J. exp. Biol. 155, 21–35 (1991)
Printed in Great Britain

RED PIGMENT CONCENTRATING HORMONE IS A MODULATOR OF THE CRAYFISH SWIMMERET SYSTEM

BY CAROLYN M. SHERFF AND BRIAN MULLONEY
Zoology Department, University of California, Davis, CA 95616, USA

Accepted 31 July 1990

Summary

The crustacean red pigment concentrating hormone (RPCH) has been localized in neurons of the crayfish abdominal nerve cord and modulates the crayfish swimmeret rhythm. An antibody to RPCH labels a small set of cell bodies and axons in each abdominal ganglion. Physiological experiments in which RPCH was perfused into the ganglia of isolated nerve cords showed that RPCH modulated the swimmeret rhythm. In nerve cords that were spontaneously producing the swimmeret rhythm, RPCH lengthened both the period and the duration of bursts of action potentials, but did not alter the phase relationships between bursts in different segments. RPCH did not initiate the swimmeret rhythm in preparations that showed intermittent or no bursting activity. We believe that RPCH is released as a neurotransmitter in the lateral neuropil, where it exerts its effects on the local swimmeret circuits.

Introduction

Red pigment concentrating hormone (RPCH) was originally isolated from the sinus gland of the prawn *Pandalus borealis* (Fernlund and Josefsson, 1968) and identified as a circulating hormone that regulated the distribution of pigment granules in chromatophores. More recently, RPCH has been shown to modulate two different motor patterns of the stomatogastric system of crabs and lobsters, where it is probably released as a neurotransmitter. RPCH can initiate the crab pyloric rhythm in silent preparations and increase the frequency of this rhythm in bursting preparations (Nusbaum and Marder, 1988). RPCH can also initiate the cardiac sac rhythm in the lobster (Dickinson and Marder, 1989). The results presented in this paper suggest that RPCH has yet another neuronal role in crustacean nervous systems, that of modulating the swimmeret rhythm of the crayfish *Pacifastacus leniusculus* Dana.

The swimmerets are paired appendages on the ventral side of the crayfish abdomen that beat during swimming. There are five pairs of swimmerets in male *Pacifastacus*, one pair located in each of the first five abdominal segments, A1–A5. RPCH-like immunoreactivity is present in neurons of the crayfish abdominal

Key words: crustacean, immunohistochemistry, neuropeptide, locomotion, pattern generation, *Pacifastacus leniusculus*.

nerve cord (A. Madsen, personal communication), in regions of neuropil to which most swimmeret neurons project. Here, we report that an antibody to RPCH labels cell bodies in the abdominal ganglia, axons in peripheral nerve 1 (N1) and axons in the connectives between ganglia. This observation suggested that RPCH might have a physiological role in behaviors that use the swimmerets.

The circuitry of the swimmeret system can produce a variety of motor patterns. The most common pattern observed in isolated nerve cords is a metachronal rhythm in which the more anterior swimmeret pairs beat with a constant phase lag relative to more posterior pairs (Hughes and Wiersma, 1960*b*; Heitler, 1985). Perfusion of RPCH into the ganglia of isolated nerve cords affected the spontaneous swimmeret rhythm by lengthening the cycle period and increasing the duration of individual bursts of action potentials in power-stroke motor neurons. The effects of RPCH are distinct from those of the other known modulators of the swimmeret rhythm, proctolin and octopamine (Mulloney *et al.* 1987; L. D. Acevedo and B. Mulloney, in preparation; L. D. Acevedo, M. E. Adams and B. Mulloney, in preparation; L. D. Acevedo, W. M. Hall and B. Mulloney, in preparation).

Materials and methods

Animals

Pacifastacus leniusculus were obtained from local suppliers and maintained in freshwater aquaria.

Immunohistochemistry

Dissection and fixation

Animals were first anesthetized by chilling on ice. The claws were then removed and the animals exsanguinated by perfusion with 50 ml of low-calcium saline (Mulloney and Hall, 1990). Slits were made in the cuticle with a razor blade on either side of the anus to provide an exit route for the blood. After exsanguination, a window was cut in the dorsal carapace of the thorax, and the animal was restrained in saline. A cannula was inserted into the sternal artery (Brown and Sherwood, 1981), through which a solution of 4% paraformaldehyde plus 2% picric acid in Dulbecco's phosphate-buffered saline (PBS, Sigma) was perfused under pressure. After 30 min of perfusion, the ventral nerve cord was dissected from the animal, pinned out in a Sylgard-lined (Corning) Petri dish containing fixative, and desheathed. The nerve cord was fixed overnight at 4°C. Nerve cords were washed in 0.1 mol l^{-1} glycine in PBS to block any remaining fixative and then washed in PBS. To increase its permeability to antibodies, the tissue was dehydrated to 80% ethanol and then rehydrated to PBS. This was followed by eight 30-min rinses in wash buffer (0.3% Triton X-100 and 5% goat serum in PBS). Forty nerve cords were processed for RPCH-immunoreactivity.

Primary antibody

We used an indirect, two-stage labeling process whereby a polyclonal anti-RPCH antibody, a gift from Arthur Madsen and Robert Elde, University of Minnesota, was first used to label the peptide, then secondary antibody, an HRP-conjugated IgG (Tago), was used to visualize the localization of the primary antibody in the abdominal nerve cord.

The anti-RPCH serum was used at a dilution of 1:1000 in wash buffer and preabsorbed with 0.3 mg ml^{-1} lobster muscle powder or lobster nerve powder overnight to reduce nonspecific background staining (Mulloney and Hall, 1990). Nerve cords were treated with 0.3 % H_2O_2 in methanol to quench any endogenous peroxidases and were then incubated in the primary antibody for 20 h at 4 °C.

Secondary antibody

The secondary antibody was a goat-anti-rabbit horseradish peroxidase (HRP)-conjugated IgG $F(ab')_2$ (Tago) diluted 1:25 in wash buffer. Nerve cords were incubated in wash buffer (eight 30-min washes) to remove excess antibody. The tissue was then incubated in the secondary antibody at 4 °C for 20 h. Nerve cords were again rinsed in wash buffer and then preincubated in 0.05 % 3,3′-diaminobenzidine (DAB) in Tris buffer for 1 h. 20 μl of 0.3 % H_2O_2 per ml DAB was then added. Where HRP was present in the tissue, the hydrogen peroxide reacted with the DAB to form a brown precipitate (Tijssen, 1985). After 30 min, the reaction was terminated by replacing the DAB solution with PBS.

