The Journal of Experimental Biology **198**, 263–271 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

IMMUNOCYTOCHEMICAL LOCALIZATION OF MULTIPLE CHOLECYSTOKININ-LIKE PEPTIDES IN THE STOMATOGASTRIC NERVOUS SYSTEM OF THE CRAB *CANCER BOREALIS*

ANDREW E. CHRISTIE[1], DAVID BALDWIN[2], GINA TURRIGIANO[1], KATHERINE GRAUBARD[2] AND EVE MARDER[1,*]

[1]*Department of Biology and Volen National Center for Complex Systems, Brandeis University, Waltham, MA 02254, USA* and [2]*Department of Zoology, NJ-15, University of Washington, Seattle, WA 98195, USA*

Accepted 12 August 1994

Summary

Three anti-cholecystokinin antibodies were used to label the stomatogastric nervous system of the crab *Cancer borealis*. Labeled tissues were examined as whole mounts using laser scanning confocal microscopy. Although each of the anti-cholecystokinin antibodies labeled a variety of structures within the stomatogastric nervous system (including somata, fibers and neuropil), the pattern of labeling produced by each antibody was distinct. These results indicate that there is a family of cholecystokinin-like molecules that are differentially distributed among a subpopulation of the neurons in the stomatogastric nervous system of *Cancer borealis*.

Key words: confocal microscopy, peptides, Crustacea, neuromodulators, *Cancer borealis.*

Introduction

Over the past three decades, the stomatogastric nervous system (STNS) of decapod crustaceans has emerged as one of the leading systems for studying neuromodulation of small, rhythmically active neural circuits. This region of the crustacean nervous system consists of four ganglia and controls the rhythmic movement of food through the foregut. One of these ganglia, the stomatogastric ganglion (STG), contains the neural circuits responsible for controlling the movements of both the gastric mill and the pyloric valve. Each of these circuits is composed of about half of the 30 or so neurons contained within the STG. Numerous studies have shown that the motor patterns produced by the gastric mill and pyloric circuits are not hard-wired, but are capable of being modulated by a variety of neuroactive substances (Harris-Warrick and Marder, 1991; Harris-Warrick *et al.* 1992; Marder and Nusbaum, 1989; Marder and Weimann, 1992). These molecules are delivered to the ganglion by the hemolymph and local release from modulatory neurons. Biochemical and immunocytochemical studies have shown that small molecule transmitters and neuropeptides are present in the input fibers that project to the STG. Over 15 distinct neuroactive substances have been localized to these axons, including serotonin (Beltz *et al.* 1984; Katz and Harris-Warrick, 1989), octopamine (Barker *et al.* 1979), dopamine (Marder, 1987), histamine (Claiborne and Selverston, 1984) and GABA (Nusbaum *et al.* 1989), as well as proctolin (Marder *et al.* 1986), red pigment concentrating hormone (RPCH)-like (Nusbaum and Marder, 1988), FMRFamide-like (Marder *et al.* 1987; Callaway *et al.* 1987; Weimann *et al.* 1993), tachykinin-like (Goldberg *et al.* 1988; Christie *et al.* 1993; Blitz e*t al.* 1994), β-pigment dispersing hormone-like (Mortin and Marder, 1991), buccalin-like (Christie *et al.* 1994), myomodulin-like (Christie *et al.* 1994) and allatostatin-like (Skiebe and Schneider, 1994) peptides.

Numerous studies have shown that the cholecystokinins (CCKs) are a diverse and widely distributed family of substances. CCK-like molecules have been identified in both vertebrate and invertebrate neural tissue (Dockary, 1976; Dockary *et al.* 1981, 1985; Favrel *et al.* 1987; Grimmelikhuijzen *et al.* 1980; Johnsen and Rehfeld, 1990; Larson and Vigna, 1983; Nachman *et al.* 1986; Nichols, 1992; Nichols *et al.* 1988; Vigna, 1985). Moreover, there is an extensive literature demonstrating that these molecules serve important roles as neurotransmitters and/or neuromodulators (Brooks and Kelley, 1985). In a recent series of reports, CCK-like peptides were shown to be present in, and powerful modulators of, the spiny lobster *Panulirus interruptus* STG (Turrigiano and Selverston, 1989, 1990, 1991; Turrigiano *et al.* 1994). In this paper, we show that CCK-like immunoreactivity is also present in the STNS of the crab *Cancer borealis*. Using

*Author for correspondence.

laser scanning confocal microscopic techniques, we have examined the staining patterns produced by three different antibodies to CCK8, two monoclonal antibodies (P. Sithigorngul, C. Cowden and A. O. W. Stretton, in preparation) and one polyclonal antibody. While the vast majority of profiles label only with a single antibody, some appear to be recognized by both a monoclonal and the polyclonal antibody. These results indicate that a family of CCK-like peptides is distributed in a differential fashion throughout the STNS of *C. borealis*. Some of this work has appeared previously in abstract form (Christie *et al.* 1991, 1992).

Materials and methods

Animals

Rock crabs, *Cancer borealis* Stimpson (*N*=91), were obtained from Neptune Lobster and Seafood Company, Boston, MA, USA, and maintained without food in aquaria containing artificial sea water at 10–12 ˚C.

Peptides

CCK8SO_4 and proctolin were purchased from Sigma. RPCH was obtained from Peninsula Laboratories. TNRNFLRFamide was synthesized by James Weimann, Brandeis University.

Antibodies

Mouse anti-CCK monoclonal antibodies C36-9H and C37-4E were gifts from Dr Anthony Stretton (University of Wisconsin, Madison, USA). Both antibodies were generated from clones produced by immunizing mice with a mixture of CCK8/bovine serum albumin conjugate and CCK8/keyhole limpet hemocyanin conjugate. For each immunogen, two coupling agents, glutaraldehyde and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC), were used to cross-link the peptide to the carrier protein. The specificity of both antibodies for CCK-like molecules has been determined (P. Sithigorngul, C. Cowden and A. O. W. Stretton, in preparation). Antibody C36-9H shows a strong recognition of CCK8 but relatively low recognition of CCK4. Antibody C37-4E recognizes CCK8 and CCK4 equally. Both C36-9H and C37-4E were used at a final dilution of 1:300.

