Introduction

For many years, localizing and sequencing neuroactive peptides has been an important area of research in both invertebrate and vertebrate neuroscience. The physiological effects and behavioral functions of neuropeptides have also been actively investigated. The crustacean stomatogastric nervous system (STNS) has proved to be a valuable preparation for these investigations at the cellular, circuit and behavioral levels. This system contains two well-characterized neural circuits, the pyloric and the gastric mill, which are located in the stomatogastric ganglion (STG) and which mediate rhythmic movements of the foregut.

The activity of these circuits can be extensively modulated by a wide array of exogenously applied compounds, including a number of peptides (Marder, 1987; Harris-Warrick et al. 1992; Skiebe and Schneider, 1994; Blitz et al. 1995). Immunocytochemical techniques have been used to localize these peptides to cell bodies within the paired commissural ganglia (CGs) and the esophageal ganglion (OG), all of which send axons to the STG. The peptides are also found in the neuropil of the STG, suggesting that they are endogenous modulators of cellular activity and circuit output.

The peptide FMRFamide was first discovered in the clam Macrocallista nimbosa (Price and Greenberg, 1977) and was subsequently shown to be part of a large family of FMRFamide-related peptides (FaRPs) occurring ubiquitously in vertebrates and invertebrates. FaRPs modulate numerous processes, including heartbeat (Painter and Greenberg, 1982; Li and Calabrese, 1987; Cuthbert and Evans, 1989; Mercier and Russenes, 1992), blood pressure (Mues et al. 1982; Barnard and Dockray, 1984), the contraction of intestinal (Holman et al. 1986; Groome et al. 1992) and exoskeletal (Evans and Myers, 1986; Pasztor and Golas, 1993; Skerrett et al. 1995; Worden et al. 1995) muscle, synaptic transmission (Walther and Schiebe, 1987; Skerrett et al. 1995) and ionic channel function (Cottrell, 1982; Brezina et al. 1985; McCarthy and Cottrell, 1984). In the stomatogastric nervous system, exogenously applied FMRFamide was shown to activate rhythmic activity in quiescent pyloric circuits in the crab Cancer irroratus (Hooper...
and Marder, 1984). Weimann et al. (1993) later demonstrated that two FMRFamide-like peptides, TNRNFLRFamide (F₁) and SDRNFLRFamide (F₂), activated both pyloric and gastric mill rhythms in the crab Cancer borealis. The latter two peptides were isolated and sequenced from Homarus americanus (Trimmer et al. 1987) and Cancer borealis (Weimann et al. 1993) and represent endogenous crustacean FaRPs. Mercier et al. (1993) isolated a similar pair of FaRPs from the crayfish (P. clarkii) nervous system which they named NF₁ (NRNFLRFamide) and DF₂ (DRNFLRFamide).

Immunocytochemical evidence has shown that FaRPs occur throughout the crustacean nervous system. FMRFamide-like immunoreactivity (FLI) was found in many regions of the lobster nervous system, including the brain, the ventral nerve cord, the sheath surrounding the ventral nerve cord, the second thoracic roots and the pericardial organs (Kobierski et al. 1987). Similarly, Mercier et al. (1991) found FLI throughout the crayfish nervous system, with the greatest concentrations occurring in the pericardial organs. The distribution of FLI in the stomatogastric nervous system has been characterized in several marine species, including Cancer irroratus (Hooper and Marder, 1984). Cancer gracilus (Callaway et al. 1987), Cancer borealis (Marder et al. 1987), Panulirus interruptus (Marder et al. 1987) and Palaemon serratus (Meyrand and Marder, 1991). Several commonalities were found across species, including the presence of FLI in cell bodies in the commissural and esophageal ganglia, in axons of the stomatogastric nerve and in the neuropil of the STG. Interesting cross-species differences also occur. For example, in most species, FLI does not occur in cell bodies in the STG or in motor axons extending from the STG. However, in Palaemon serratus, 2–3 cell bodies in the STG and several axons extending from the STG contained FLI.