Controls

Two types of control experiments were performed to determine that the label observed was due to the presence of the anti-RPCH and was not an artifact, and also that the primary antibody was actually binding to RPCH or an RPCH-like substance. In the first control, the immunolabeling procedure was carried out as described above except that the anti-RPCH was omitted from the primary antibody solution. In the second, the primary antibody solution was preincubated with 10^{-6} mol l^{-1} RPCH at room temperature for 2 h prior to tissue incubation.

Clearing and sectioning

These wholemounts were dehydrated in ethanol, cleared with methyl salicylate, and viewed with a light microscope. Selected ganglia were rehydrated to 0.1 mol l^{-1} sodium cacodylate in PBS and postfixed in 2 % osmium tetroxide in 0.1 mol l^{-1} sodium cacodylate in the refrigerator for 3 h at 4 °C. They were then dehydrated to 100 % acetone and embedded in Spurr's plastic. 25 μm and 50 μm frontal sections and cross-sections were cut with glass knives on a Sorval JB-4 microtome.

Physiology

Crayfish were exsanguinated with physiological saline (Van Harreveld, 1936;

Mulloney and Hall, 1990). The ventral abdominal nerve cords with attached nerves were removed, keeping the ventral arteries intact. Isolated nerve cord preparations were used in 23 experiments. The isolated cords were pinned out in Sylgard-lined Petri dishes, ventral side up. Impulses in the posterior branches of the swimmeret nerves (N1s) of abdominal ganglia A2–A5 were monitored with extracellular stainless-steel pin electrodes and were simultaneously displayed on an oscilloscope screen and recorded on tape with a Neurocorder DR-886 (Neurodata Instruments Corp.). The recorded data could be played back later onto paper using a Gould ES 1000 electrostatic recorder.

The nerve cords were maintained at 18–20°C by superfusion with cold saline. Saline and RPCH (Peninsula) solutions were perfused into the abdominal ganglia through a cannula inserted into the ventral artery, anterior to the abdominal ganglia. This artery sends arterioles into each abdominal ganglion, allowing solutions to be perfused into the ganglia without the diffusional barriers present with bath application (Brown and Sherwood, 1981).

A 10^{-5} mol l^{-1} solution of RPCH was made by dissolving RPCH in saline containing 0.05 % dimethyl sulfoxide (DMSO). Dissolving RPCH in pure DMSO and then diluting to a 0.05 % DMSO, 10^{-5} mol l^{-1} RPCH stock solution yielded similar physiological effects; there was no difference in the ability of the peptide to dissolve under the two conditions. Perfusion with 0.05 % DMSO in crayfish saline did not alter the motor pattern.

Semi-intact preparations were used in three experiments to observe the movements of the swimmerets in response to various doses of RPCH. In these preparations, the swimmerets and their musculature were kept intact by removing the entire ventral abdominal cuticle with the nerve cord. The cord was perfused in the same manner as the isolated preparations. Bursts of action potentials in the N1s were recorded with suction electrodes.

Analysis of the extracellular recordings from both isolated and semi-intact experiments was done by digitizing integrated records of the bursts of action potentials recorded from a power-stroke branch of each N1 (Mulloney *et al.* 1987). Cycle period, burst duration and cycle phase relative to power-stroke bursts in the fifth abdominal segment were analyzed using the PD computer programs (Mulloney and Hall, 1987). Period was defined as the time interval between the onset of two consecutive bursts in one segment (Fig. 1). Duration was the length of the burst in seconds. Latency was the time delay between the onset of a burst in one segment and the onset of a power-stroke burst in ganglion five. Phase was defined as the ratio of latency to period.

Results

RPCH-like immunohistochemistry

RPCH-like immunoreactivity occurred in cell bodies and axons of the abdominal nervous system of *Pacifastacus*. The pattern of staining was repeated in each of the ganglia, and was the same in males and females. The labeling was abolished

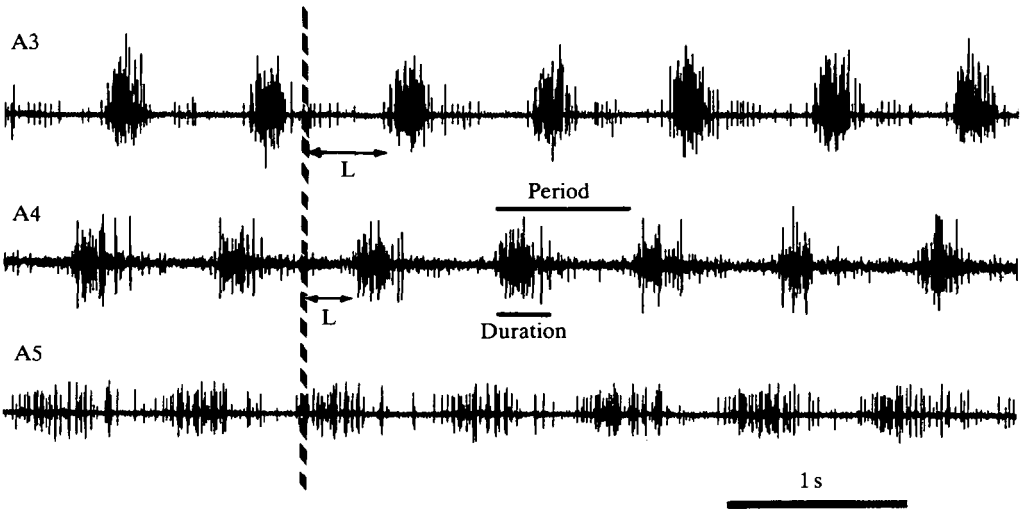

Fig. 1. Extracellular recordings from posterior branches of the swimmeret nerves (N1s) of abdominal ganglia A3–A5. Cycle period, burst duration and burst latency (L) are labeled. Latency is the delay of the onset of a burst in N1 of one segment relative to the onset of a burst in N1 of A5 (as indicated by dashed line).

both when the primary antibody was omitted and when the anti-RPCH was preabsorbed with authentic RPCH prior to tissue incubation (Fig. 2). Five clusters of cell bodies with RPCH-like immunoreactivity occurred in the ventral cell body layer of each ganglion (Fig. 3A). A cluster of three cells, ranging from 30 to 50 μm in diameter, was located anteriorly on the midline. A bilaterally symmetrical pair of clusters containing between two and four small cells each (10–30 μm in diameter) occurred midway between the midline and the lateral edges of the ganglion, anterior to N1. Finally, a bilateral pair of large cells, between 50 and 60 μm in diameter, occurred at the lateral margins of the ganglion just anterior to the base of N1.