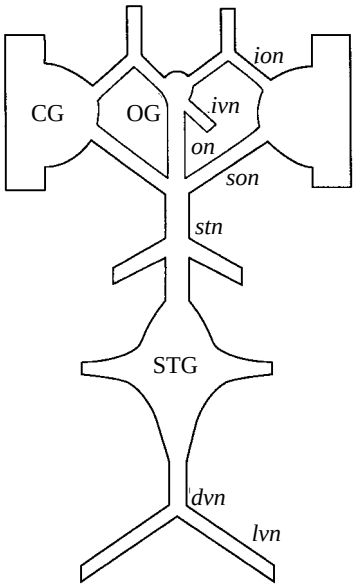

Fig. 1. A schematic representation of the stomatogastric nervous system of *Cancer borealis*. The stomatogastric nerve (*stn*) is the only input from the crustacean central nervous system to the stomatogastric ganglion (STG). The paired superior oesophageal nerves (*son*) and the single oesophageal nerve (*on*) connect with the *stn*, linking the commissural ganglia (CGs) and the oesophageal ganglion (OG) with the STG. The paired inferior oesophageal nerves (*ion*) connect the OG to both CGs; and the inferior ventricular nerve (*ivn*) links the OG to the supraoesophageal ganglion, commonly referred to as the brain. The circumoesophageal connectives (*coc*) provide connections between each CG and both the supraoesophageal ganglion and the thoracic nervous system. The dorsal ventricular nerve (*dvn*) and the paired lateral ventricular nerves (*lvn*s) contain the axons of STG motor neurons that innervate the muscles of the foregut.

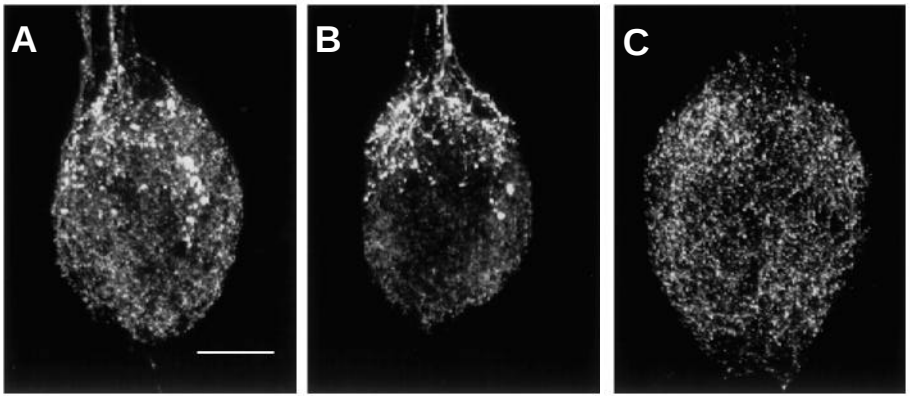

Fig. 2. CCK-like immunoreactivity in the STG of the crab *Cancer borealis*. In all cases, CCK-like immunoreactivity is evident throughout the neuropil, but not in any STG somata. Immunoreactive fibers enter the STG through the *stn*. (A) Laser scanning confocal micrograph of Rhodamine-labeled CCK-like immunoreactivity as revealed using the polyclonal antibody 243-4. The image is a maximum projection of 33 optical sections taken at 2 μm intervals. (B) Laser scanning confocal micrograph of FITC-labeled CCK-like immunoreactivity as revealed using monoclonal antibody C36-9H. The image is a maximum projection of 26 optical sections taken at 2 μm intervals. (C) Laser scanning confocal micrograph of FITC-labeled CCK-like immunoreactivity as revealed using monoclonal antibody C37-4E. The image is a maximum projection of 36 optical sections taken at 2 μm intervals. All images are presented at the same scale. Orientation as in Fig. 1. Scale bar, 100 μm.

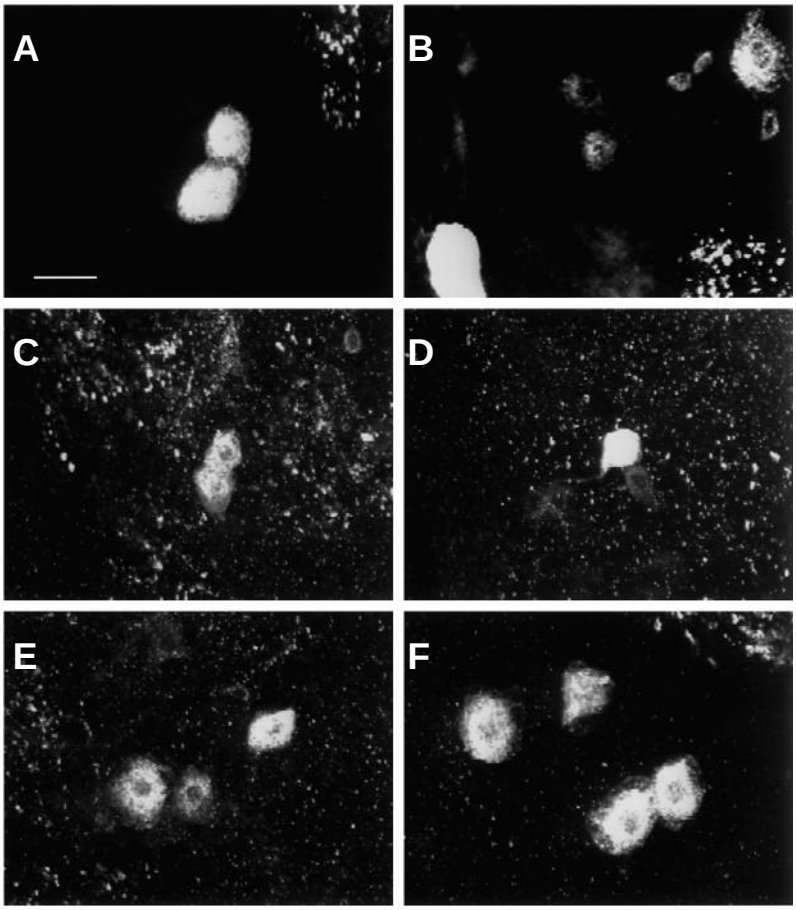

Fig. 3. CCK-like immunoreactivity in the CG of the crab *Cancer borealis*. (A,B) Laser scanning confocal micrographs of two clusters of somata containing CCK-like peptide (in the same CG) as revealed by the polyclonal antibody 243-4 and a Rhodamine-conjugated secondary antibody. The images are maximum projections of 18 optical sections (A) and 43 optical sections (B) taken at 1 μm intervals. (C,D) Laser scanning confocal micrographs of two clusters of Rhodamine-labeled CCK-like immunopositive somata in a different CG as revealed using monoclonal antibody C36-9H. The images are maximum projections of 16 optical sections (C) and 14 optical sections (D) taken at 1 μm intervals. (E,F) Laser scanning confocal micrographs of Rhodamine-labeled CCK-like immunoreactivity in two clusters of neurons in a C37-4E-labeled CG. The images are maximum projections of eight optical sections (E) and nine optical sections (F) taken at 1 μm intervals. All the images are presented at the same scale. Scale bar, 50 μm.

