experiments (Fig. 9, 10) gill contractions were monitored with a displacement transducer (Schaevitz, DCD200) attached to the distal end of the gill.

The abdominal ganglion was pinned to a raised sylgard platform, taking care not to injure or stretch nerves to the periphery. The caudal artery to the ganglion was cannulated by the procedure described in chapter II. The anterior aorta was tied off proximal to the abdominal ganglion artery, isolating the cannulated vessel to the ganglion from the rest of the preparation. The ganglionic infusion fluid was buffered ASW. The rate of arterial infusion was 2.5 μL/min where indicated.

L10 was identified, and its neuronal activity was recorded, using the procedures described in the previous chapter. All experiments were conducted at room temperature (19-22 °C).

Changes in the activity of the heart were difficult to quantify because of the tendency of the preparation to run down over the 3 to 5 hour period of the experiments. Long-term stability of the heart was maximized by raising the oxygen tension (pO_2 > 100 mmHg) of fluids infused into the heart, and by avoiding the use of muscle relaxants (isotonic $MgCl_2$) or anesthetics during dissection. In some

experiments, the addition of hemocoel blood to the infusion medium improved longevity of the preparation, but it also tended to increase baseline variations in heart activity. Heart beat frequency and amplitude were strongly affected by the amount of fluid entering the heart during diastole, so it was necessary to carefully adjust and maintain a constant infusion pressure throughout the duration of the experiments.

RESULTS

In the previous chapter we found that eliminating blood flow to the abdominal ganglion produced the strongest and most readily reversible changes in the activity of ganglionic neurons. With the exception of the results in Figure 10, therefore, all of the gill and heart responses described in this paper were evoked by turning on, or turning off, the flow of oxygenated seawater into the cannulated caudal artery of the abdominal ganglion (hereafter referred to as "ganglionic circulation"). In the present study, we observed two types of physiological responses to reduced ganglionic circulation: (1) transient increase in contractile activity of the gill and pericardium, and (2) long-term excitation of the heart. These responses are discussed separately below.

Response of Gill and Pericardium

The most immediate and reliable response to reduced ganglionic circulation was strong, multiple contractions of the gill (Fig. 9 A). These usually began within 0 to 3 minutes after turning off the flow of oxygenated seawater into the ganglionic artery. Generally, both the frequency and amplitude of gill contractions increased for several minutes after this time. This response was not observed, however, when circulation to the ganglion was restored. Strong contractions of the gill always gave rise to large surges of blood

that passed directly through the heart and into the arterial system. As shown in Figure 9 A and B, these pressure surges were not always evident in our recordings because of the necessity of cannulating the gill vein to infuse circulatory fluids into the heart. In experiments where the gill vein was cannulated more distally (Fig. 9 A), contractions of the gill during respiratory pumping caused a simultaneous and short-lived increase in vascular (gastroesophogeal artery) pressure, and usually a momentary decrease in frequency and amplitude of heart beats. When the gill vein was cannulated near the base of the gill (Fig. 9 B), gill contractions did not register as synchronous pulses of vascular pressure increase. However, in this configuration it was possible to observe a second mode of increasing cardiac output. In addition to contracting the gill, Int II discharges stimulate delayed (10-20 sec) contractions of the pericardial membrane. Pericardial contractions force blood out of the heart and into the arterial system, and simultaneously force fluid out of the pericardial cavity and into the kidney (Kandel, '76). Both of these actions should facilitate circulation through the cardiac system and thus contribute to increased cardiac output.

This stereotypic pattern of the gill and pericardial contraction may be a general mechanism for abruptly increasing cardiac output. A similar pattern of activation was observed (Fig. 10 A) when blood flow into the heart was terminated. In this case, however, the response was triggered by sensors in the heart or pericardial tissues since it was abolished by severing the pericardial nerve to the

abdominal ganglion.

Response of the Heart

The heart usually showed a biphasic response when ganglionic circulation was turned off. In the first 0 to 5 minutes of terminating the infusion of seawater into the abdominal ganglion artery, heart rate usually decreased for a brief period due to synaptic inhibition from Int II during respiratory pumping. Figure 11 shows the temporal correlation between Int II inhibition of the heart excitor interneuron L10, and the brief period of decreased frequency and strength of heart contractions.

If ganglionic circulation remained off, spontaneous activity of the heart gradually increased and remained high for the period of interrupted flow. Figure 12 shows three examples of the types of responses we observed. Generally, both frequency and strength of heart contractions increased over a 10 to 30 min period of reduced ganglionic circulation, as represented by example 2 in this figure. In some experiments however, only the amplitude (example 1) or frequency (example 3) of heart beats increased. In each case these responses were reversed when circulation to the ganglion was restored.

The time course of the heart's response to decreased ganglionic circulation is very similar to that of the heart excitor interneuron L10 (see Chapter II). This raises the possibility that the heart's

response may be mediated, in part, by the effects of decreased circulation on L10. To test this possibility we hyperpolarized L10 to reversibly eliminate its excitatory effect on the heart during the onset of a response (Fig. 13). When infusion of the ganglion artery was turned off, L10 activity and the rate and amplitude of heart beats began to increase. When L10 was abruptly hyperpolarized several minutes later, the rate of increase in frequency of heart beats was reduced while amplitude continued to increase. When hyperpolarization of L10 was removed, the cell continued to discharge in strong bursts and heart beat frequency and amplitude increased once again. The excitatory responses of L10 and the heart were partially reversed when circulation to the ganglion was restored. These results suggest that the heart response is mediated partly by the effect of ganglionic circulation on L10 but that other mechanisms must also contribute to excitation of cardiac activity.