The midline cells labeled clearly in most preparations, and the lateral cell body pair labeled in the majority of preparations. Compared to axons, the cell bodies were difficult to label, especially the cluster located between the midline cluster and the lateral pair. Since they were frequently unlabeled, these cells may have contained lower concentrations of the RPCH-like peptide. When these cell bodies did show RPCH-like immunoreactivity, the staining was faint. A composite drawing (Fig. 3E) shows the positions of all the immunoreactive cell bodies.

Three bilateral groups of immunoreactive axons ran through the connectives of the nerve cord and sent processes into each ganglion. One group formed a bilateral pair of intertwined cables, each with five axons, that ran laterally along the hemiconnective (Fig. 3E). These axons ascended at the anterior of each ganglion to run dorsally through the ganglion's lateral dorsal tract (Skinner, 1985*a*) and then descended again, posterior to each ganglion, to run along the lateral margins of the connective. Two of these axons can be seen in each of the lateral dorsal tracts of an

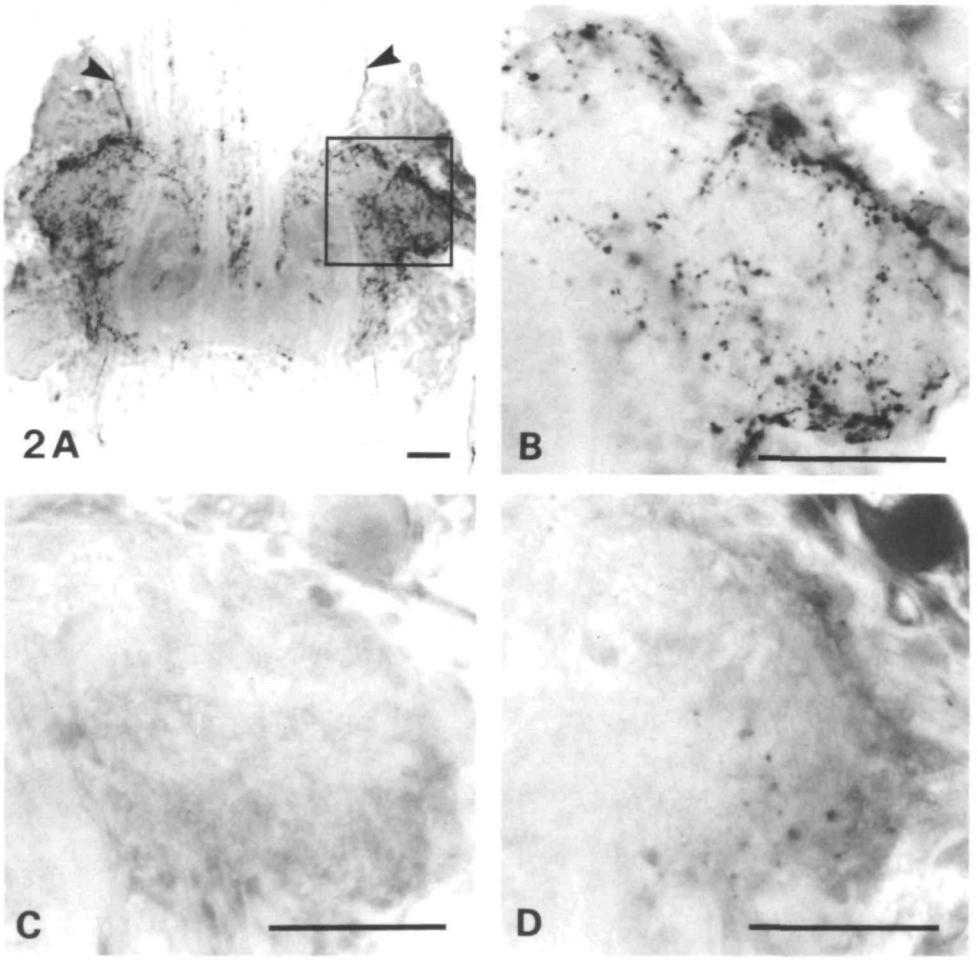

Fig. 2. RPCH-like immunoreactivity compared to controls. (A) A 50 μm frontal section of a third abdominal ganglion at the level of the lateral neuropil. The ganglion was labeled with anti-RPCH serum and visualized with an HRP-conjugated secondary antibody. Arrowheads indicate axons of the ventral lateral tract. (B) Higher magnification of the right lateral neuropil, the boxed area in A. (C) A similar frontal section cut through the lateral neuropil of a ganglion whose primary incubation omitted the anti-RPCH. (D) A similar frontal section cut through the lateral neuropil of a ganglion labeled with anti-RPCH serum that had been preincubated with 10^{-6} mol l^{-1} RPCH. No labeling was visible in either control. Scale bars, 100 μm.

A3 in Fig. 3D. A ganglion cross-section, showing the group of five, can be seen in Fig. 4. A second group of three axons ran through the center of each hemiconnective, diverging at the anterior of the ganglion, one axon continuing through the ventral lateral tract (Fig. 3C,E) and two through the dorsal intermediate tract (Skinner, 1985*a*) of each ganglion (Fig. 3D,E). The third group was a bilateral pair of axons that traveled medially and ventrally in the connectives and in the median

ventral tracts (Skinner, 1985*a*) through the ganglia (Fig. 3B,E). All of these axons branched extensively in each ganglion and appeared to send branches into the lateral neuropil (Skinner, 1985*b*). Two axons with RPCH-like immunoreactivity projected into each of the swimmeret nerves (N1s).