Antibody 243-4, a rabbit polyclonal antibody, was raised against a glutaraldehyde-cross-linked CCK8/bovine thyroglobulin conjugate (Turrigiano and Selverston, 1991). This serum shows strong recognition of a variety of CCK-like peptides (CCK8, caerulian peptide and gastrin). As with the two monoclonal antibodies, 243-4 was also used at a final dilution of 1:300.

Since the use of these three anti-CCK antibodies is novel in either decapod crustaceans (C36-9H and C37-4E) or *C. borealis* (243-4), preabsorption controls were conducted. In these experiments, each antibody was incubated with $CCK8SO_4$, proctolin, RPCH or TNRNFLRFamide for 2 h at room temperature prior to application to tissue. Incubation of each of the CCK antibodies with 10^{-7} mol l^{-1} $CCK8SO_4$ completely abolished staining (N=4 preparations for each antibody), whereas preincubation with the same or higher concentrations of proctolin, RPCH or TNRNFLRFamide (10^{-7} to 10^{-4} mol l^{-1}) had no effect [N=4 for each antibody at each peptide concentration (10^{-7} mol l^{-1}, 10^{-6} mol l^{-1}, 10^{-5} mol l^{-1} and 10^{-4} mol l^{-1})].

Secondary antibodies were goat anti-mouse and goat anti-rabbit affinity-purified IgGs labeled either with FITC or with Rhodamine (Boehringer-Mannheim). All secondary antibodies were used at a final dilution of 1:25.

Whole-mount immunocytochemistry

Prior to fixation and staining, the entire STNS was dissected from each animal (Selverston and Moulins, 1987). The STNS (Fig. 1) consists of the STG, the oesophageal ganglion (OG) and the paired commissural ganglia (CGs). There are 28–32 neurons

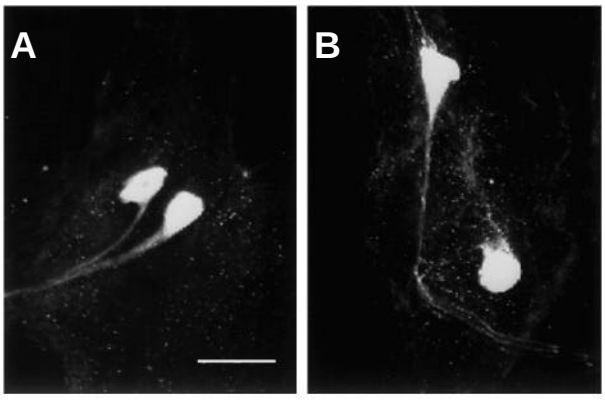

Fig. 4. CCK-like immunoreactivity in the OG of *Cancer borealis*. (A) Laser scanning confocal micrograph of Rhodamine-labeled CCK-like immunoreactivity as revealed using the polyclonal antibody 243-4. The image is a maximum projection of 23 optical sections taken at 2 μm intervals. (B) Laser scanning confocal micrograph of Rhodamine-labeled CCK-like immunoreactivity in another preparation as revealed using monoclonal antibody C37-4E. The image is a maximum projection of 29 optical sections taken at 2 μm intervals. No CCK-like immunoreactivity is seen in this ganglion using monoclonal antibody C36-9H. Both images are presented at the same scale. Scale bar, 100 μm.

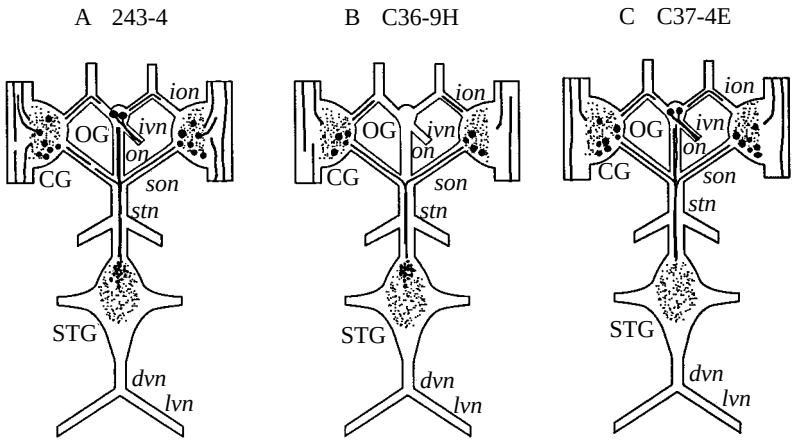

Fig. 5. Schematic representation of CCK-like immunoreactivity in the ganglia and nerves of the *Cancer borealis* STNS. (A) Distribution of 243-4-immunopositive structures in the STNS. (B) Distribution of C36-9H-immunopositive structures in the STNS. (C) Distribution of C37-4E-immunopositive structures in the STNS. Abbreviations as in Fig. 1.

in the STG, approximately 18 in the OG and approximately 400 in each CG (V. Kilman, unpublished results). A network of nerves connects the four ganglia to the musculature of the foregut.

Tissues were processed for immunocytochemistry as whole mounts using indirect immunofluorescence methods modified from Beltz and Kravitz (1983). The entire STNS was dissected in chilled (approximately 4 ˚C) physiological saline (440 mmol l^{-1} NaCl; 11 mmol l^{-1} KCl; 26 mmol l^{-1} $MgCl_2$; 13 mmol l^{-1} $CaCl_2$; 11 mmol l^{-1} Trizma base; 5 mmol l^{-1} maleic acid; pH 7.4–7.6), fixed overnight with 4 % paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.3–7.4) and subsequently rinsed six times over approximately 6 h in a solution of 0.1 mol l^{-1} sodium phosphate (pH 7.2) containing 0.3 % Triton X-100 (P-Triton). Fixation, and all subsequent processing, was carried out at approximately 4 ˚C. The incubation in primary antibody (or antibodies in the case of double labels) was performed in P-Triton for 18–48 h (10 % goat normal serum was added to all reactions to reduce nonspecific binding). Tissues were again rinsed six times in P-Triton over approximately 6 h. Secondary antibody incubation was also carried out in P-Triton. For double-label experiments,