Comparative studies of the stomatogastric nervous system are important as they reveal which features are conserved and which vary from species to species, perhaps as a result of adaptive evolution. This information offers insight into how the stomatogastric nervous system and its modulatory inputs evolved and, hence, contributes to our knowledge of how motor systems in general evolved (Katz and Tazaki, 1992). To date, most studies of peptidergic modulation of STG circuits have focused on large marine Crustacea; little is known about circuit modulation in their freshwater relative, the crayfish. The aim of the present study was to initiate a description of peptidergic modulation of the crayfish pyloric circuit by characterizing the distribution of FLI in the stomatogastric nervous system and by investigating the effects of FaRPs on pyloric motor output. We found that the distribution of FLI in the crayfish stomatogastric nervous system is similar, but not identical, to that reported in other crustaceans. We also found that several FaRPs (including F₁, DF₂ and NF₁) reliably excited certain pyloric neurons and produced a modest increase in pyloric cycle frequency. A fourth FaRP isolated from cockroach tissue, leucomyosuppression (LMS), inhibited pyloric cycling, and FMRFamide itself had no effect on pyloric output.

### Materials and methods

#### Animals

Crayfish (Procambarus clarkii) were obtained from Carolina Biological Supply and maintained in circulating freshwater tanks. We used adult animals of both sexes weighing 20–55 g for immunocytochemical (48 animals used) and electrophysiological (34 animals used) experiments.

#### Immunocytochemistry

Whole-mount immunocytochemistry was performed as previously described by Lange et al. (1988) and Mercier et al. (1991). Dissection of the stomatogastric nervous system was...
performed in cold (4°C) physiological saline (NaCl, 210 mmol l⁻¹; KCl, 5.4 mmol l⁻¹; CaCl₂, 10 mmol l⁻¹; MgCl₂, 2.4 mmol l⁻¹; NaHCO₃, 2 mmol l⁻¹; pH 7.4). The entire nervous system, including the stomatogastric ganglion, the paired commissural ganglia, the esophageal ganglion and the connecting nerves, was fixed in 2% paraformaldehyde in Millonig’s buffer (120 mmol l⁻¹ NaH₂PO₄, 1% D-glucose, 0.005% CaCl₂, buffered with NaOH to pH 7.4) for 12 h at 4°C. Tissues were washed for 1–2 h in PBS (10 mmol l⁻¹ phosphate buffer, 0.9% NaCl, pH 7.2) and then incubated for 1 h in 4% Triton X-100 (2% bovine serum albumin, BSA, 2% normal goat serum, NGS, 4% Triton X-100 in PBS) at 21°C. We used a commercially available primary antiserum generated in rabbits immunized with FMRFamide conjugated to bovine thyroglobulin (Incstar, Stillwater, MN). The antiserum was diluted 1:1000 with PBS containing 2% BSA, 0.4% Triton X-100 and 2% NGS, and incubated for 48 h at 4°C. Tissues were washed in PBS for 4 h and then incubated in 1:200 secondary antisera (goat anti-rabbit IgG labelled with fluorescein, FITC, or Texas Red, TRSC; Jackson ImmunoResearch, West Grove, PA) in 10% NGS in PBS for 18 h at 4°C. Following washes in PBS for 1–2 h, tissue was mounted in 5% n-propyl gallate in glycerol, and viewed and photographed using an Olympus epifluorescence microscope equipped with fluorescein (excitor filter, band pass 460–500 nm; barrier filter, band pass 510–550 nm) and Texas Red (excitor filter, band pass 530–550 nm; barrier filter, band pass 590–650 nm) optics.

To investigate the specificity of the antisera for FMRFamide-like peptides, we preincubated the primary antibody with either FMRFamide (10⁻⁵ mol l⁻¹; Sigma) or unrelated peptides (10⁻⁴ mol l⁻¹ proctolin or 10⁻⁴ mol l⁻¹ substance P). All peptides were incubated for 3 h at room temperature (21°C) before they were applied to the tissue. To assess method specificity, we eliminated the primary antibody from our staining sequence and incubated some tissue samples in the antibody diluent alone.

A double-labelling technique was used to determine the axonal projections of certain neurons labeled with the anti-FMRFamide antibody (Marder et al. 1987). For this technique, the cut end of a nerve was surrounded with a Vaseline well containing a 15% solution of Lucifer Yellow-CH (Sigma) in distilled water. The preparation was incubated at 4°C for approximately 12 h, allowing the Lucifer Yellow to backfill the nerve. The Lucifer Yellow was then washed out, and the preparation was fixed in 2% paraformaldehyde and processed for immunocytochemical labeling of FMRFamide-like peptides using the techniques described above and a Texas-Red-conjugated secondary antibody. The Lucifer Yellow labeling was viewed with the fluorescein filter set, and the anti-FMRFamide antibody was viewed with the Texas Red filter set. The use of two filter sets eliminated cross-talk between the channels and allowed single- and double-labeled structures to be distinguished clearly.