Of the five types of neuropil that occur in these ganglia, RPCH-immunoreactive processes were seen only in the lateral neuropil (Figs 2A,B, 3E) and along the outer anterior surface of the horseshoe neuropil (Fig. 3A,E). There was no staining in the core of the horseshoe neuropil or in either of the midline neuropils.

Physiology

The regions of lateral neuropil in the crayfish abdominal ganglia contain axon branches of many of the motor neurons and interneurons of the swimmeret system. Paul and Mulloney (1985*a*,*b*) recorded from swimmeret interneurons and then filled them with Lucifer Yellow. All these interneurons branched into the lateral neuropil. Intersegmental interneurons that are involved in the coordination of the swimmeret rhythm between ganglia also branch into the lateral neuropil (Paul and Mulloney, 1986). Backfills of N1s indicate that the motor neurons that project to the swimmerets have branches in the lateral neuropil and that sensory projections from the swimmerets also branch in the lateral neuropil (Leise and Mulloney, 1984; W. M. Hall and B. Mulloney, unpublished results).

Since RPCH-like immunoreactivity occurred in regions of the abdominal nerve cord known to contain the neural circuitry that generates the swimmeret motor pattern (Leise and Mulloney, 1984; Mulloney *et al.* 1990), we tested the ability of authentic RPCH to affect the crayfish swimmeret rhythm.

The swimmeret motor pattern associated with the swimming behavior consists of the metachronal beating of the swimmerets of the second to the fifth abdominal segments. The swimmerets beat with alternating power strokes and return strokes produced by alternating bursts of action potentials in the power-stroke and return-stroke motor neurons. Each hemiganglion contains the neural circuitry for the generation of this rhythm in its corresponding swimmeret. The power-stroke and return-stroke motor neurons project from each hemiganglion to the ipsilateral swimmeret through the first nerve (N1). In any one segment, the left and right power-stroke bursts are simultaneous, and they alternate with simultaneous left and right return-stroke bursts, such that the pair of swimmerets in each segment beat in phase with each other. There is a constant phase lag between the beating of the swimmerets in different segments; the more posterior swimmerets lead the more anterior swimmerets.

Nerve cords isolated from the crayfish often produce this swimmeret rhythm (Hughes and Wiersma, 1960*a*,*b*). In the isolated nerve cord of *P. leniusculus*, the period of spontaneous swimmeret rhythm ranges between 0.3 s and 1.0 s, with an average of 0.66±0.10 s. The burst of action potentials in each group of power-stroke motor neurons lasts for about half the cycle, as can be seen for A4 in Table 1 (control). The pattern is similar in each of the abdominal ganglia 2–5. During

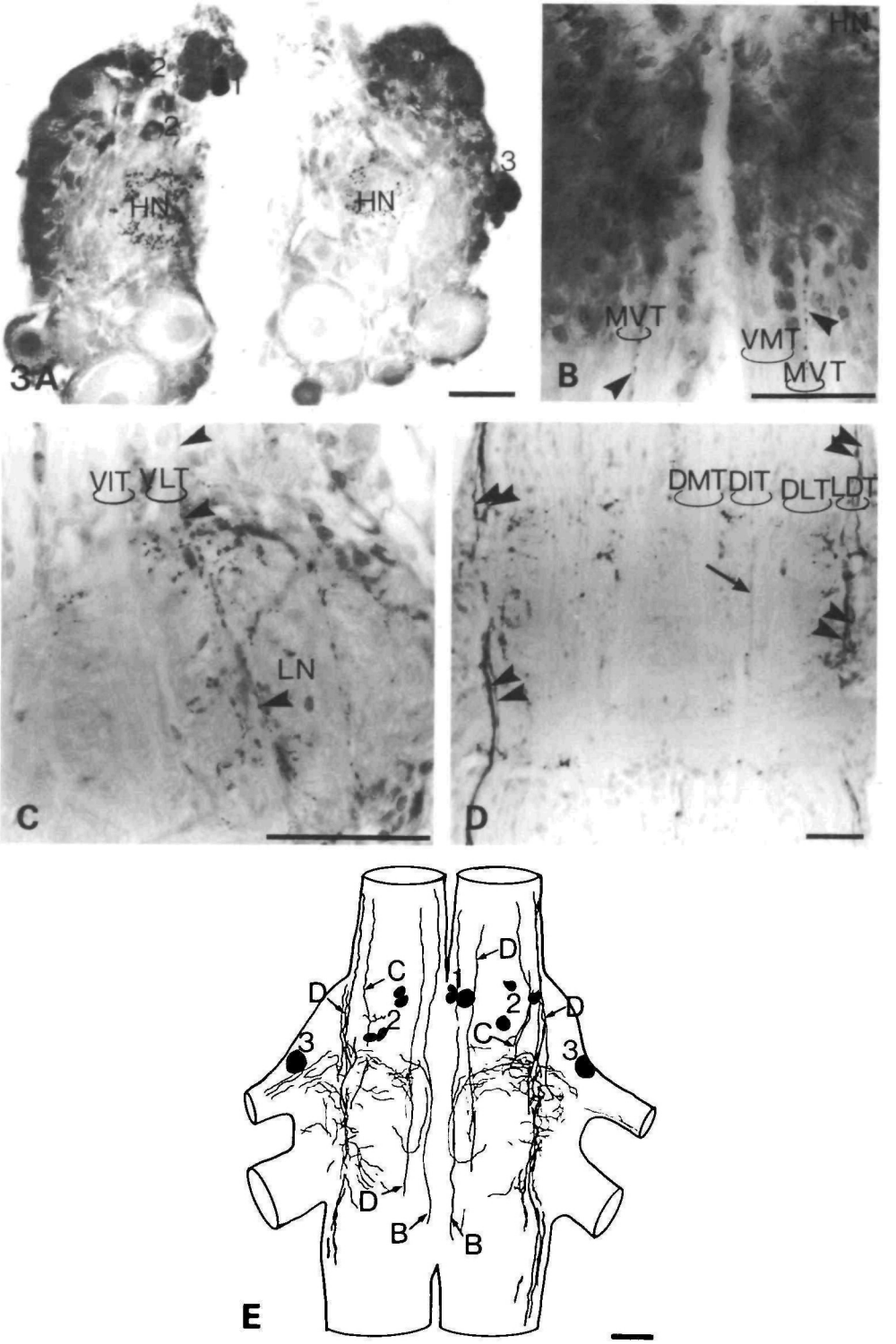
1
2
2
HN
3A
HN
3
HN
MVT
B
VMT
MVT
VIT VLT
LN
C
DMT DIT DLT LDT
D
C
D
2
3
D
B
1
D
2
C
D
3
B
E