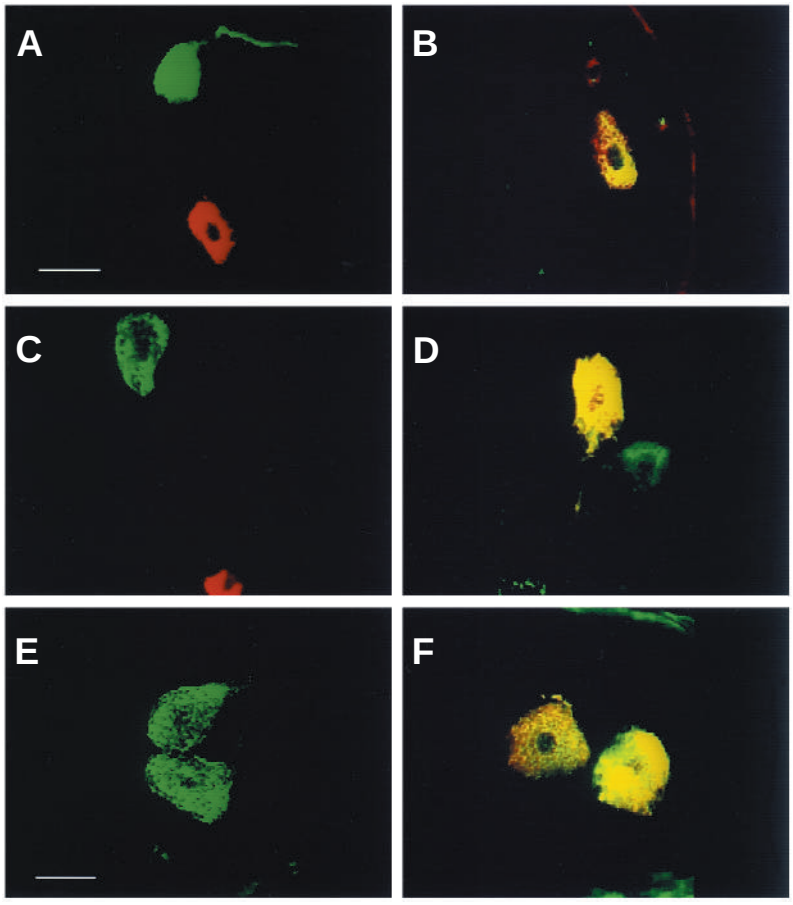

Fig. 6. Co-localization of CCK-like immunoreactivities in the CG and OG. (A–D) This series of micrographs is a merged pseudocolor composite of simultaneously collected FITC- and Rhodamine-labeled images from single focal planes. In each micrograph, green codes for profiles showing 243-4 single labeling, red codes for profiles exhibiting either C36-9H or C37-4E immunoreactivity and yellow codes for structures showing immunoreactivity for both 243-4 and either C36-9H or C37-4E. (A) An example of a CG neuron showing no 243-4/C36-9H co-localization. (B) An example of a CG neuron that exhibits both 243-4 and C36-9H immunoreactivities. (C) Examples of somata showing unique labeling for 243-4 and C37-4E. (D) An example of a neuron showing both 243-4 and C37-4E immunoreactivities in close apposition to a C37-4E uniquely labeled somata. (E) A 243-4/C36-9H double-labeled preparation showing only 243-4 immunoreactivity in a pair of OG somata. This image represents a merged pseudocolor maximum projection of 20 optical section taken at 1 μm intervals. (F) A 243-4/C37-4E double-labeled preparation showing that the neurons in the OG that exhibit 243-4 immunoreactivity and the pair that exhibits C37-4E immunoreactivity have the same somata. This image represents a merged pseudocolor maximum projection of 11 optical sections taken at 1 μm intervals. All images are presented at the same scale. Scale bars, 50 μm.

a cocktail of goat anti-mouse and goat anti-rabbit IgG was employed. After incubation with secondary antibody (12–24 h), each preparation was rinsed six times at 1 h intervals in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.2). Following the final rinse, ganglia were mounted on glass coverslips using a solution of 80 % glycerine and 20 % 20 mmol l^{-1} sodium carbonate, pH 9.5. Fragments of glass coverslips were placed near each preparation before covering to minimize compression of the tissue. All preparations were viewed with a Biorad MRC 600 laser scanning confocal microscope equipped with a krypton/argon mixed gas laser and the standard YHS [for Rhodamine (excitor filter, 568 nm DF10; dichroic reflector, 585 nm DRLP; emission filter, 585 nm EFLP)] or BHS [for FITC (excitor filter, 488 nm DF10; dichroic reflector, 510 nm LP; emission filter, 515 nm LP)] filter blocks provided by the manufacturer. For double-label preparations, the K1/K2 filter set (K1: 488 and 568 nm dual excitation, dual dichroic reflector; K2: dichroic, DR 560 nm LP; green emission filter, 522 nm DF35; red emission filter, 585 nm EFLP) supplied by the manufacturer was employed.

Data analysis

Comos (Biorad) and NIH Image (software for the Macintosh available *via* anonymous ftp from: zippy.nimh.nih.gov) were used for all measurements of cell number and diameter. Kaleidagraph software (Synergy Software) was used for all statistical computations. All measurements are given as the mean value ± one standard deviation. As all measurements are taken from fixed fluorescently labeled tissue, sizes are given only to permit comparison between the antibodies used and do not necessarily reflect the true size of the structures described.