DISCUSSION

In Aplysia there are two pumps, the heart and gill, which force blood into circulation when they contract. When the gill contracts, blood which has entered the gill from the hemocoel is forced into the heart via the efferent branchial vein. Strong contractions of the gill, like those associated with respiratory pumping, force blood through the heart and into the arterial system thus contributing directly to cardiac output. Respiratory pumping is coordinated by a group of coupled interneurons (Int II) within the abdominal ganglion (Kupfermann et al., '69, '70, '74; Mayeri et al., '71; Byrne, '83), so factors that increase the frequency or intensity of Int II discharges also cause a transient increase in cardiac output. Longer-term changes in cardiac output are controlled by another interneuron, L10, which acts synaptically through cardiac motorneurons to increase the frequency and strength of heart contractions (Koester et al., '73, '74; Mayeri et al., '74). The results of this study show that decreased circulation through the abdominal ganglion directly effects the activity of both the gill and the heart in a manner that would increase cardiac output. In the intact animal this would increase blood flow to the abdominal ganglion, thus neutralizing the excitatory effects of decreased circulation.

As diagrammed in Figure 14, both of the transient (gill) and long-term (heart) mechanisms for increasing cardiac output appear to

involve direct actions of ganglionic circulation on interneurons in the abdominal ganglion. The initial response to reduced circulation was an increase in the frequency and intensity of the periodic discharges of Int II (gill pumping). This occurred within minutes after ganglionic blood flow was turned off and appeared to be a particularly effective mechanism of forcing an immediate and strong increase in cardiac output. Pericardial contractions occurring in conjunction with gill pumping also caused surges in blood flow from the heart. Contraction of the pericardium has the added effect of forcing fluid out of the pericardial space and into the renal system (Kandel, '76), thus reducing the resistance to blood flow into the heart. Activation of gill and pericardial contractions appears to be a general mechanism for compensating decreased cardiac performance since reduced blood flow to the heart also stimulated the same pattern of activity. In other experiments (not shown), we observed that L10 and some of the neurosecretory white cells, were also strongly stimulated when blood flow into the heart was reduced. This reflex is mediated by the pericardial nerve (it did not occur when the nerve was cut), suggesting that information from peripheral sensors is integrated into the Int II mediated response.

The second and more sustained action of reduced ganglionic circulation was to stimulate activity of the heart excitor interneuron, L10 (Fig. 14). L10 excites the heart through its connections with cardiovascular motorneurons, so a sustained increase in L10 activity should increase cardiac output. L10 spike rate and

heart rate increased gradually over a 10 to 30 minute period following reduced ganglionic circulation and were largely reversed when flow resumed. The slowness of the L10 response suggests that this component of the feedback regulating mechanism operates over extended periods of time (several minutes), and is insensitive to small or quick changes in cardiovascular function. This is consistent with the L10 effect on the heart, which is itself slow (Koester et al., '73, '74). In this regard, Int II mediated gill pumping may be more important for short-term regulation of cardiac output.

We observed a strong correlation between L10's response to decreased ganglionic circulation and the response of the heart, suggesting that they may be functionally related. Removal of L10 from the circuit during the response to reduced circulation (Fig. 13), did not abolish the heart response, however, suggesting that other mechanisms are involved. In chapter II we found that other ganglionic neurons which send axons into the pericardial region (eg. L3, white cells R3-R13) were excited by reduced ganglionic circulation. These cells might be involved in mediating some of the cardiac response.

To our knowledge, this is the first report of a feedback mechanism in which blood flow, operating directly on the activity of identified central neurons, produces a compensatory response in the activities of organs controlling circulation. These effects of ganglionic circulation constitute only one component in the feedback mechanism that regulates cardiovascular activity in this animal. Our results suggests that peripheral sensory mechanisms may also

contribute to cardiovascular control by affecting the activity of the heart and by regulating the amount of blood injected into the heart by gill contractions. Thus, several mechanisms may be involved in contributing to circulatory homeostasis.

Fig. 8. Position of the abdominal ganglion and its caudal artery relative to major vessels exiting the heart. The ganglion is drawn ventral surface up, and is enlarged approximately 2-fold relative to the vasculature to emphasize the position of the heart excitor interneuron, L10, near the ganglionic artery.