Fig. 3. RPCH-like immunoreactivity in frontal sections of abdominal ganglia. The planes of the sections range from ventral in A to dorsal in D. (A) A 25 μm section of A5 through the ventral cell body layer showing (1) two of the three midline cell bodies, (2) two of the cells located midway between the midline and the lateral edges of the ganglion and (3) one of the lateral cell bodies. HN, horseshoe neuropil. (B) Dorsal to A, a 50 μm section through A2. Arrowheads indicate the pair of RPCH-immunoreactive axons in the medial ventral tract (MVT). VMT, ventral medial tract. (C) Dorsal to B, a 50 μm section through A3. Arrowheads indicate one continuous axon in the ventral lateral tract (VLT) that can be seen branching into the lateral neuropil (LN). VIT, ventral intermediate tract. (D) A 50 μm section, very dorsal in A3. Arrowheads indicate two axons on both the right and left sides of the ganglion that run in the lateral dorsal tracts (LDT). These axons enter the ganglion anteriorly, weave out of the plane of the section, and reappear in the posterior portion of the section. The arrow indicates one of the immunoreactive axons in the dorsal intermediate tract (DIT). DMT, dorsal medial tract; DLT, dorsal lateral tract. (E) *Camera lucida* drawing of A3 and the typical staining pattern observed in wholemount. Cell bodies are labeled as in A. Axons shown in B–D are labeled. Scale bar, 100 μm.

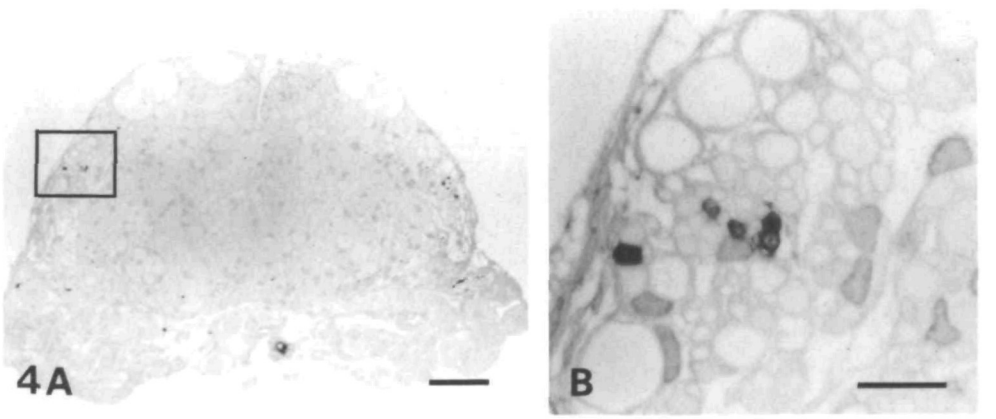

Fig. 4. RPCH-like immunoreactive lateral axons. (A) A 10 μm cross-section through the anterior end of A3. Scale bar, 100 μm. (B) A magnified view of the boxed area in A, showing one of the two groups of five lateral axons. Scale bar, 20 μm.

generation of the swimmeret rhythm, the onset of bursts in each segment is delayed by roughly one-quarter of a cycle relative to the next posterior segment (Table 1, control and Fig. 5, control). Administering RPCH through the ventral artery caused a lengthening of both cycle period and burst duration in the power strokes of ganglia 2–5 (Fig. 6). In contrast, there was no significant change in phase; although the latency of the onset of bursts in anterior ganglia relative to posterior ganglia was lengthened, the ratio of latency to period remained the same. Responses to perfused RPCH were calculated to occur within 20 s from the time the solution arrived at the first abdominal ganglion. Partial recovery from

Table 1. *The mean period, burst duration and phase from A4 in isolated nerve cord preparations during perfusion of physiological saline (control) and 10^{-6} mol l^{-1} RPCH*

	Control	10^{-6} mol l^{-1} RPCH	% Change
Period (s)	0.658±0.130	0.951±0.220	+45
Duration (s)	0.346±0.089	0.500±0.167	+45
Phase	0.273±0.142	0.275±0.070	+0.7

Column at far right indicates (10^{-6} mol l^{-1} RPCH−control)/(control).
Values are mean±S.D., N=13.

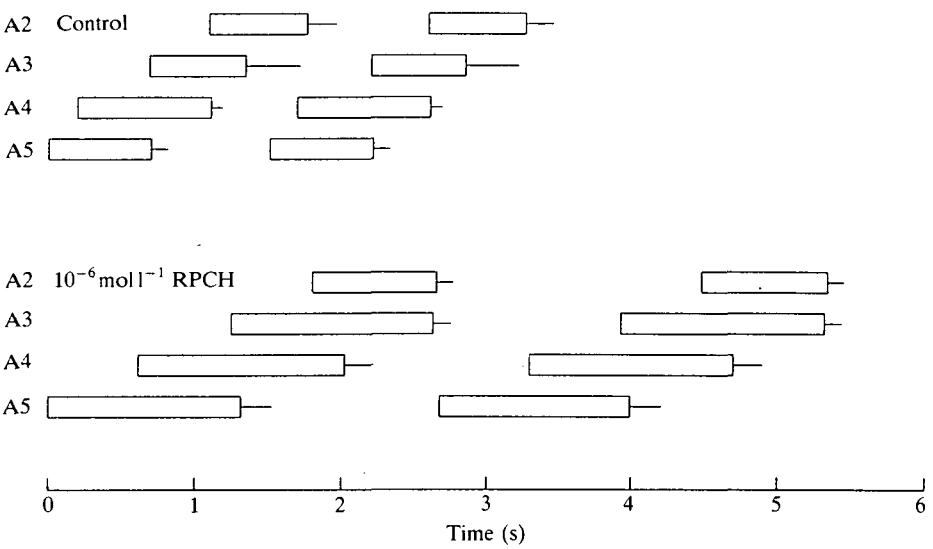

Fig. 5. Box plots of two cycles of power-stroke activity in A2–A5 during perfusion with saline (control) and perfusion with 10^{-6} mol l^{-1} RPCH in one experiment. Each box represents an average burst of impulses calculated from the means of 40 consecutive bursts. Each box begins at the mean latency on the left and extends for the mean burst duration. Bars at the right of boxes indicate +S.D.