Results

CCK-like immunoreactivity in the STG

Although none of the CCK antibodies used in this study

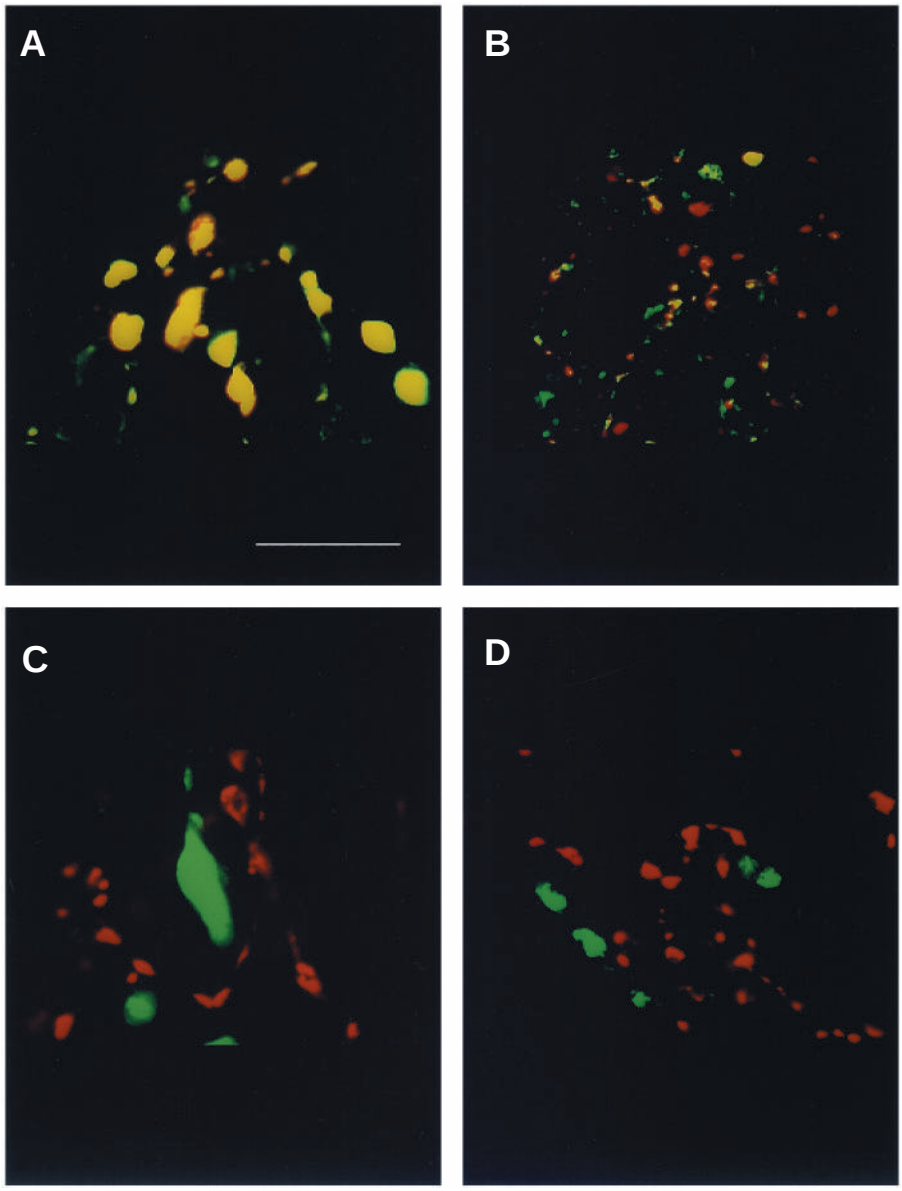

Fig. 7. Co-localization of CCK-like immunoreactivities in the STG neuropil. All images represent merged pseudocolor composites of simultaneously collected FITC- and Rhodamine-labeled images from single focal planes. Pseudocolor representation is the same as in Fig. 6. (A) An example of a filled region with 'large' varicosities showing that all varicosities greater than 10 μm in mean cross-sectional diameter exhibit co-localization for both 243-4 and C36-9H immunoreactivities. (B) An example of a region of a 243-4/C36-9H-labeled ganglion in which most varicosities are less than 10 μm in mean cross-sectional diameter. While some of the varicosities show co-localization for both immunoreactivities, others are uniquely labeled by one or the other antibody. (C,D) Two examples of staining from a 243-4/C37-4E-labeled ganglion showing that the profiles labeled by each antibody are distinct. All images are presented at the same scale. Scale bar, 25 μm.

labels any of the 28–32 neuronal somata found in the STG, each gives rise to intense labeling within the neuropil of the ganglion (Fig. 2). With each antibody (243-4, *N*=8 ganglia; C36-9H, *N*=6 ganglia; C37-4E, *N*=7 ganglia), immunoreactive fibers can be seen to descend through the stomatogastric nerve (*stn*) from the anteriorly located CGs and/or OG and to ramify within the STG, where they terminate. Since the fibers labeled by each of the antibodies generally travel as one or more tightly associated fascicles, it is difficult to determine accurately the number of fibers stained by each of the antibodies. However, in several preparations, antibodies 243-4 (*N*=4 of eight ganglia) and C36-9H (*N*=4 of six ganglia) can be seen to label at least four fibers, while only two fibers are routinely seen in C37-4E-labeled ganglia (*N*=5 of seven ganglia).

Within the STG neuropil, each of the patterns of labeling is composed of immunopositive neurites and varicosities. Striking differences are seen in the size of the neuropilar varicosities labeled by each of the antibodies. With C37-4E, measurements of the major cross-sectional diameter of sample populations of labeled profiles show that essentially all of the varicosities are less than 10 μm in diameter (hereafter referred to as 'small' profiles). Similar measurements taken from ganglia labeled with 243-4 and C36-4E show that, in addition to these small profiles, a population of varicosities greater than 10 μm in diameter (hereafter referred to as 'large' profiles), including a significant number of profiles greater than 15 μm, is also present. While the small profiles stained by all three antibodies are distributed throughout the entire peripheral neuropil, the large profiles are concentrated at the anterior end of this structure. This differential distribution is particularly evident in the C36-9H-labeled ganglion shown in Fig. 2B.

CCK-like immunoreactivity in the commissural and oesophageal ganglia

Approximately 40 of the 50 or so axons that project to the STG in *C. borealis* originate in the paired CGs (Coleman *et al.* 1992). Antibodies 243-4, C36-9H and C37-4E all label complements of neuronal somata and neuropil within the CGs (Fig. 3). 243-4-labeled ganglia (*N*=16) show 5–10 immunopositive somata (mean 7±1 somata) as well as an intensely stained neuropilar plexus (Fig. 3A,B). The labeled somata ranged in size from 12 to 76 μm in diameter (mean diameter 36±15 μm, *N*=115 somata). The staining pattern seen with C36-9H (*N*=12 ganglia) contains 2–6 labeled somata (mean 4±1 somata), which ranged in size from 14 to 59 μm (mean diameter 32±11 μm, *N*=45 somata) as well as an intensely labeled neuropil (Fig. 3C,D). 5–12 immunopositive somata, ranging in size from 13 to 52 μm (mean diameter 36±9 μm, *N*=103 somata) and a large neuropilar plexus are seen in C37-4E-labeled ganglia (*N*=14 ganglia; Fig. 3E,F).

With all anti-CCK labels, fibers can be seen to exit the CG by way of the superior oesophageal nerves (*son*), to pass through these nerves and to enter the STG *via* the *stn.* All anti-CCK labels also show immunopositive fibers in the inferior oesophageal nerves (*ion*). In preparations labeled with 243-4 and C37-4E, these fibers can be seen to project into the oesophageal nerve (*on*). No labeled fibers are seen in the *on* with C36-9H. All three of the CCK antibodies stain fibers in the circumoesophageal connectives (*coc*) that connect each CG with both the supraoesophageal ganglion (the 'brain') and the thoracic nervous system. A feature seen in 243-4- and C37-4E-labeled preparations, but absent in those stained with C36-9H, is fibers in the inferior ventricular nerve (*ivn*), a second connection between the STNS and the brain.

In addition to the STG inputs originating in the CGs, several OG neurons have been shown to project to the STG (Nusbaum and Marder, 1989; Coleman *et al.* 1992). While C36-9H labels no structures in the OG (*N*=6 ganglia), 243-4 (*N*=8 ganglia) and C37-4E (*N*=7 ganglia) each stain a pair of somata which range in diameter from 43 to 66 μm (mean diameter 56±6 μm, *N*=16 somata) and from 42 to 67 μm (mean diameter 56±8 μm, *N*=14 somata), respectively (Fig. 4). These somata send projections into the *ivn* and do not project to the STG.