**Electrophysiology**

Standard electrophysiological techniques were used to record extracellular signals from stomatogastric nerves. The STG, CGs, OG, connecting nerves and motor nerves, including the dorsal ventricular nerve (dvn), lateral ventricular nerves (lvns) and medial ventricular nerves (mvns), were dissected from the animal and pinned in a Sylgard-lined Petri dish. Extracellular recordings were made with glass-tipped suction electrodes or pin electrodes placed in contact with a nerve and then isolated from the rest of the bath with Vaseline. In actively cycling preparations, spikes from individual pyloric cells could be identified by their firing pattern within the pyloric rhythm.

**Fig. 2. Whole-mount view of FMRFamide-like immunoreactivity in the commissural ganglia (CG) of Procambarus clarkii.** (A) The entire neuropil region and large (longer arrow) and smaller (shorter arrow) somata on the surface of the ganglion are stained. (B) Two nerves, the superior esophageal nerve (son) and the inferior esophageal nerve (ion), extend from the CG towards the stomatogastric nervous system. The son contained several densely stained axons; the ion stained less densely and contained two densely stained axons (arrows). Scale bars, 100 μm.
characteristic of crayfish (Anderson and Kushner, 1987) and other crustaceans (Johnson and Hooper, 1992). Data were recorded on VCR tape and analyzed using a MacLab hardware interface/software system. In some preparations, activity in the stomatogastric nerve (sten) was blocked by placing a Vaseline well around the sten and CGs, and replacing the saline in the well with isotonic sucrose. Peptides were bath-applied by switching the inflow of the superfusion system that continuously supplied the preparation with fresh, cooled saline (18–20°C). A Vaseline well was placed around the STG so that the peptides contacted only this ganglion. The bath volume was approximately 3 ml, and the superfusion rate was 5–8 ml min⁻¹. FMRFamide, proctolin, substance P, NF₁ and DF₂ were obtained from Sigma (St Louis, MO, USA); F₁ and LMS were obtained from Peninsula Laboratories (Belmont, CA, USA). The amino acid sequences of these peptides are given in Table 1.

**Results**

*Immunocytochemical localization of FMRFamide-like peptides*

The stomatogastric ganglion of *P. clarkii* resembles that of the larger reptantian crustaceans. It is located within the ophthalmic artery and contains a central neuropil surrounded by cell bodies, most of which are motor neurons that control

![Figure 3](https://example.com/figure3.png)

**Fig. 3.** FMRFamide-like immunoreactivity in the esophageal ganglion (OG), inferior ventricular nerve (ivn) and stomatogastric nerve (sten) of *Procambarus clarkii*. (A) The OG contains a large, brightly stained soma, but shows no distinct neuropil staining. (B) The ivn (lying across another nerve in the figure) contains two elongated, stained somata. (C) Two stained somata are present at the junction of the sons and the sten. Scale bars, 50 μm.
FMRFamide-like peptides in the crayfish stomach muscles. In crayfish, the STG contains 27–29 somata which range in diameter from 25 to 100 μm (Meiss, 1975). The STG receives input from the rest of the nervous system via the stn. Most motor axons leaving the STG are initially carried in the unpaired dorsal ventricular nerve (dvn), which branches posteriorly into smaller nerves that innervate individual stomach muscles. Several small nerves exit the STG directly and, in several larger species, these nerves innervate adjacent gastric and cardiac muscles (Maynard and Dando, 1974; Selverston et al. 1976). In P. clarkii, bright staining occurred in the neuropil of the STG, but not in the cell bodies which surround the neuropil (Fig. 1). The stn contained at least five stained axons, but accurate counts could not be obtained from the immuno-stained images. The dvn and all posterior motor axons contained no FMRFamide-like immunoreactivity (FLI). However, several small axons extending laterally from the STG were stained in all preparations (Fig. 1). Typically, each STG had one or two stained axons on each side which, in some preparations, branched after leaving the ganglion. Preparations stained with Methylene Blue revealed that these axons run in the bilateral anterior cardiac dilator nerves (acdn), which innervate cardiac muscles in crayfish (Eitner et al. 1996).