these effects was observed within 5 s to 1 min of perfusion of saline, and complete recovery was usually observed within 5–15 min.

The effects of RPCH on the rhythm were dose-dependent. The threshold concentration was about 10^{-7} mol l^{-1} RPCH, and a significant response of both period and duration was observed at 10^{-6} mol l^{-1} RPCH (Fig. 7). On average, both period and duration were lengthened by 45 % in the presence of 10^{-6} mol l^{-1} RPCH (Table 1). The changes from control values of period and duration were both significant at the 95 % confidence level at 10^{-6} mol l^{-1} RPCH. As the concentration of RPCH was increased to 10^{-6} mol l^{-1} or higher, the rhythm

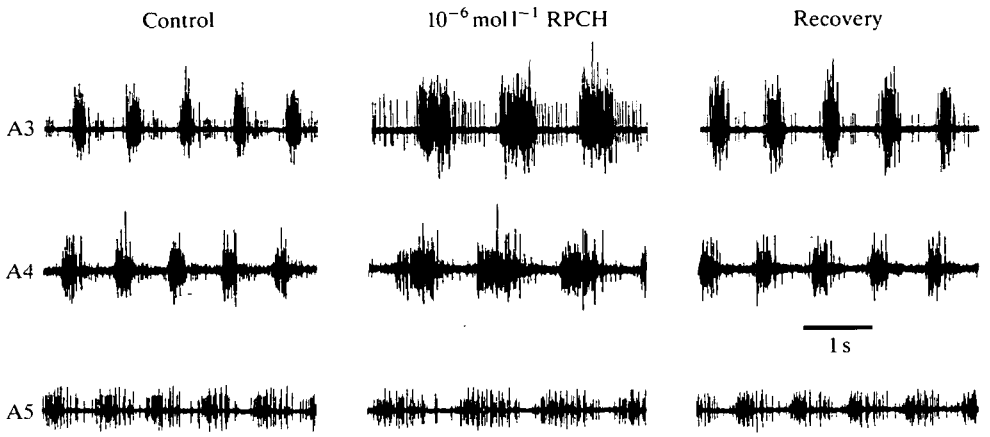

Fig. 6. Extracellular recordings of bursts in posterior branches of N1 for ganglia A3–A5 during perfusion of saline or 10^{-6} mol l^{-1} RPCH through the ventral artery. The RPCH effects could be reversed by subsequent perfusion with saline.

became less consistent. As the period and burst duration continued to lengthen, bursts often failed to occur in one or more segments. When 10^{-5} mol l^{-1} RPCH was perfused, prolonged bursts occurred in each segment. With increasing concentrations of RPCH, the effects of RPCH on the mean period and mean duration became more variable between preparations. This increase, which became increasingly apparent at doses above threshold, was probably due to the individual differences between animals.

To determine whether RPCH could affect the swimmeret system through afferent pathways from the swimmerets, we perfused RPCH through the ventral artery of preparations in which the swimmerets and their musculature were intact. The effects of RPCH on these preparations were qualitatively the same as those on isolated nerve cords. Quantitative differences in RPCH response between isolated and semi-intact preparations occurred because the period of the spontaneous rhythm in semi-intact preparations was often less than that in isolated cords. RPCH tended to have a greater effect on isolated preparations with a slow spontaneous rhythm than on isolated cords with a faster rhythm. Thus, the faster semi-intact preparations showed, on average, a weaker response to RPCH than did the isolated nerve cord preparations.

Discussion

In many systems, one complement of neurons is used to perform different tasks at different times. In the chick, one group of motor neurons can be coordinated to produce either the hatching or the walking motor patterns (Bekoff *et al.* 1975, 1987). In the stomatogastric system of crustaceans, spontaneous motor patterns, also generated by the same set of neurons, can be switched from one to another in isolated preparations by superfusion with neuropeptides. The peptide proctolin