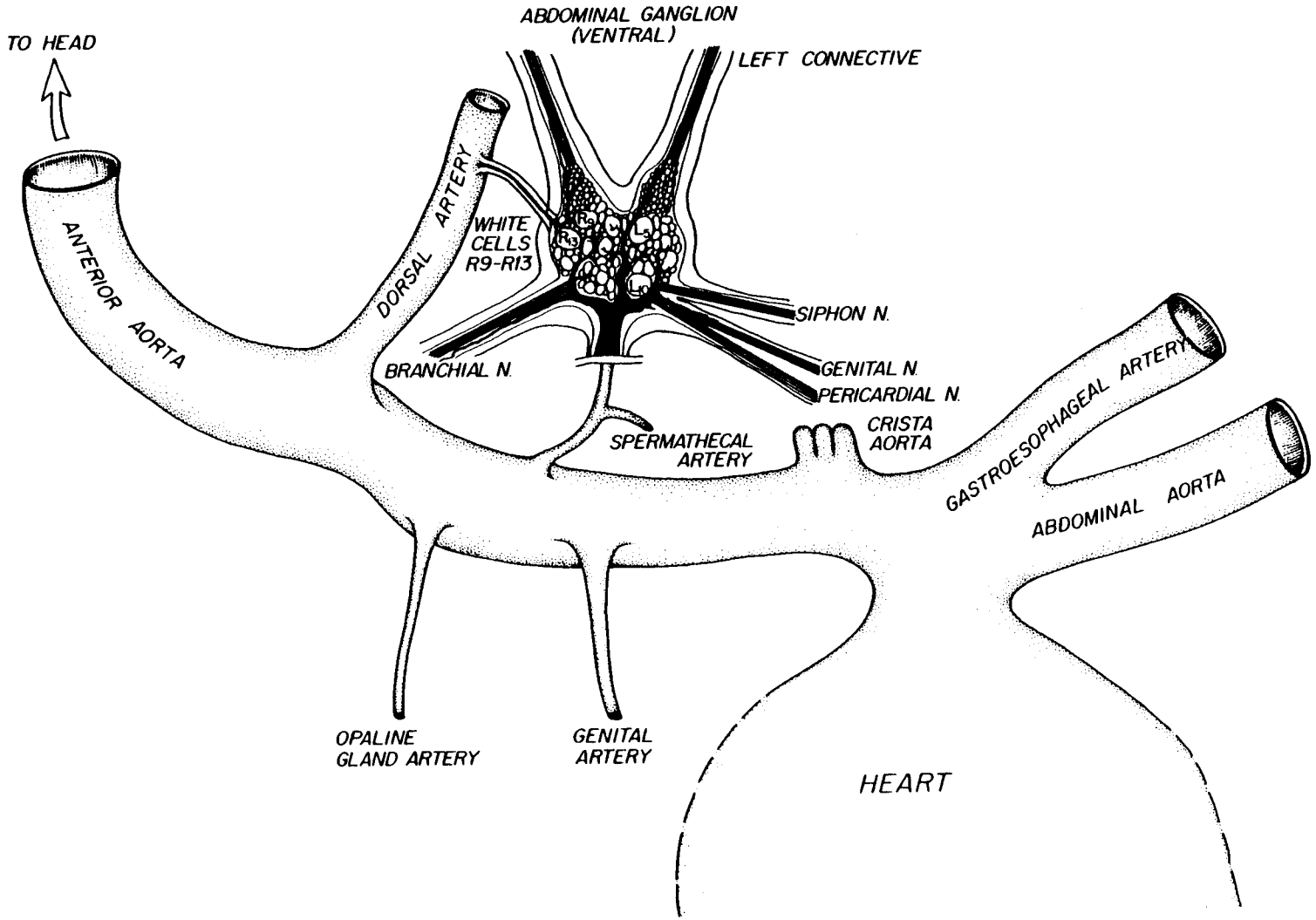
ABDOMINAL GANGLION
(VENTRAL)
TO HEAD
LEFT CONNECTIVE
ANTERIOR AORTA
DORSAL ARTERY
WHITE
CELLS
R9-R13
SIPHON N.
BRANCHIAL N.
GENITAL N.
PERICARDIAL N.
SPERMATHECAL
ARTERY
CRISTA
AORTA
GASTROESOPHAGEAL ARTERY
ABDOMINAL AORTA
OPALINE
GLAND ARTERY
GENITAL
ARTERY
HEART

Figure 8

Fig. 9. The response of the gill and pericardium to decreased ganglionic circulation. A: When a constant flow (2.5 uL/min) of oxygenated seawater into the ganglion artery was turned off, the first observable response of cardiac and respiratory organs was strong, multiple contractions of the gill (monitored by a displacement transducer). Gill contractions force blood through the heart into the major arteries, which is recorded here as a coincident increase in vascular pressure (monitored in gastroesophageal artery). B: Contractile activity of the pericardium (large pressure surges labeled with *) also increased in association with contraction of the gill. In this recording, the gill vein was cannulated near the heart so contractions of the gill did not cause surges in vascular pressure.