The paired CGs are located in the circumesophageal connectives that run between the brain and the subesophageal ganglion. Each CG contains several hundred cell bodies, some of which project to the STG via the superior esophageal nerves (sons) or inferior esophageal nerves (ions) (Coleman et al. 1992). Fig. 2A illustrates the pattern of FLI in the commissural ganglion of P. clarkii. The neuropil was densely stained, and stained cell bodies can be seen on the surface and edges of the neuropil. Each ganglion contained 2–5 large stained cells (approximately 40 μm in diameter) and 8–20 smaller cells (mean 14 cells; N=46 ganglia). The distribution of stained cell bodies was variable, and distinctive individual cells could not be recognized from preparation to preparation. The sons and ions both contained stained axons. In most preparations, one or two axons could be seen in the ions; the sons appeared to contain three or four axons and always stained much more brightly than the ions (Fig. 2B). The circumesophageal connectives contained many stained fibers, some of which had small varicosities along their length.

The OG is connected to the commissural ganglia via the two ions, and in P. clarkii contains approximately 10 cell bodies (Spirito, 1975). In all preparations, the OG contained a large, brightly stained cell body (Fig. 3A). In four of the 24 ganglia examined, one or two additional smaller cells were also

Table 2. Effects of DF2 on the number of spikes per burst and firing frequency in pyloric neurons

<table>
<thead>
<tr>
<th>Neuron</th>
<th>Spikes per burst</th>
<th>Firing frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DF2</td>
</tr>
<tr>
<td>PD</td>
<td>5.7±2.9</td>
<td>5.6±2.5</td>
</tr>
<tr>
<td>LP</td>
<td>8.2±3.7</td>
<td>18.2±7.5*</td>
</tr>
<tr>
<td>PY</td>
<td>21.8±10.7</td>
<td>24.0±12.8</td>
</tr>
<tr>
<td>VD</td>
<td>10.5±4.4</td>
<td>31.3±7.2*</td>
</tr>
</tbody>
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Values are means ± s.d.; spikes were counted in 10 consecutive bursts for each preparation (N=6).

Spike frequency, was calculated by dividing the number of spikes within each burst by the burst duration.

Control, normal saline; DF2, 10^-6 mol l^-1.

Data were recorded 10 min after the onset of peptide application.

*P<0.05; †P<0.01 (Student’s t-test).
stained, although more faintly than the large cell. In some cases, an axon could be seen extending from the large cell toward the stn. There was no staining of the neuropil in the esophageal ganglion. In *P. clarkii*, the inferior ventricular nerve (*ivn*) joins the stn posterior to the OG approximately midway between the ganglion and the junction of the *sons* with the *stn*. Two cell bodies were present in this nerve (Fig. 3B). The location of these was variable, but they were typically situated together midway between the *stn* and the brain.

The junction of the *sons* with the *stn* is considered to be an extension of the OG in some species, and in lobsters several cell bodies are located in this region (Kushner, 1979; Claiborne and Ayers, 1987). Fig. 3C shows the *son–stn* junction in *P. clarkii*. In all the preparations we examined (*N*=48), two stained cell bodies were present at this junction. The cells were approximately 30–40 μm in diameter and contained clearly visible nuclei. In some preparations, axons could be seen extending from these cells, but the direction taken by these axons could not reliably be determined using anti-FMRFamide immunchemistry alone. However, Lucifer Yellow backfills of the *stn* stained these cells, indicating that their axons extend into the *stn* and presumably into the STG. Fig. 4 shows a preparation in which a Lucifer Yellow backfill of the *stn* was combined with immunostaining for FMRFamide using a Texas-Red-conjugated secondary antibody. Fig. 4A shows the preparation viewed with fluorescein optics, and Fig. 4B shows the same preparation viewed with Texas Red optics. Lucifer Yellow backfills of the *ivn* (*N*=3) and *sons* (*N*=4) did not stain the *son–stn* cell bodies clearly. Fig. 5 provides a schematic summary of the distribution of FLI in the complete STNS of *P. clarkii*.

Preincubation of the primary antiserum with FMRFamide (10^{-5} mol l^{-1}) completely abolished staining in all preparations tested (*N*=4). In contrast, preincubation with the unrelated peptides proctolin (10^{-5} mol l^{-1}; *N*=3) and substance P (10^{-3} mol l^{-1}; *N*=3) had no effect on staining. Similarly, preincubation with bovine pancreatic polypeptide did not effect staining of crayfish tissue (Mercier *et al*. 1991). Preparations incubated in secondary antibody alone were completely unstained.

