The Journal of Experimental Biology **198**, 2431–2439 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
JEB9908

MATRIX OF NEUROMODULATORS IN NEUROSECRETORY STRUCTURES OF THE CRAB *CANCER BOREALIS*

ANDREW E. CHRISTIE*, PETRA SKIEBE† AND EVE MARDER‡
Department of Biology and Volen Center, Brandeis University, Waltham, MA 02254, USA

Accepted 15 September 1995

Summary

The crustacean stomatogastric ganglion, which is situated in the ophthalmic artery, can be modulated by both intrinsically released molecules and hormones. In the crab *Cancer borealis*, over a dozen neuroactive compounds have been identified in the input axons that project into the stomatogastric neuropil. However, little is known about the modulator content of the two major neurohemal organs, the sinus glands and the pericardial organs, in this crab. We now report the results of a series of immunocytochemical experiments designed to identify putative neurohormones in these tissues. We find that the majority of modulators present in the input axons of the stomatogastric ganglion are also present in at least one of the neurohemal organs. Specifically, allatostatin-like, buccalin-like, cholecystokinin-like, FLRFamide-like, GABA-like, locustatachykinin-like, myomodulin-like, proctolin-like, red pigment concentrating hormone-like and serotonin-like immunoreactivities are all present in both the stomatogastric neuropil and at least one of the neurohemal organs. Thus, these substances are likely to serve a dual role as both local and hormonal modulators of the stomatogastric network. Two other substances, β-pigment dispersing hormone and crustacean cardioactive peptide, are not present in the stomatogastric neuropil, but β-pigment dispersing hormone immunoreactivity is present in the sinus glands and crustacean cardioactive peptide immunoreactivity is present in the pericardial organs. It is likely that crustacean cardioactive peptide exerts its influence on the stomatogastric neural circuit *via* hormonal pathways. Double-labeling experiments show that the patterns of modulator co-localization present in the stomatogastric neuropil are different from those in the neurosecretory organs, suggesting that few rules of co-localization hold across these tissues.

Key words: neurohormone, immunoreactivity, neuropeptides, stomatogastric ganglion, Crustacea, crab, *Cancer borealis*.

Introduction

Recent work on the stomatogastric ganglion (STG) has shown that this portion of the crustacean nervous system is an excellent preparation for studying the neuromodulatory control of a small neural circuit (Harris-Warrick *et al.* 1992). The STG is situated in the ophthalmic artery and controls the rhythmic movements of the gastric mill and pyloric regions of the foregut. Like most neural circuits, the STG is modulated by a variety of neuroactive agents that are delivered to the ganglion by both input axons and the hemolymph (Marder, 1987; Harris-Warrick *et al.* 1992; Skiebe and Schneider, 1994; Blitz *et al.* 1995). In one of the most well-studied crustacean species, the crab *Cancer borealis*, over 12 distinct neuroactive molecules have been identified in the input axons that project to the STG (Marder *et al.* 1994). Among these substances are γ-aminobutyric acid (GABA; Nusbaum *et al.* 1989), serotonin (5-HT; Beltz *et al.* 1984; Katz *et al.* 1989), histamine (Christie, 1995) and dopamine (Marder, 1987) as well as neuropeptides identical to or related to allatostatin (Skiebe and Schneider, 1994), buccalin (Christie *et al.* 1994*a*), cholecystokinin (CCK; Christie *et al.* 1995), SDRNFLRFamide and TNRNFLRFamide (FLRFamide; Marder *et al.* 1987; Weimann *et al.* 1993), locustatachykinin (Goldberg *et al.* 1988; Blitz *et al.* 1995), myomodulin (Christie *et al.* 1994*a*), proctolin (Marder *et al.* 1986) and red pigment concentrating hormone (RPCH; Nusbaum and Marder, 1988). Several studies have shown that the modulatory inputs that project to the STG often contain more than one neuroactive substance (Katz *et al.* 1989; Nusbaum and Marder, 1989; Christie *et al.* 1993, 1995; Skiebe and Schneider, 1994; A. E. Christie, B. J. Norris, M. J. Coleman, I. Cournil, M. P. Nusbaum and E. Marder, in preparation).