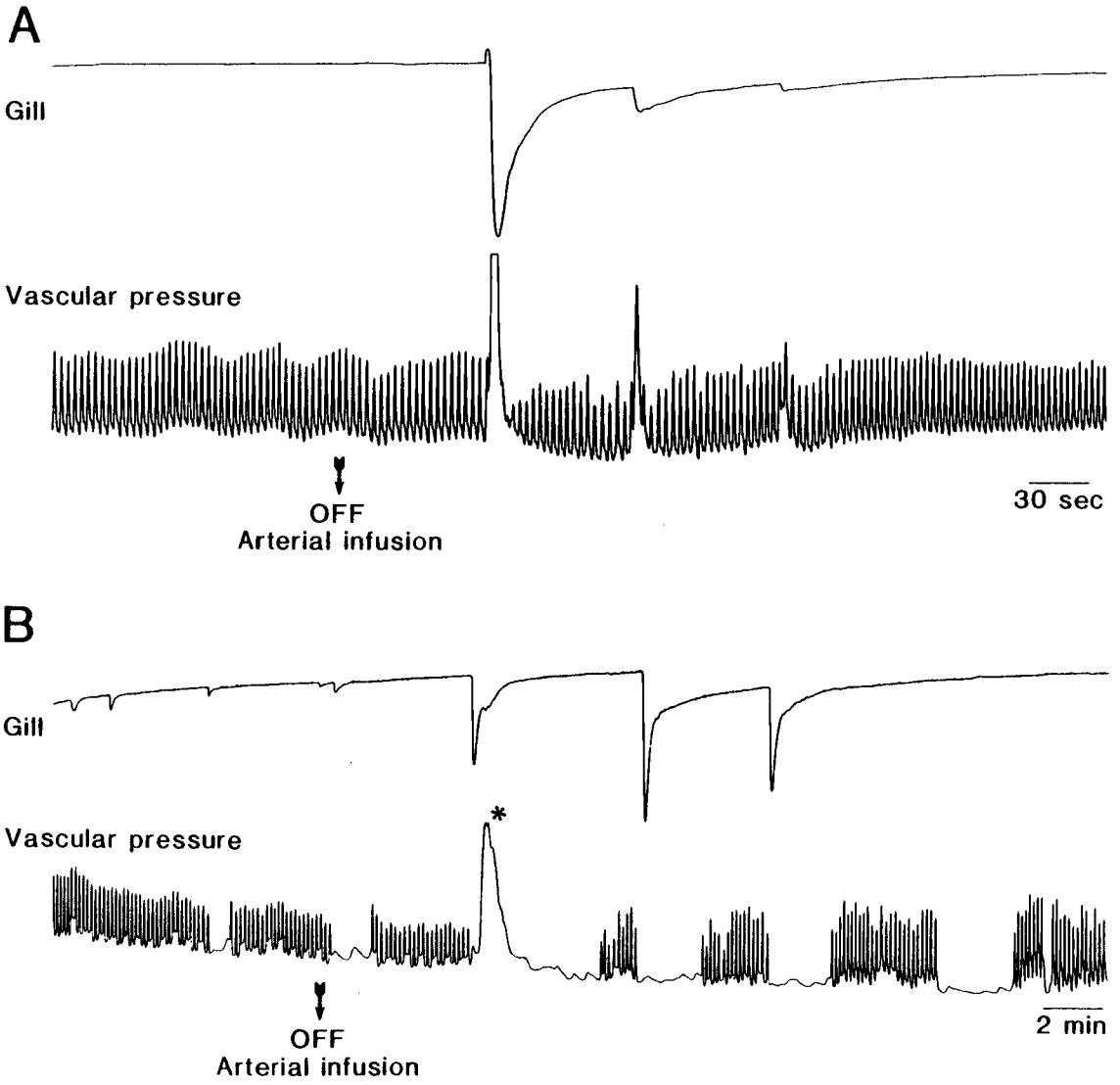
A
Gill
Vascular pressure
OFF
Arterial infusion
30 sec
B
Gill
Vascular pressure
*
OFF
Arterial infusion
2 min

Figure 9

Fig. 10. Contractile activity of the gill and pericardium was also stimulated when blood flow to the heart was decreased. A: Frequency and amplitude of heart contractions (monitored as pressure in gastroesophageal artery) decreased following reduction of blood flow into the heart. Gill contractions generally occurred within the first 1 to 2 minutes of reduced cardiac infusion. B and C: Gill contractile activity was often synchronized with delayed contractions of the pericardium which are shown in C (expanded trace from B) as large surges in arterial pressure several seconds after gill contraction.