**Effects of FMRFamide-related peptides (FaRPs) on the pyloric motor pattern**

The STG contains two central pattern generators, one controlling the pyloric region of the stomach and the other controlling the gastric mill. We recorded the effects of FaRPs on the pyloric rhythm by bath-applying peptides over the STG while monitoring pyloric cell activity with extracellular electrodes on the *ivn* and *mvn* motor nerves (see arrows in Fig. 5). The *ivn* contains axons from three different classes of motor neurons: the pyloric dilators (PD), and the pyloric (PY) and lateral pyloric (LP) cells. The *mvn* contains the axon from the ventral dilator (VD) neuron, but not the axon of the inferior cardiac neuron (IC) (Meiss, 1975). Both of these nerves also contain axons from gastric motor neurons; however, our present analysis focused only on pyloric cells. The pyloric motor pattern consists of alternating bursts of activity in these cells (Fig. 6, top trace). The motor pattern period varied between 1 and 12 s (mean 3.1 s; *N*=16) and, in many preparations, was interrupted by rhythmic bursts from gastric neurons. In the remaining preparations (*N*=18), pyloric cycling was absent or irregular, with the pyloric rhythm sometimes represented by activity in only one or two cell types.

Fig. 6 shows a preparation with an initial cycle period of approximately 3 s, and with most of the cycle period occupied by an extended PY burst. In this and all other preparations, the peptides DF2, F1 and NF1 increased the activity of some neurons, most strikingly that of the LP and VD cells. In most preparations, the peptides also caused an increase in cycle frequency (Fig. 7). Overall, this increase was statistically significant, but it did not occur in all individual preparations as cycle frequency was sometimes unchanged or slightly slowed by the peptides. In Fig. 7, the diagonal line indicates no change.
in cycle frequency resulting from peptide \(10^{-6}\text{mol} \cdot \text{L}^{-1}\) application; points above the line indicate an increase in cycle frequency and points below the line indicate a decrease in cycle frequency. When the initial cycle frequency was especially slow (below 0.25 cycles s\(^{-1}\)), the peptides always enhanced cycle frequency, but at higher initial cycle frequencies, responses to the peptides were more variable. Reductions in cycle frequency were due to increased burst durations of

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**Fig. 6.** Response of the pyloric rhythm to bath application of FMRFamide-related peptides (FaRPs). Data are from a single nonsucrose-blocked stomatogastric nervous system. Control: during perfusion with normal saline, the pyloric motor pattern cycled slowly and regularly, with most of the cycle period occupied by the PY burst. The \(lvn\) trace shows three cell types (LP, PY, PD) firing in sequence; the \(mvn\) trace shows rhythmic bursts of a single cell, the VD cell. \(DF_2 (10^{-6})\), \(F_1 (10^{-6})\), \(NF_1 (10^{-6})\): bath application of each peptide at \(10^{-6}\text{mol} \cdot \text{L}^{-1}\) increased impulse activity in several neurons. Data were recorded 10 min following the onset of peptide application, and there was a 30 min wash between the application of each peptide. Wash: data were recorded 15 min following the onset of normal saline perfusion. \(lvn\), lateral ventricular nerve; \(mvn\), medial ventricular nerve; LP, lateral pyloric neuron; PY, pyloric neuron; PD, pyloric dilator neuron; VD, ventricular dilator neuron.
preparations tested with DF2, NF1 and F1, respectively. Peptide change in cycle frequency. Squares, triangles and circles indicate after the onset of peptide application. The diagonal line indicates no increase in cycle frequency.

The single preparation (represents the mean of ten cycles recorded in saline and peptide in a function of the cycle frequency in normal saline. Each data point in the LP and VD cells was between 10^{-8} mol l^{-1} and 10^{-7} mol l^{-1}) and an increase in bursting at high concentrations (10^{-6} mol l^{-1}). However, in no case did the peptide produce a complete pyloric rhythm. Instead, cycling consisted of an irregular mixture of pyloric and gastric cell activity. Because pyloric cells could not definitively be identified in such recordings and because their activity was no longer regulated by AB/PD bursts, we did not include data from sucrose-blocked preparations in our quantitative analysis.