*Present address: Department of Neuroscience, 215 Stemmler Hall, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.
†Present address: Institute for Neurobiology, Free University of Berlin, Konigin Luise-Strassse 28/30, D-14195, Berlin, Germany.
‡Author for correspondence.

Like locally released modulators, hormones delivered to the ganglion *via* the hemolymph are capable of exerting a modulatory influence on the STG (Turrigiano and Selverston, 1990). In decapod crustaceans, neurohormones are known to be released into the hemolymph from the pericardial organ (PO), located in the pericardial cavity surrounding the heart, and from the sinus gland (SG), located in the eyestalks (Cooke and Sullivan, 1982). While a variety of neuromodulatory agents have been identified in the POs and SGs of other crustacean species (Cooke and Sullivan, 1982; Stangier *et al.* 1988; Dircksen *et al.* 1987, 1988; Dircksen, 1992, 1994; Keller, 1992), little is known about the hormone complement of these structures in *C. borealis*. In this paper, we demonstrate that many of the neuromodulatory substances found in the input axons of the STG are also present in the POs and/or SGs. Additionally, crustacean cardioactive peptide (CCAP), which is found in neurosecretory structures of other crustacean species (Stangier *et al.* 1988), is found in the POs of *C. borealis* and β-pigment dispersing hormone (β-PDH) is present in the sinus gland of *C. borealis.* We find that few of the patterns of co-localization present in the STG input axons are preserved in the neurosecretory organs. Taken collectively, our data suggest that many neuroactive substances may serve a dual function as both an intrinsically released modulator and a hormonal modulator in the *C. borealis* STG. Moreover, few rules of co-localization apply across these tissues. Some of these data have appeared previously in abstract form (Christie *et al.* 1994*b*).

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 artificial seawater aquaria at 10–12 ˚C.

Peptides

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

Antibodies

All of the antibodies used in this study have been previously used in studies of the crustacean stomatogastric nervous system and/or other crustacean tissues. The references that follow the description of each antibody describe the development of the antibody and the specificity of its staining in crustacean nervous tissue. The anti-buccalin used was a rabbit polyclonal antiserum raised against buccalin A (Miller *et al.* 1992; Christie *et al.* 1994*a*). This antibody was employed at a final dilution of 1:300. The anti-myomodulin was a rabbit polyclonal antiserum raised against myomodulin C (Miller *et al.* 1991; Christie *et al.* 1994*a*), used at a final dilution of 1:300. For the localization of proctolin immunoreactivity, a rabbit anti-proctolin polyclonal antibody (Davis *et al.* 1989) was used at a concentration of 1:300. This antibody stains identical structures seen with other proctolin antibodies in the stomatogastric nervous system (Marder *et al.* 1986). FLRFamide-like immunoreactivity was examined using a rabbit anti-FMRFamide polyclonal antibody (671M; Marder *et al.* 1987) at a dilution of 1:200. While this antibody was generated against FMRFamide, it shows equal or greater recognition for extended FLRFamide-like peptides (Marder *et al.* 1987), which have been purified from *C. borealis* and make up the largest component of the RFamide-related peptides in *C. borealis* (Weimann *et al.* 1993). Biochemical studies failed to find any FMRFamide in these animals (Marder *et al.* 1987; Weimann *et al.* 1993). Additionally, in other crustacean species, all of the peptides thus far characterized in this family have been extended FLRFamide peptides (Trimmer *et al.* 1987; Mercier *et al.* 1993; Keller, 1992). Together these data indicate that the staining obtained with this antiserum is due to the presence of one or more of the family of FLRFamide peptides, and we hereafter will refer to this antibody as an anti-FLRFamide antibody. The anti-RPCH was a rabbit polyclonal antibody (Madsen *et al.* 1985; Nusbaum and Marder, 1988). This antibody was used at a concentration of 1:200. Locustatachykinin-like immunoreactivity was examined using a rat anti-substance-P monoclonal antibody (Accurate) at a final dilution of 1:300 (Goldberg *et al.* 1988). This antibody has broad specificity for a wide range of tachykinin-like peptides. We now believe that the anti-substance P antibody labels peptides more closely related to the insect tachykinins than to vertebrate tachykinins for reasons discussed in detail in Blitz *et al.* (1995), and therefore call this immunoreactivity locustatachykinin-like immunoreactivity (Blitz *et al.* 1995). 5-HT-like immunoreactivity was studied using a rat anti-5-HT monoclonal antibody (Accurate) at 1:200 (Beltz *et al.* 1984; Katz *et al.* 1989). CCK-like immunoreactivity was studied using two mouse monoclonal antibodies, anti-$CCK_{C36\text{-}9H}$ and anti-$CCK_{C37\text{-}4E}$, and one rabbit polyclonal antibody, anti-$CCK_{243\text{-}4}$, all generated against mammalian CCK8 (Turrigiano and Selverston, 1991; Christie *et al.* 1995; P. Sithigorngul, C. Cowden and A. O. W. Stretton, in preparation). Previous work has shown that each of these antibodies recognizes a different set of CCK-like molecules in *C. borealis* (Christie *et al.* 1995). All of the CCK antibodies were used at final concentrations of 1:300. The anti-β-pigment dispersing hormone (β-PDH) antibody used in this study was a rabbit polyclonal antibody (Dircksen *et al.* 1987; Mortin and Marder, 1991), used at a final dilution of 1:500. The anti-crustacean cardioactive peptide (CCAP) antibody used was a rabbit polyclonal antibody (Stangier *et al.* 1988) and was used at a final dilution of 1:500. The anti-GABA antibody was a rabbit polyclonal antibody (Sigma) and was used at 1:500 (A. E. Christie and M. P. Nusbaum, unpublished results). The anti-allatostatin was a rabbit polyclonal antibody (Skiebe and Schneider, 1994) and was used at a final dilution of 1:500.