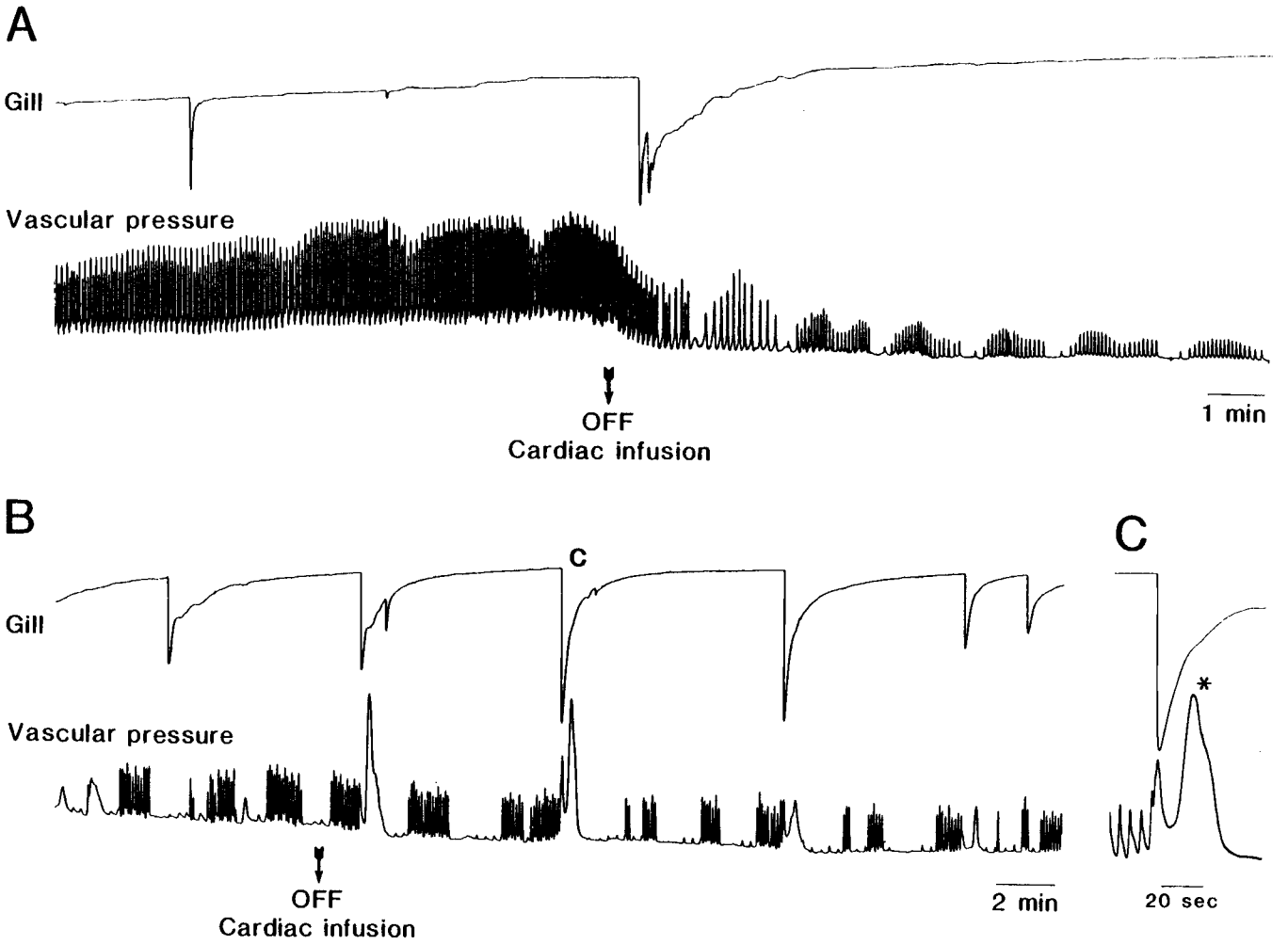
A
Gill
Vascular pressure
OFF
Cardiac infusion
1 min
B
C
Gill
Vascular pressure
OFF
Cardiac infusion
2 min
20 sec
*

Figure 10

Fig. 11. Transient inhibition of cardiac activity following a reduction in ganglionic circulation. B: Plots of heart rate and L10 spike rate for the experiment in (A) show that inhibition of the heart and L10 occurred simultaneously. (open symbols - arterial infusion on, closed symbols - arterial infusion off)