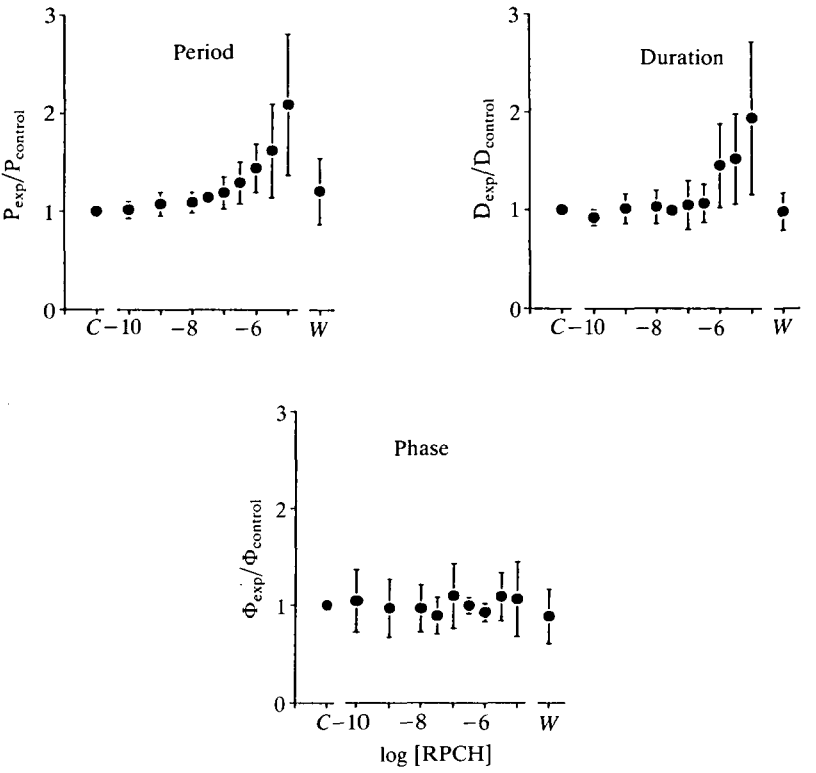

Fig. 7. Dose–response curves for period, duration and phase of the swimmeret rhythm in A4 power-stroke motor neurons as a function of the RPCH concentration (measured in $mol\,l^{-1}$). Values on the ordinate represent experimental values/the original control value. The first control is *C*; the value obtained after RPCH was washed out is *W*. Error bars represent standard deviation, $N=13$.

can initiate the pyloric rhythm (Hooper and Marder, 1987), while RPCH can initiate both the pyloric rhythm (Nusbaum and Marder, 1988) and the cardiac sac rhythm (Dickinson and Marder, 1989). In the stomatogastric system, neuromodulators not only change the state of a preparation from silent to active but also modulate the intensity and frequency of the pattern.

Our results suggest that RPCH, or an RPCH-like substance, modulates the spontaneous motor pattern of the crayfish swimmeret system. The effects of RPCH differ from those of the other known modulators of this system. Proctolin and octopamine can initiate the swimmeret rhythm in silent preparations and terminate the rhythm in bursting preparations (Mulloney *et al.* 1987). Results from our laboratory (Mulloney *et al.* 1987; L. D. Acevedo, M. E. Adams and B. Mulloney, in preparation; L. D. Acevedo, W. M. Hall and B. Mulloney, in preparation; L. D. Acevedo and B. Mulloney, in preparation) indicate that some of the command neurons described by Wiersma and Ikeda (1964) use proctolin and octopamine as neurotransmitters. Stimulation of the excitatory command

neurons induces the swimmeret rhythm in silent preparations, an excitation that is mimicked by perfusion with proctolin. Stimulation of inhibitory command neurons terminates activity in bursting preparations, an inhibition that is mimicked with perfusion of octopamine. In contrast, perfusion of RPCH into the abdominal ganglia merely slows the rhythm. The differences in responses to these different modulators suggest that these substances have different sites of action within the pattern-generating network.

The lengthening of the period of the rhythm and the duration of each burst translates into a slowing of the swimmeret rhythm. For perfused concentrations of RPCH of up to about 10^{-6} mol l^{-1}, the phasing of the rhythm between segments is unaltered. Period and duration increase by the same percentage such that bursts of action potentials occupy the same fraction of the period as they did in the absence of RPCH (the duty cycle remains constant). These effects differ from those of octopamine in that RPCH does not inhibit all activity of the swimmeret motor neurons, as octopamine does (L. D. Acevedo and B. Mulloney, in preparation). With high doses of RPCH ($\geq 10^{-6}$ mol l^{-1}), the bursts of action potentials in the N1s still occur; however, they occur infrequently and are extremely protracted (up to 1.3 s at 10^{-5} mol l^{-1} RPCH). Furthermore, these bursts may occur in only one segment, indicating a failure in the intersegmental coordinating mechanism. The disruption of the rhythm at high concentrations of RPCH may be due to the inability of the circuit to operate at frequencies below about 0.5–1 Hz.

The pattern of RPCH-immunoreactive cell bodies and axons differed from the distributions of other neurotransmitters that have been mapped with antibodies in these ganglia: proctolin (Siwicki and Bishop, 1986; Bishop *et al.* 1982; L. D. Acevedo, M. E. Adams and B. Mulloney, in preparation), γ-aminobutyric acid (Mulloney and Hall, 1990), histamine (B. Mulloney and W. M. Hall, in preparation), FMRFamide (C. M. Sherff, unpublished results) and serotonin (Beltz *et al.* 1984). RPCH-immunoreactive cell bodies are found only in the anterior half of each ganglion, and neuropil labeling is restricted to the lateral neuropil and to the very anterior of the horseshoe neuropil. Proctolin-like immunoreactivity is found in both the lateral and the horseshoe neuropils and in a different set of cell bodies (L. D. Acevedo, M. E. Adams and B. Mulloney, in preparation). The difference in neuropil staining suggests different sites of action for these two modulators. Since RPCH can neither initiate nor terminate the swimmeret rhythm, it is not involved with the swimmeret system at the command level.

Anti-RPCH labeling in the abdominal nerve cord is concentrated in the lateral neuropil. The lateral neuropil is distinguished both physiologically, as the location of the swimmeret rhythm-generating circuitry (Paul and Mulloney, 1985*a*,*b*; Heitler and Pearson, 1980), and anatomically, as a region of extensive axon branching and synapsing (Skinner, 1985*b*). Though we have not yet carried out any electron microscopy to determine whether these processes actually form synapses in this region, under the light microscope labeling in the lateral neuropil is dense and appears as a population of thin, branching processes. The immunohistochemi-

cal localization, the physiology, and the dose–response characteristics of RPCH indicate that an RPCH-like substance is a neuromodulator in this system. Although we do not know where the RPCH-like substance is released or where the receptors to this peptide are located, the RPCH-like immunoreactivity results are consistent with a transmitter role for an RPCH-like peptide.

In addition to changing the output of a neural circuit from one motor pattern to another, as in the cases of chick locomotion, the various crustacean stomatogastric rhythms and crayfish swimming, neuropeptides can also vary parameters such as frequency and burst duration within a given motor pattern. This has been seen in the case of proctolin in the stomatogastric system (Hooper and Marder, 1987; Dickinson and Marder, 1989) and in the crayfish swimmeret system. Red pigment concentrating hormone appears to be one of these peptides, altering the burst period and duration within the swimmeret rhythm.