Our prior experience with the stomatogastric nervous system of *C. borealis* failed to indicate any cross-reactivity of these

reagents and suggested that, although some of these antibodies label classes of molecules (e.g. anti-FLRFamide, etc.), each appears to be labeling a unique set of antigens. However, because there is always the possibility that different peptides may be present in the sinus gland or pericardial organ and in the stomatogastric nervous system, we did an additional series of preabsorption controls in the pericardial organ for those antibodies used in double-labeling experiments that are known to label families of molecules. In these experiments, the antiserum was preincubated with the peptide for 2 h at room temperature prior to application to the pericardial organs. FLRFamide-like staining was not blocked by 10^{-5} mol l^{-1} $CCK8SO_4$ (*N*=3) or RPCH (*N*=3). RPCH-like staining was not blocked by 10^{-5} mol l^{-1} TNRNFLRFamide (*N*=3) or $CCK8SO_4$ (*N*=3). Proctolin-like staining was not blocked by 10^{-5} mol l^{-1} substance P (*N*=3). CCK-like (C-36) staining was not blocked by preincubation with TNRNFLRFamide or RPCH. The staining in the pericardial organs generated by each of the above-mentioned antisera was abolished by preabsorption with 10^{-7} mol l^{-1} of the peptide used to generate the antiserum (*N*=2 preparations for each antibody; TNRNFLRFamide was used to block anti-FLRFamide).

The secondary antisera were goat anti-mouse, goat anti-rabbit and goat anti-rat affinity-purified IgGs labeled with either fluorescein or rhodamine. All secondary antisera were purchased from Boehringer-Mannheim and used at final dilutions of 1:25.

Whole-mount immunocytochemistry

Pericardial organs (POs) and eyestalks were processed for immunocytochemistry as whole mounts using indirect immunofluorescence methods modified from Beltz and Kravitz (1983). Animals were cold-anesthetized by packing in ice for 15–30 min prior to dissection. All tissue 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 in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.3–7.4) containing either 4 % paraformaldehyde (for anti-5-HT, anti-GABA, anti-CCK_{C36-9H}, anti-CCK_{C37-4E}, anti-CCK_{243-4}, anti-RPCH, anti-FLRFamide, anti-proctolin, anti-substance P, anti-β-PDH, anti-myomodulin and anti-buccalin) or 4 % paraformaldehyde and 1 % 1-ethyl 3(3 dimethylaminopropyl)-carbodiimide (EDAC; for anti-CCAP), 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 a temperature of approximately 4 ˚C. The incubation in primary antibody (or antibodies in the case of double-labeling experiments) was performed in P-Triton for 18–72 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 incubations were also carried out in P-Triton. For double-labeling experiments, a cocktail of goat anti-mouse and goat anti-rabbit or goat anti-rat 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, tissues were mounted between glass coverslips using a solution of 80 % glycerin, 20 % 20 mmol l^{-1} sodium carbonate, pH 9.5.