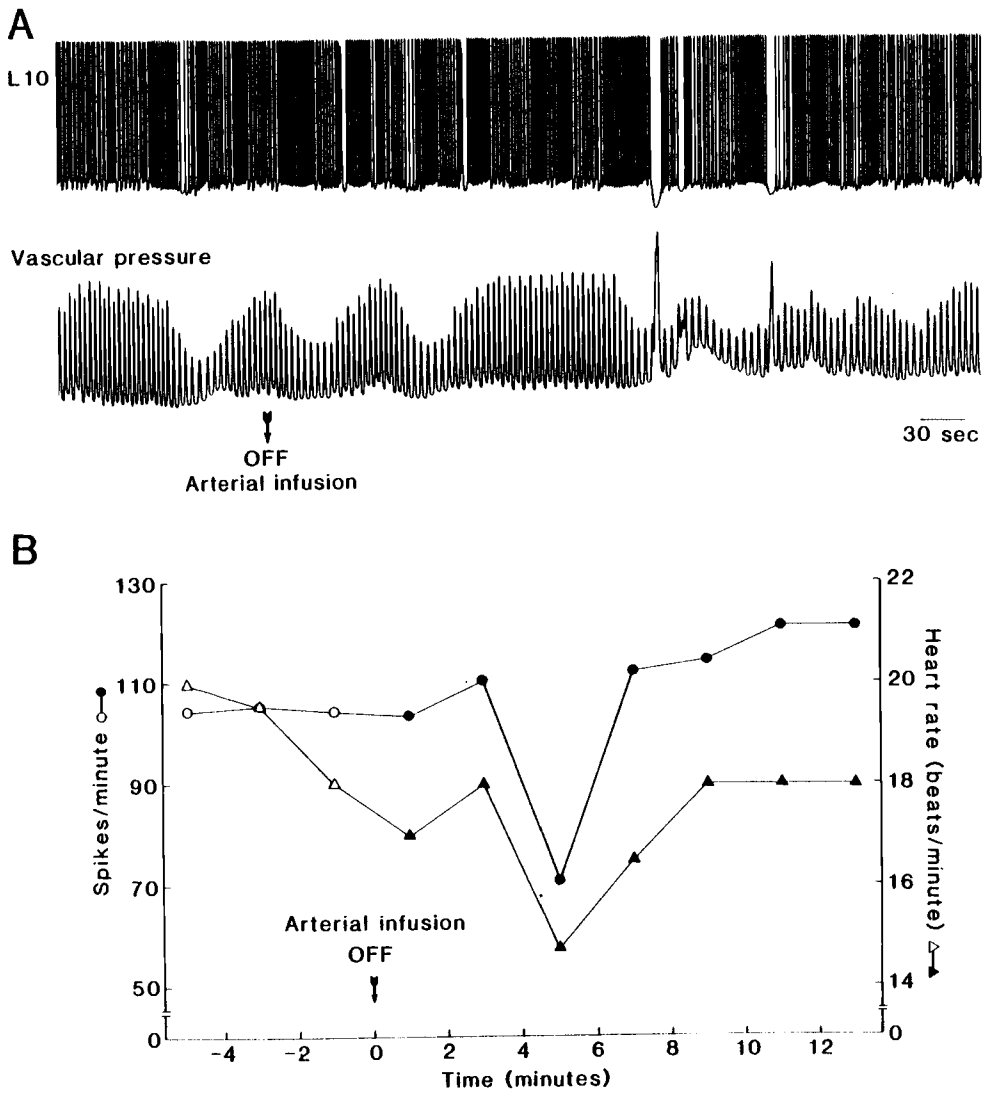
A
L10
Vascular pressure
OFF
Arterial infusion
30 sec
B
130
110
90
70
50
0
Spikes/minute
22
20
18
16
14
0
Heart rate (beats/minute)
Arterial infusion
OFF
-4
-2
0
2
4
6
8
10
12
Time (minutes)

Figure 11

Fig. 12. Long-term excitation of the heart in the absence ganglionic circulation. A: Strength of heart contractions, (monitored as the amplitude of pressure pulses in the gastroesophageal artery), gradually increased when ganglionic circulation was turned off. In two of the experiments (traces 1 and 2), the response was reversed when ganglionic circulation was turned on again. B: Changes in heart rate observed for the three experiments shown in A. When ganglionic circulation was turned off, heart rate gradually increased until circulation was turned on again.

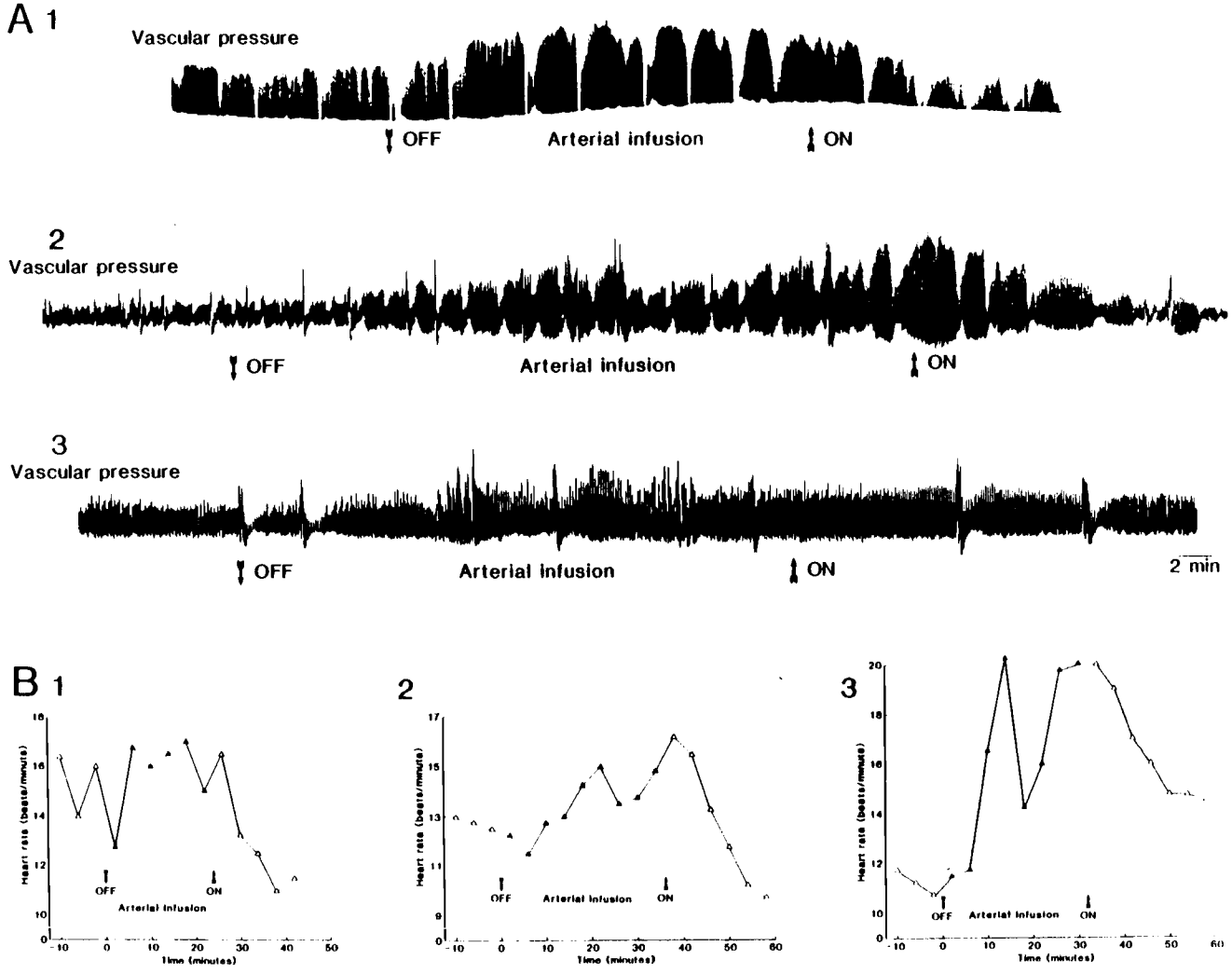
A 1
Vascular pressure
OFF
Arterial infusion
ON
2
Vascular pressure
OFF
Arterial infusion
ON
3
Vascular pressure
OFF
Arterial infusion
ON
2 min
B 1
Heart rate (beats/minute)
OFF
ON
Arterial infusion
Time (minutes)
2
Heart rate (beats/minute)
OFF
Arterial infusion
ON
Time (minutes)
3
Heart rate (beats/minute)
OFF
Arterial infusion
ON
Time (minutes)