The pattern of immunoreactivity seen with each of the three CCK antibodies in the *C. borealis* STNS is summarized schematically in Fig. 5.

Each antibody detects a distinct subset of CCK-like immunoreactive structures

To ascertain the extent to which the structures that are labeled by either of the monoclonal antibodies are contained within the set of structures stained by the polyclonal serum, double-label experiments were conducted. In these experiments, tissue was incubated with a primary antibody cocktail containing either 243-4 and C36-9H or 243-4 and C37-4E. These studies reveal that the majority of structures labeled by each monoclonal antobody are stained uniquely by that antibody. Likewise, most of the profiles labeled by 243-4 show no labeling with either of the monoclonal antibodies. As can be seen in Fig. 6A,B, several CG neurons do show co-localization of 243-4 and C36-9H immunoreactivities. In ganglia stained with 243-4/C36-9H cocktails (*N*=6), co-localization was present in 1–3 somata (mean 2±1 somata), which ranged in diameter from 21 to 66 μm (mean diameter 40±16 μm, *N*=12 somata) and were most commonly seen immediately adjacent to the *son*. Similarly, some of the CG neuropil and 1–2 fibers in each *coc* can be seen to contain both 243-4 and C36-9H immunoreactivities.

An identical situation exists in CGs stained using 243-4/C37-4E cocktails (Fig. 6C,D). In these ganglia (*N*=6), 2–4 somata (mean 3±1 somata), a subset of the neuropil and several *coc* fibers can be seen to exhibit both 243-4 and C36-9H immunoreactivities. Somata exhibiting this pattern of co-localization, as with those immunopositive for both 243-4 and C36-9H, range in diameter from 17 to 64 μm (mean diameter 39±11 μm) and are also found immediately adjacent to the *son.* In the OG (*N*=5 ganglia), 243-4 and C37-4E label the same pair of neurons (Fig. 6E,F).

Within the STG neuropil (*N*=5 ganglia), all of the stained

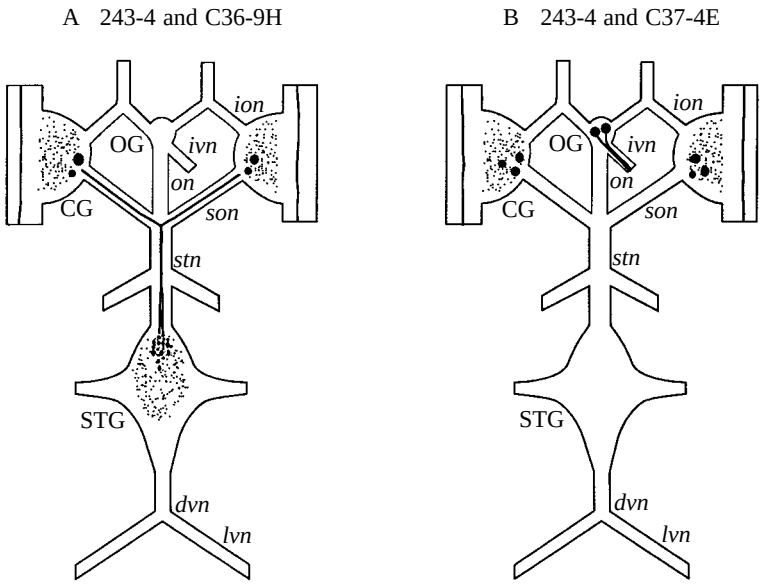

Fig. 8. Schematic representation of STNS structures exhibiting co-localization of (A) 243-4 and C36-9H and (B) 243-4 and C37-4E immunoreactivities. Abbreviations as in Fig. 1.

varicosities greater than 10 μm in major cross-sectional diameter exhibit both 243-4 and C36-9H immunolabeling (Fig. 7A), as do some of the smaller profiles (Fig. 7B). All of the large varicosities can be seen to arise from two double-labeled input fibers that travel to the STG from the CGs *via* the *son*. No profiles within the STG neuropil (*N*=5 ganglia) show a co-localization of 243-4 and C37-4E immunoreactivities (Fig. 7C,D).

Fig. 8 summarizes in schematic form the distribution of structures that exhibit 243-4/C36-9H (Fig. 8A) and 243-4/C37-4E (Fig. 8B) colocalization.

Discussion

Recent reports have shown that peptides similar to CCK are found in the STG and neurohemal organs of several crustacean species, including the spiny lobster *P. interruptus* and the American lobster *H. americanus* (Turrigiano and Selverston, 1989, 1990, 1991; Turrigiano *et al.* 1994). In *P. interruptus*, five distinct CCK-like moieties (peaks A–E) were isolated from the pericardial organs, with two additional species (peaks F–G) being found only in the STG. In the one species of crab investigated, *Cancer antenarius*, no CCK-like immunoreactivity was found in the STG; however, immunoreactivity was present in neurohemal structures (Turrigiano and Selverston, 1991). On the basis of these findings, it was postulated that, in the STG of lobsters, CCK-like peptides function as both neurohormones and local modulators, while in the crab, they serve only a neurohormonal role. Our findings suggest that, in at least *C. borealis*, a local modulatory function is also likely.

In studying the distribution of CCK-like peptides in the *C. borealis* STNS, we have employed three antibodies, each generated against mammalian CCK8. While all show strong and specific recognition for CCK-like peptides, each stains a unique set of structures within the STNS. The most likely explanation for these different patterns of distribution is that each antibody is recognizing a different CCK-like peptide(s) and that these distinct peptides are differentially distributed within the STNS. Using the same monoclonal antibodies employed in our study, P. Sithigorngul, C. Cowden and A. O. W. Stretton (in preparation) obtained similar results in the nematode *Ascaris suum.* As the presence of multiple CCK-like peptides has been documented in a number of organisms, it will be interesting to see whether the antibodies employed in our study can be used to elucidate differential distributions of CCK-like peptides in these other systems.

Families of related peptides have been described in many systems. Two recently documented examples are the buccalin and myomodulin families of peptides. Both the buccalins and the myomodulins were originally isolated from the accessory radula closer nerve–muscle system of the marine mollusc *Aplysia californica* (Cropper *et al.* 1987*a*,*b*, 1988; Miller *et al.* 1993*a*,*b*). Nineteen distinct buccalin-like peptides (buccalin A–S) and nine distinct myomodulin-like peptides (myomodulins A–I) are present in *A. californica* (Miller *et al.* 1993*a*,*b*). In the *C. borealis* STNS, two FMRFamide-like peptides, TNRNFLRFamide and SDRNFLRFamide, have recently been biochemically isolated (Weimann *et al.* 1993).