In contrast to the effects of DF2, F1 and NF1, FMRFamide had no effect on pyloric cell activity (Fig. 10). We tested FMRFamide at concentrations between 10^{-7} and 10^{-5} mol l^{-1}, and in all six preparations tested we observed no changes in impulse activity or cycle period. Application of leucomyosuppression (LMS; 10^{-6} mol l^{-1}), however, did consistently alter pyloric cell activity. This peptide disrupted the pyloric rhythm in actively cycling preparations and reduced tonic activity in sucrose-blocked preparations (Fig. 10). These effects were observed within 10 min of the start of LMS perfusion and were at least partially reversed within 15 min of the start of the wash-out. The threshold for activity changes in LP and VD cells was 10^{-7} mol l^{-1} LMS (Fig. 8C,D). The data in Fig. 8C,D were from a single preparation and were recorded 10 min after the initial application of LMS. When LMS was allowed to remain in the bath for longer (more than 20 min), activity in the LP cell ceased completely in some preparations, leaving only tonic PY spikes (as in Fig. 10).

**Discussion**

The stomatogastric nervous system of crayfish and other crustaceans consists of four ganglia, three of which (the paired CGs and the OG) contain cells which project to and modulate the activity of cells within the fourth ganglion, the STG. The STG, in turn, generates two motor patterns that drive movements of the gastric mill and pyloric regions of the stomach. We have mapped the distribution of FLI in the crustacean species, can be compared with descriptions of FLI found both similarities with and differences from other parts of the nervous system of Panulirus interruptus (Marder et al. 1987) and Homarus americanus (Kobierski et al. 1987), and the shrimp Palaemon
The Incstar primary antibody we used is different from those used by previous STG researchers (i.e. antisera 231 and 671). However, the Incstar antibody stained the same structures as antisera 231 and 671 in *P. serratus* (P. Meyrand, personal communication), suggesting that the differences we report among species are indeed species-dependent and are not due solely to differences in primary antibody specificity.

In all species examined thus far, processes within the central neuropil of the STG are densely stained and the *stn* contains stained axons. In *Cancer* species and *P. interruptus*, no staining was observed in somata within the STG or in axons exiting the ganglion (Marder et al. 1987; Callaway et al. 1987). In contrast, Meyrand and Marder (1991) found two or three stained somata in the shrimp STG and also observed staining in two axons exiting the STG via the *dvn*. The staining pattern in *P. clarkii* resembles that of lobsters and crabs in that no FLI occurred in STG somata. However, unlike any other species described to date, FLI was detected in axons branching directly from the STG. We have not yet determined the location of the somata that give rise to these axons, but they do not appear to be located in the STG. Possibly, the somata location is in the CGs or OG, suggesting that a cell or cells in an anterior ganglion may be able to modulate the activity of stomatogastric muscles directly.

The paired commissural ganglia, each of which contains approximately 400 neurons, typically show FLI in both somata and neuropil. In the crab *C. borealis*, FLI is present in 10–20 somata located around the neuropil, which is also densely stained (Marder et al. 1987). Similar somata and neuropil staining was observed in *C. gracilis* (Callaway et al. 1987), *C. irroratus* (Hooper and Marder, 1984) and *P. interruptus* (Marder et al. 1987). In *P. clarkii*, each commissural ganglion contained 12–22 stained somata of varying sizes. Most of these cells which project to the stomatogastric nervous system do so through the *son*, which was always more brightly stained than the *ion*. The latter nerve, however, did reliably contain one or two stained axons. This pattern of FLI resembles that of the

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**Fig. 8.** Dose-dependent effects of DF2 and LMS on the number of LP and VD spikes produced per burst in *Procambarus clarkii*. Bars indicate the mean number of spikes per burst in ten consecutive bursts from the ongoing pyloric rhythm. Error markers are the standard deviation of the mean. The lowest concentration (10^{-9}mol l^{-1}) was presented first and the highest concentration was presented last, with a 30 min wash between each peptide application. (A,B) DF2 produces a dose-dependent increase in the number of spikes per burst in the LP (A) and VD (B) cells. Data for both cells are from the same preparation. (C,D) LMS produces a dose-dependent decrease in the number of spikes per burst in the LP (C) and VD (D) cells. Data from both cells are from the same preparation (but a different preparation from that used in A and B). See Fig. 6 for abbreviations. C, control; W, wash.
lobster *H. americanus* in which the *ion* contains one or two stained axons and the *son* contains four (Kobierski et al. 1987).