Figure 12

Fig. 13. The effect of L10 activation on the heart's response. A: When ganglionic circulation was turned off, the frequency of L10 bursting (* indicates 3 examples) increased, as did amplitude of heart contraction (vascular pressure monitor in gastroesophageal artery). The response was largely reversed when circulation was restored 55 minutes later. B: During the response interval, hyperpolarization of L10 by direct current injection slowed the rate of increase in frequency of heart beats. L10 spike rate decreased when ganglionic circulation was turned on again while heart rate remained at an elevated level.

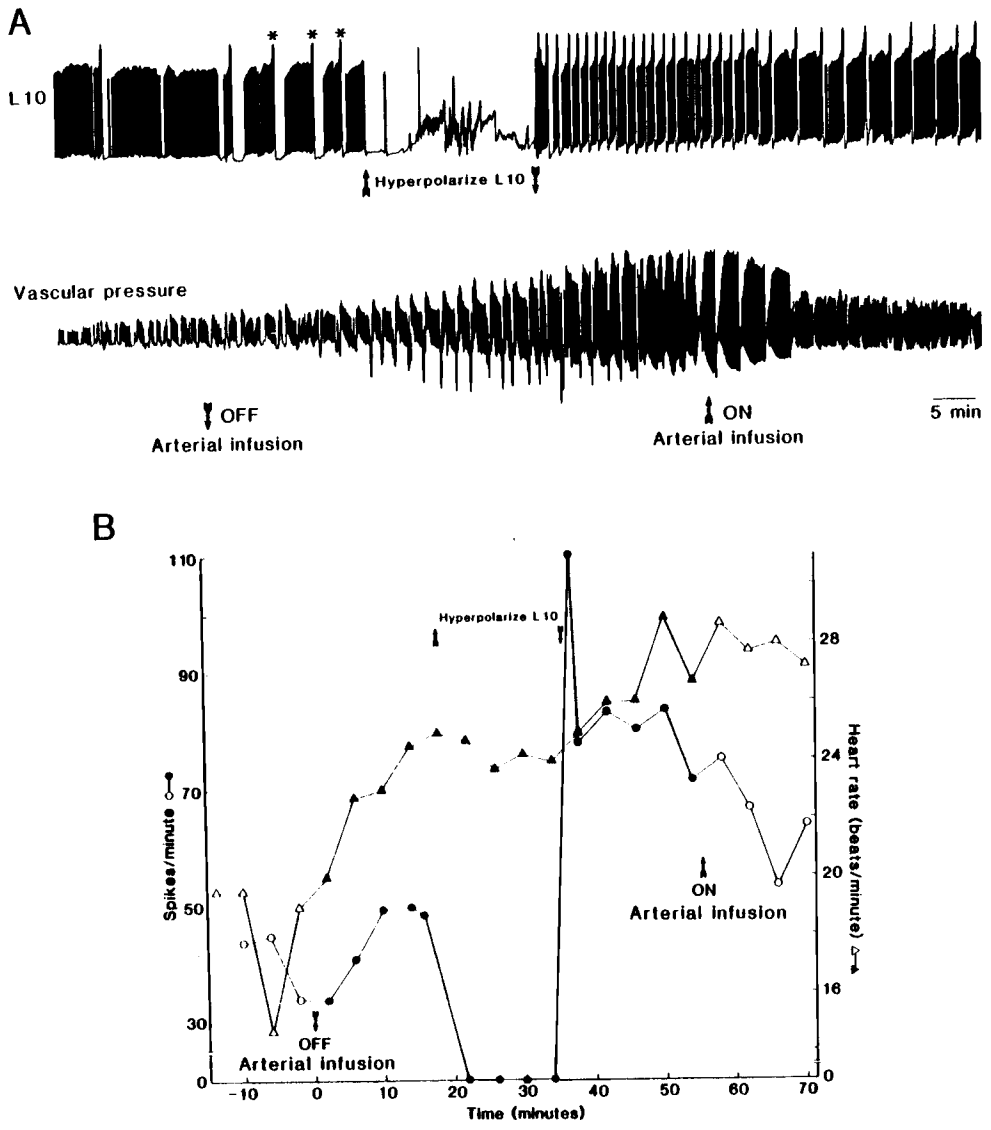
A
L10
Hyperpolarize L10
Vascular pressure
OFF
Arterial infusion
ON
Arterial infusion
5 min
B
110
90
70
50
30
0
Spikes/minute
Hyperpolarize L10
28
24
20
16
0
Heart rate (beats/minute)
ON
Arterial infusion
OFF
Arterial infusion
-10
0
10
20
30
40
50
60
70
Time (minutes)