The detection of a family of differentially distributed CCK-like peptides raises the question of the individual function of each of the related molecules. While the differential distribution of the CCK-like peptides points towards the possibility that each peptide elicits a distinct repertoire of behavioral outputs, it is possible that some or most of the molecules share a common function. All physiological studies of CCK-like peptide modulation of the STG have been carried out on the spiny lobster *P. interruptus*. Physiological application of one of the partially purified *P. interruptus* peptides, peak E, to the STG of this animal has shown that this native crustacean peptide is a more powerful modulator of the STG than is $CCK8SO_4$ itself (Turrigiano *et al.* 1994). The

physiological actions of the other partially purified *P. interruptus* peaks have yet to be determined. A series of experiments designed to determine the actions of CCK-like molecules on the STG motor patterns of *C. borealis* is planned, as are the isolation and characterization of the native CCK-like peptides from this animal.

We thank Dr A. Stretton (University of Wisconsin, Madison) for the anti-CCK monoclonal antibodies C36-9H and C37-4E and Ms Simona Retter for her participation in some preliminary experiments using the monoclonal anti-CCK antibodies. Research supported by NS 17813 (E.M.), NIH Shared Instrument Grant RR05615 (Brandeis University), NS15697 (K.G.) and a grant from the Human Frontiers Science Program Organization. E.M. gratefully acknowledges the support of the W. M. Keck Foundation.

References

BARKER, D. L., KUSHNER, P. D. AND HOOPER, N. K. (1979). Synthesis of dopamine and octopamine in the crustacean stomatogastric nervous system. *Brain Res*. **161**, 99–113.

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

BELTZ, B. A. AND KRAVITZ, E. A. (1983). Mapping of serotonin-like immunoreactivity in the lobster nervous system. *J. Neurosci*. **3**, 585–602.

BLITZ, D. M., CHRISTIE, A. E., MARDER, E. AND NUSBAUM, M. P. (1994). Distribution and effects of tachykinin-like peptides in the stomatogastric nervous system of the crab, *Cancer borealis. J. comp. Neurol.* (in press).

BROOKS, P. A. AND KELLEY, J. S. (1985). Cholecystokinin as a potent excitant of neurons of the dentate gyrus of rats. *Ann. N.Y. Acad. Sci.* **448**, 361–374.

CALLAWAY, J. C., MASINOVSKY, B. AND GRAUBARD, K. (1987). Co-localization of SCP_B-like and FMRFamide-like immunoreactivities in crustacean nervous systems. *Brain Res.* **405**, 295–304.

CHRISTIE, A. E., BALDWIN, D., GRAUBARD, K. AND MARDER, E. (1992). A comparison of the microstructure of cholecystokinin-like immunoreactivity in the stomatogastric ganglion of three species of decapod crustacea. *Soc. Neurosci. Abstr.* **18**, 471.

CHRISTIE, A. E., HALL, C., OSHINSKY, M. AND MARDER, E. (1994). Buccalin-like and myomodulin- like peptides in the stomatogastric ganglion of the crab *Cancer borealis. J. exp. Biol*. **193**, 337–343.

CHRISTIE, A. E., NORRIS, B. J., COLEMAN, M. J., MARDER, E. AND NUSBAUM, M. P. (1993). Neuropil arborization and transmitter complement of a modulatory neuron. *Soc. Neurosci. Abstr.* **19**, 931.

CHRISTIE, A. E., TURRIGIANO, G. G., RETTER, S., NECKAMEYER, W. S. AND MARDER, E. (1991). Immunocytochemical identification of multiple CCK-related peptides in the stomatogastric nervous system of several species of decapod crustacea. *Soc. Neurosci. Abstr*. **17**, 278.

CLAIBORNE, B. J. AND SELVERSTON, A. I. (1984). Histamine as a neurotransmitter in the stomatogastric nervous system of the spiny lobster. *J. Neurosci*. **4**, 708–721.

COLEMAN, M. J., NUSBAUM, M. P., COURNIL, I. AND CLAIBORNE, (1992). Distribution of modulatory inputs to the stomatogastric ganglion of the crab, *Cancer borealis*. *J. comp. Neurol*. **325**, 581–594.

CROPPER, E. C., LLOYD, P. E., REED, W., TENENBAUM, R., KUPFERMANN, I. AND WEISS, K. R. (1987*a*). Multiple neuropeptides in cholinergic motor neurons of *Aplysia*: evidence for modulation intrinsic to the motor circuit. *Proc. natn. Acad. Sci. U.S.A.* **84**, 3486–3490.

CROPPER, E. C., MILLER, M. W., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1988). Structure and action of buccalin: a modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of *Aplysia californica*. *Proc. natn. Acad. Sci. U.S.A.* **85**, 6177–6181.

CROPPER, E. C., TENENBAUM, R., KOLKS, M. A. G., KUPFERMANN, I. AND WEISS, K. R. (1987*b*). Myomodulin: a bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **84**, 5438–5468.

DOCKARY, G. J. (1976). Immunocytochemical evidence of cholecystokinin-like peptides in brain. *Nature* **264**, 568–570.

DOCKARY, G. J., DESMOND, H., GAYTON, R. J., JONSSON, A., RAYBOULD, H., SHARKEY, K. A., VARRO, A. AND WILLIAMS, R. G. (1985). Cholecystokinin and gastrin forms in the nervous system. *Ann. N.Y. Acad. Sci*. **448**, 32–43.

DOCKARY, G. J., DUVE, H. AND THORPE, A. (1981). Immunocytochemical characterization of gastrin/cholecystokinin-like peptides in the brain of the blowfly, *Calliphora vomitoria. Gen. comp. Endocr*. **45**, 491–496.

FAVREL, P., VAN-WORMHOUDT, A., STUDLER, J. M. AND BELLOWS, C. (1987). Immunochemical and biochemical characterization of gastrin/CCK-like peptides in *Palaemon serratus* (Crustacea Decapoda): intermolt variations. *Gen. comp. Endocr*. **65**, 363–372.

GOLDBERG, D., NUSBAUM, M. P. AND MARDER, E. (1988). Substance P-like immunoreactivity in the stomatogastric nervous tissue of the crab *Cancer borealis* and the lobsters *Panulirus interruptus* and *Homarus americanus. Cell Tissue Res*. **252**, 515–522.

GRIMMELIKHUIJZEN, C. J. P., SUNDLER, F. AND REHFELD, F. (1980). Gastrin/CCK-like immunoreactivity in the nervous system of Coelenterates. *Histochem*. **69**, 61–66.