The *ions* project to the esophageal ganglion which, in *P. clarkii*, typically contained one large stained soma. The identity of this soma is unknown, but it appears to be the largest soma in the OG. In other species, a large unpaired soma in the OG has been identified as CD1, a motor neuron that innervates cardiac sac muscles adjacent to the STG (Claiborne and Ayers, 1987). If the large stained soma in the crayfish OG proves to be CD1, it would be a likely source of the stained axons that branch laterally from the STG. In some preparations, one or two smaller cells in the OG were also stained more faintly. Little neuropil staining was present in this ganglion. The *ivn*, a nerve connecting the esophageal region with the brain, contained two somata usually situated adjacent to each other. Similarly, stained somata occur in the esophageal ganglion of *C. borealis* (four somata), *P. interruptus* (two somata) (Marder et al. 1987) and *C. gracilis* (four somata) (Callaway et al. 1987). The *ivns* of *P. interruptus* and *Homarus gammarus* each contain two cells, termed *ivn*-through fibers (Dando and Selverston, 1972) and PS neurons (Cazalets et al. 1990a,b), respectively. The PS neurons, which act to modulate stomatogastric motor patterns and temporarily create a motor pattern that generates swallowing, show FLI (Meyrand et al. 1994). The *ivn*-through fibers contain histamine, and their effects on STG circuits differ from the actions of PS cells (Claiborne and Selverston, 1984a,b). The relationship between the crayfish *ivn* cells and the cells in *Homarus gammarus* and *Panulirus interruptus* is uncertain at present as the functional properties of the crayfish cells have not yet been determined. However, the presence of FLI in both the PS and the crayfish neurons, and their common location in the *ivn*, raises the possibility that the lobster PS cells and the pair of cells in the crayfish *ivn* are homologous.

A distinctive feature in the crayfish was the presence of two stained somata in the *son* at the junction of the *sons* with this nerve. Although neuropil staining occurs at this junction in *Cancer borealis* and *Panulirus interruptus* (Marder et al. 1987), in no other species have stained somata been observed at this location. Because these cells were unusual, we used Lucifer Yellow backfills combined with Texas-Red-labelled immunostaining to determine the projection patterns of these cells. Lucifer Yellow backfills from the *son* clearly labeled two somata at the *son–stn* junction. Visualization with optics for Texas Red revealed the same two somata at the *son–stn* junction (Fig. 4A,B). These data indicate that the *son–stn* cells project to the STG and presumably contribute to the immunostaining in the STG neuropil. In some immunostained preparations, axons could be seen extending a short distance from the *son–stn* cells, and they appeared to project towards the *sons* and esophageal ganglion. However, our Lucifer Yellow backfills of the *son* and *ivn* failed to confirm these observations. In some preparations, especially from the *son* backfills, many axons were stained, and the dense fluorescence at the *son–stn* junction may have obscured the filled somata if they were present.

The three excitatory FaRPs we tested were isolated from lobster (F1; Trimmer et al. 1987) or crayfish (DF2 and NF1; Mercier et al. 1993) neurosecretory tissue. In our experiments, all three peptides reliably excited certain pyloric neurons. The strongest effects were seen in the LP and VD cells, in which both the number of spikes per burst and the firing frequency increased significantly during peptide exposure. Intraburst firing frequency also increased in the PY cells. The activity of the PD cells was not increased by any of the FaRPs. In fact, in some preparations, the number of PD spikes per burst appeared to decrease during peptide wash-ins, although these changes were not significant. The PD neurons are electrically coupled to the AB (anterior burster) interneuron. The AB/PD group make up the circuit pacemaker and their synchronous bursts determine pyloric cycling frequency. The lack of PD activation by the peptides suggests that these cells and the AB cell are not strongly excited by the peptides. This suggestion is consistent with our observation that a complete pyloric rhythm was not elicited in sucrose-blocked preparations.