Figure 13

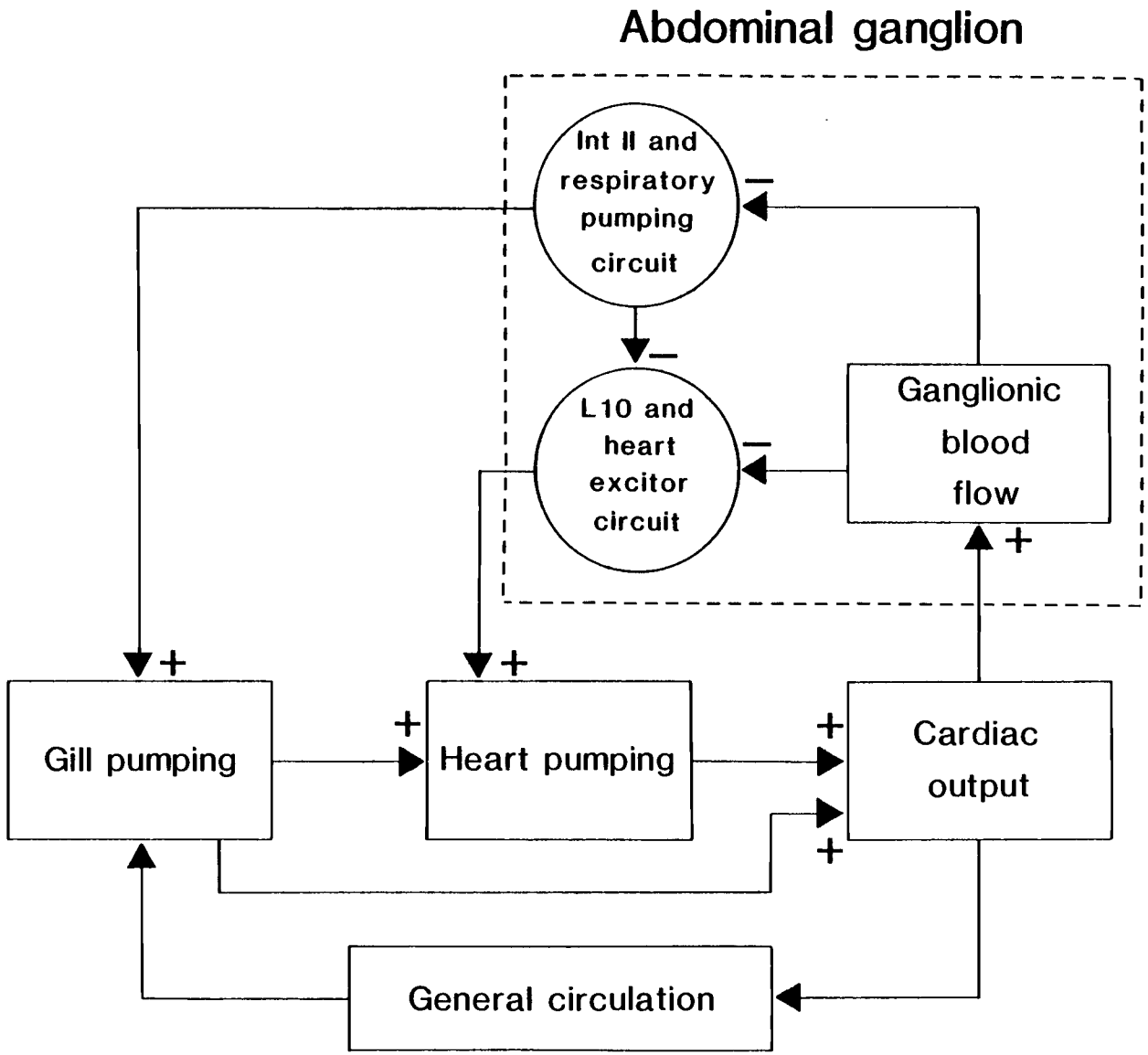

Fig. 14. Central feedback regulation of cardiac output in Aplysia

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