We thank Arthur Madsen and Robert Elde for the gift of the RPCH antibody. This research was supported by NSF grant BNS-87 19397 and NIH grant NS 26472 to BM. We thank Martin Wilson and Karen Sigvardt for reading the manuscript critically and Larisa Acevedo and Wendy Hall for technical advice and assistance.

References

BEKOFF, A., NUSBAUM, M. P., SABICHI, A. L. AND CLIFFORD, M. (1987). Neural control of limb coordination. I. Comparison of hatching and walking motor output patterns in normal and deafferented chicks. *J. Neurosci.* **7**, 2320–2330.

BEKOFF, A., STEIN, P. S. G. AND HAMBURGER, V. (1975). Coordinated motor output in the hindlimb of the 7-day chick embryo. *Proc. natn. Acad. Sci. U.S.A.* **72**, 1245–1248.

BELTZ, B. S., EISEN, J. J., FLAMM, R., HARRIS-WARRICK, R. M., HOOPER, S. AND MARDER, E. (1984). Serotonergic innervation and modulation of the stomatogastric ganglion of three decapod crustaceans. *J. exp. Biol.* **109**, 35–54.

BISHOP, C. A., O'SHEA, M. AND WINE, J. J. (1982). Immunological location of the peptide proctolin and serotonin in identifiable neurons of cockroaches and crayfish. *Neurosci. Abstr.* **8**, 188.12.

BROWN, S. K. AND SHERWOOD, D. N. (1981). Vascularization of the crayfish abdominal nerve cord. *J. comp. Physiol.* **143**, 93–101.

DICKINSON, P. S. AND MARDER, E. (1989). Peptidergic modulation of a multioscillator system in the lobster. I. Activation of the cardiac sac motor pattern by the neuropeptides proctolin and red pigment-concentrating hormone. *J. Neurophysiol.* **61**, 833–844.

FERNLUND, P. AND JOSEFSSON, L. (1968). Chromactivating hormones of *Pandalus borealis*. Isolation and purification of the 'red-pigment-concentrating hormone'. *Biochim. biophys. Acta* **158**, 262–273.

HEITLER, W. J. (1985). Motor programme switching in the crayfish swimmeret system. *J. exp. Biol.* **114**, 521–549.

HEITLER, W. J. AND PEARSON, K. G. (1980). Non-spiking interactions and local interneurones in the central pattern generator of the crayfish swimmeret system. *Brain Res.* **187**, 206–211.

HOOPER, S. L. AND MARDER, E. (1987). Modulation of the lobster pyloric rhythm by the peptide proctolin. *J. Neurosci.* **7**, 2097–2112.

HUGHES, G. M. AND WIERSMA, C. A. G. (1960*a*). Neuronal pathways and synaptic connections in the abdominal cord of the crayfish. *J. exp. Biol.* **37**, 291–307.

HUGHES, G. M. AND WIERSMA, C. A. G. (1960*b*). The co-ordination of swimmeret movements in the crayfish, *Procambarus clarkii*. *J. exp. Biol.* **37**, 657–670.

LEISE, E. M. AND MULLONEY, B. (1984). Identifiable neuropilar areas in crayfish abdominal ganglia integrate different motor activities. *Am. Zool.* **24**, 432.

MULLONEY, B., ACEVEDO, L. D. AND BRADBURY, A. G. (1987). Modulation of the crayfish swimmeret rhythm by octopamine and the neuropeptide proctolin. *J. Neurophysiol.* **58**, 584–597.

MULLONEY, B., ACEVEDO, L. D., CHRACHRI, A., HALL, W. M. AND SHERFF, C. M. (1990). A confederation of neural circuits: Control of swimmeret movements by a modular system of pattern generators. In *Frontiers in Crustacean Neurobiology* (ed. K. Wiese, W. D. Krenz, J. Tautz, H. Reichert and B. Mulloney), pp. 439–447. Basel: Birkhauser Verlag.

MULLONEY, B. AND HALL, W. M. (1987). The PD programs: a method for the quantitative description of motor patterns. *J. Neurosci. Meth.* **19**, 47–59.

MULLONEY, B. AND HALL, W. M. (1990). GABAergic neurons in the crayfish nervous system: An immunocytochemical census of the segmental ganglia and stomatogastric system. *J. comp. Neurol.* **291**, 383–394.

NUSBAUM, M. P. AND MARDER, E. (1988). A neuronal role for a crustacean red pigment concentrating hormone-like peptide: neuromodulation of the pyloric rhythm in the crab, *Cancer borealis*. *J. exp. Biol.* **135**, 165–181.

PAUL, D. H. AND MULLONEY, B. (1985*a*). Local interneurons in the swimmeret system of the crayfish. *J. comp. Physiol.* **156**, 489–502.

PAUL, D. H. AND MULLONEY, B. (1985*b*). Nonspiking local interneuron in the motor pattern generator for the crayfish swimmeret. *J. Neurophysiol.* **54**, 28–39.

PAUL, D. H. AND MULLONEY, B. (1986). Intersegmental coordination of swimmeret rhythms in isolated nerve cords of crayfish. *J. comp. Physiol.* **158**, 215–224.

SIWICKI, K. K. AND BISHOP, C. A. (1986). Mapping of proctolinlike immunoreactivity in the nervous systems of lobster and crayfish. *J. comp. Neurol.* **243**, 435–453.

SKINNER, K. (1985*a*). The structure of the fourth abdominal ganglion of the crayfish, *Procambarus clarkii*. I. Tracts in the ganglionic core. *J. comp. Neurol.* **234**, 168–181.

SKINNER, K. (1985*b*). The structure of the fourth abdominal ganglion of the crayfish, *Procambarus clarkii*. II. Synaptic neuropils. *J. comp. Neurol.* **234**, 182–191.

TIJSSEN, P. (1985). *Practice and Theory of Enzyme Immunoassays*. Amsterdam: Elsevier.

VAN HARREVELD, A. (1936). A physiological solution for freshwater crustaceans. *Proc. Soc. exp. Biol. Med.* **34**, 428–432.

WIERSMA, C. A. G. AND IKEDA, K. (1964). Interneurons commanding swimmeret movements in the crayfish, *Procambarus clarkii*. *Comp. Biochem. Physiol.* **12**, 509–525.