In our experiments, the FaRPs were applied only to the STG. Hence, it is clear that the peptides can affect STG neurons (or

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**Fig. 9.** Effects of bath-applied DF2 (10^{-6} mol L^{-1}) on pyloric phase relationships. Boxes indicate the mean onset and duration of the activity of each cell type. Data for each preparation are means of ten consecutive bursts from ongoing pyloric rhythms in nonsucrose-blocked preparations. Data were normalized to 1.0, indicating the percentage of the cycle period at which each cell begins and ends its activity in control (normal) saline and DF2. Left-hand error markers indicate the standard deviation of the mean (S.D.) for activity onsets; right-hand markers indicate the S.D. for duty cycles (the proportion of time each cell is active). In the presence of DF2, the LP duty cycle was significantly increased and the onset of activity in the PY was significantly delayed; *P<0.05, N=5. See Fig. 6 for abbreviations.
presynaptic terminals) directly rather than altering the activity of STG neurons indirectly via changes in OG or CG cell activity. However, our present results do not establish which pyloric neurons are direct targets of FaRPs. Some of the effects we observed in the LP, VD and PY cells may have been indirect effects of peptide actions on other pyloric or gastric neurons. It seems likely, for example, that the delay in the onset of PY activity was due to activation of the LP cell (which inhibits PY cells), rather than to a direct action of DF2 on the PY cell. Experiments with isolated cells (e.g. Flamm and Harris-Warrick, 1986) would be necessary to establish definitively how FaRPs directly affect individual pyloric cells. Our physiological experiments did not reveal any striking differences among the three peptides. This may indicate that they interact with the same receptor, but additional studies would be necessary to confirm this.

FaRPs (F1 and a second lobster peptide, F2; SDRNFLRFamide) also excited pyloric cells in the crab C. borealis (Weimann et al. 1993). As in P. clarkii, F1 and F2 significantly increased the number of spikes per burst in crab VD cells and elicited LP bursting in quiescent preparations. The peptides caused small and nonsignificant changes in the duty cycles and phase relationships of crab pyloric neurons, whereas we observed a modest but significant change in the LP duty cycle and a delay in the onset of the PY burst relative to the PD burst. Also in contrast to the crayfish, complete and regular pyloric rhythms were elicited in sucrose-blocked crab preparations. Weimann et al. (1993) suggest that the AB
neuron is likely to be a direct FaRP target in the crab STG; our results do not offer strong support for this conclusion in crayfish. Relative to *C. borealis*, the threshold concentration needed to elicit a change in pyloric cell activity was high in *P. clarkii* (between 10^{-8} and 10^{-7} mol l^{-1} in crayfish compared with 10^{-12} to 10^{-11} mol l^{-1} in the crab). Although DF2 is an endogenous crayfish peptide, it is possible that the receptors on pyloric neurons would respond with higher affinity to a different, as yet unsequenced, FaRP. Alternatively, the FaRPs may be released differently in the crayfish and the crab STG. In the crayfish, the FMRFamide-like projections may form discrete synapses with certain neurons, allowing a higher endogenous concentration of peptide to be present directly adjacent to these neurons.

In contrast to the excitatory effects of F1, DF2 and NF1, FMRFamide had no observable effects on crayfish pyloric cells, and LMS consistently inhibited the pyloric motor pattern. In a previous study, FMRFamide was shown to have excitatory effects on pyloric neurons in *Cancer irroratus* (Hooper and Marder, 1984). In this crab species, FMRFamide increased the activity of the PY cells and increased the frequency of the pyloric motor pattern, with these effects elicited at a relatively high concentration of FMRFamide (10^{-5} – 10^{-4} mol l^{-1}). The lack of response to FMRFamide in crayfish pyloric neurons suggests that the FaRP receptor (or receptors) may differ between *C. irroratus* and *P. clarkii*, with the receptors of the latter requiring an extended RNFLRFamide peptide to induce cell activation.

The peptide LMS was isolated from head extracts of the cockroach *Leucophaea maderae* (Holman et al. 1986) and shares the carboxy-terminal ending FLRFamide with the excitatory FaRPs discussed above. The effects of LMS have not previously been described in the stomatogastric nervous system but, interestingly, LMS inhibited spontaneous contractions of the hindgut in the cockroach. In our experiments, LMS reversibly inhibited the pyloric motor pattern, indicating that this peptide is functionally distinct from the other FaRPs and presumably acts through a distinct receptor. With the exception of allatostatin peptides (Skiebe and Schneider, 1994), most neuropeptide modulators of STG circuits are excitatory, and LMS is thus the second peptide found to have inhibitory actions in the STG. Preliminary data (Mercier, 1995) indicate that a decapptide showing some sequence homology with LMS is associated with the crayfish hindgut. Further studies are needed to determine whether LMS occurs endogenously in the STNS of crayfish and to establish the direct cellular targets of this peptide in the STG.

References