HARRIS-WARRICK, R. M. AND MARDER, E. (1991). Modulation of neural networks for behavior. *A. Rev. Neurosci*. **14**, 39–57.

HARRIS-WARRICK, R. M., MARDER, E., SELVERSTON, A. I. AND MOULINS, M. (eds) (1992). *Dynamic Biological Networks: The Stomatogastric Nervous System*. Cambridge, MA: MIT Press.

JOHNSEN, A. H. AND REHFELD, J. F. (1990). Cionin: a disulfotyrosyl hybrid of cholecystokinin and gastrin from the neural ganglion of the protochordate *Ciona intestinalis*. *J. biol. Chem*. **265**, 3054–3058.

KATZ, P. S., EIGG, M. H. AND HARRIS-WARRICK, R. M. (1989). Serotonergic/cholinergic muscle receptor cells in the crab stomatogastric nervous system. I. Identification and characterization of the gastropyloric receptor cells. *J. Neurophysiol.* **62**, 558–570.

LARSON, B. A. AND VIGNA, R. S. (1983). Species and tissue distribution of cholecystokinin/gastrin-like substances in some invertebrates. *Gen. comp. Endocr.* **50**, 469–475.

MARDER, E. (1987). Neurotransmitters and neuromodulators. In *The Crustacean Stomatogastric System* (ed. A. I. Selverston and M. Moulins), pp. 263–300. Berlin: Springer-Verlag.

MARDER, E., CALABRESE, R. L., NUSBAUM, M. P. AND TRIMMER, B. A. (1987). Distribution and partial characterization of FMRFamide-like peptides in the stomatogastric nervous system of the rock crab,

Cancer borealis and the spiny lobster, *Panulirus interruptus. J. comp. Neurol*. **259**, 150–163.

MARDER, E., HOOPER, S. L. AND SIWICKI, K. K. (1986). Modulatory action and distribution of the neuropeptide proctolin in the crustacean stomatogastric nervous system. *J. comp. Neurol*. **243**, 454–467.

MARDER, E. AND NUSBAUM, M. (1989). Peptidergic modulation of the motor pattern generators in the stomatogastric ganglion. In *Perspectives in Neural Systems and Behavior* (ed. T. J. Carew and D. B. Kelly), pp. 73–91. New York: Liss.

MARDER, E. AND WEIMANN, J. M. (1992). Modulatory control of multiple task processing in the stomatogastric nervous system. In *Neurobiology of Motor Progamme Selection* (ed. J. Kien, C. McCrohan and B. Winslow), pp. 3–19. New York: Pergamon Press.

MILLER, M. W., BEUSHAUSEN, S., CROPPER, E. C., EISINGER, K., STAMM, S., VILIM, F., VITEK, A., ZAJC, A., KUPFERMANN, I., BROSIUS, J. AND WEISS, K. R. (1993*a*). The buccalin-related neuropeptides: isolation and characterization of an *Aplysia* cDNA clone encoding a family of peptide cotransmitters. *J. Neurosci*. **13**, 3346–3357.

MILLER, M. W., BEUSHAUSEN, S., VITEK, A., STAMM, S., KUPFERMANN, I., BROSIUS, J. AND WEISS, K. R. (1993*b*). The myomodulin-related neuropeptides: characterization of a gene encoding a family of peptide cotransmitters in *Aplysia*. *J. Neurosci.* **13**, 3358–3367.

MORTIN, L. I. AND MARDER, E. (1991). Differential distribution of β-pigment dispersing hormone (β-PDH)-like immunoreactivity in the stomatogastric nervous system of five species of decapod crustaceans. *Cell Tissue Res*. **265**, 19–33.

NACHMAN, R. J., HOLMAN, G. M., HADDON, W. R. AND LING, N. (1986). Leucosulphakinin, a sulfated insect neuropeptide with homology to gastrin and cholecystokinin. *Science* **234**, 71–73.

NICHOLS, R. (1992). Isolation and expression of the *Drosophila* drosulfakinin neural peptide gene product, DSK-I. *Molec. cell. Neurosci*. **3**, 342–347.

NICHOLS, R., SCHNEUWLY, S. A. AND DIXON, J. E. (1988). Identification and characterization of a *Drosophila* homologue to the vertebrate neuropeptide cholecystokinin. *J. biol. Chem*. **263**, 12167–12170.

NUSBAUM, M. P., COURNIL, I., GOLOWASCH, J. AND MARDER, E. (1989). Modulating rhythmic motor activity with a dual-transmitter neuron. In *Neural Mechanisms of Behavior* (ed. J. Erber, R. Menzel, H.-J. Pfluger and D. Todt), pp. 228. Stuttgart: Georg Thieme Verlag.

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

NUSBAUM, M. P. AND MARDER, E. (1989). A modulatory proctolin containing neuron (MPN). I. Identification and characterization. *J. Neurosci.* **9**, 1591–1599.

SELVERSTON, A. I. AND MOULINS, M. (1987). *The Crustacean Stomatogastric Nervous System*. Berlin, Heidelberg, New York: Springer.

SKIEBE, P. AND SCHNEIDER, H. (1994). Allatostatin peptides in the crab stomatogastric nervous system: inhibition of the pyloric motor pattern and distribution of allatostatin-like immunoreactivity. *J. exp. Biol*. **194**, 195–208.

TURRIGIANO, G. G. AND SELVERSTON, A. I. (1989). Cholecystokinin-like peptide is a modulator of a crustacean central pattern generator. *J. Neurosci*. **9**, 2486–2501.

TURRIGIANO, G. G. AND SELVERSTON, A. I. (1990). A cholecystokinin-like hormone activates a feeding-related neural circuit in lobster. *Nature* **344**, 866–868.

TURRIGIANO, G. G. AND SELVERSTON, A. I. (1991). Distribution of cholecystokinin-like immunoreactivity within the stomatogastric nervous systems of four species of decapod crustacea. *J. comp. Neurol*. **305**, 164–176.

TURRIGIANO, G. G., VAN WORMHOUDT, A., OGDEN, L. AND SELVERSTON, A. I. (1994). Partial purification, tissue distribution and modulatory activity of a crustacean cholecystokinin-like peptide. *J. exp. Biol*. **187**, 181–200.

VIGNA, S. R. (1985). Cholecystokinin and its receptors in vertebrates and invertebrates. *Peptides* **6**, 283–287.

WEIMANN, J. M., MARDER, E., EVANS, B. AND CALABRESE, R. L. (1993). The effects of SDRNFLRFamide and TNRNFLRFamide on the motor patterns of the stomatogastric ganglion of the crab *Cancer borealis*, *J. exp. Biol*. **181**, 1–26.