Data collection

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 fluorescein (excitor filter, 488 nm DF10; dichroic reflector, 510 nm LP; emission filter, 515 nm LP)] filter blocks provided by the manufacturer. For double-labeled preparations, the manufacturer-supplied 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) filter set was employed. All micrographs were printed using a Sony Mavigraph color video printer.

Results

Neurohormones in the sinus gland

In decapod crustaceans, hormones released from several peripheral neurosecretory sites (Fig. 1) have been shown to modulate the output of more centrally located neurons, including neurons contained within the stomatogastric ganglion (STG; Turrigiano and Selverston, 1990). Primary among these peripheral neurosecretory tissues are the sinus glands (SGs), located in the eyestalks, and the pericardial organs (POs), located on the walls of the pericardial cavity. As is schematically illustrated in Fig. 1, hormones released from both the SGs and the POs are delivered to the STG through a semi-closed hemolymph system. Specifically, hormone-containing hemolymph is delivered to the STG *via* the ophthalmic artery, in which the STG is located.

In brachyuran crabs, such as *C. borealis*, the SG is recognizable as an iridescent white structure located just below the connective tissue sheath of the optic ganglia (Cooke and Sullivan, 1982). In size, the SG is approximately 500 μm long, 300 μm wide and 100 μm thick (Fig. 2A). The majority of neurons that project to the SG are located in the X-organ (XO), a loosely associated group of several hundred somata present in the medulla terminalis ganglion of the eyestalk (Cooke and Sullivan, 1982). The XO somata project to the SG *via* the sinus gland nerve. While it is postulated that 90 % of the inputs to the crustacean SG are from the XO somata, several additional input axons project to the SG from somata located in the supraoesophageal ganglion, commonly referred to as ‘the brain’, and other more posteriorly located ganglia (Cooke and Sullivan, 1982).

Of the 14 antibodies used in this study, only anti-allatostatin (*N*=8), anti-β-PDH (*N*=8), anti-proctolin (*N*=8) and anti-RPCH (*N*=8) give rise to labeling in the *C. borealis* SG (Fig. 2). In

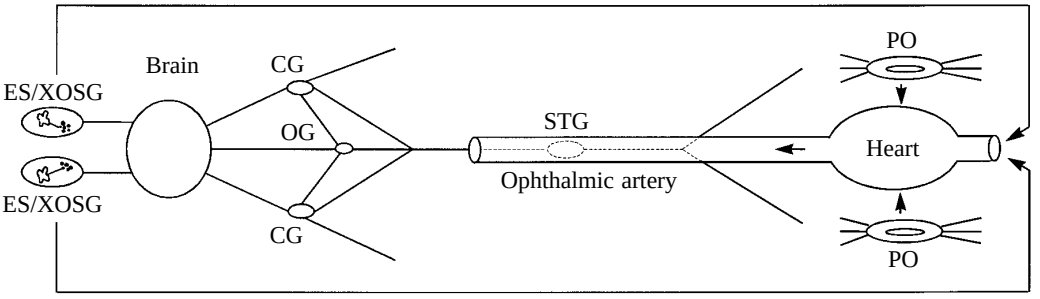

Fig. 1. Schematic representation of neurohormonal pathways to the stomatogastric ganglion (STG). The X-organ/sinus gland complex (XOSG) and the pericardial organs (PO) are the major neurohemal structures in the crab. Hormones released into the hemolymph by both the XOSGs, located in the eyestalk (ES), and the POs, located in the pericardial chamber, are pumped by the heart through the ophthalmic artery to the stomatogastric ganglion. Neurohormones and modulator-containing axons [which project to the STG from the more anteriorly located commissural ganglia (CG), oesophageal ganglion (OG) and supraoesophageal ganglion (brain)], have been shown to influence the motor output of the STG neural circuit.

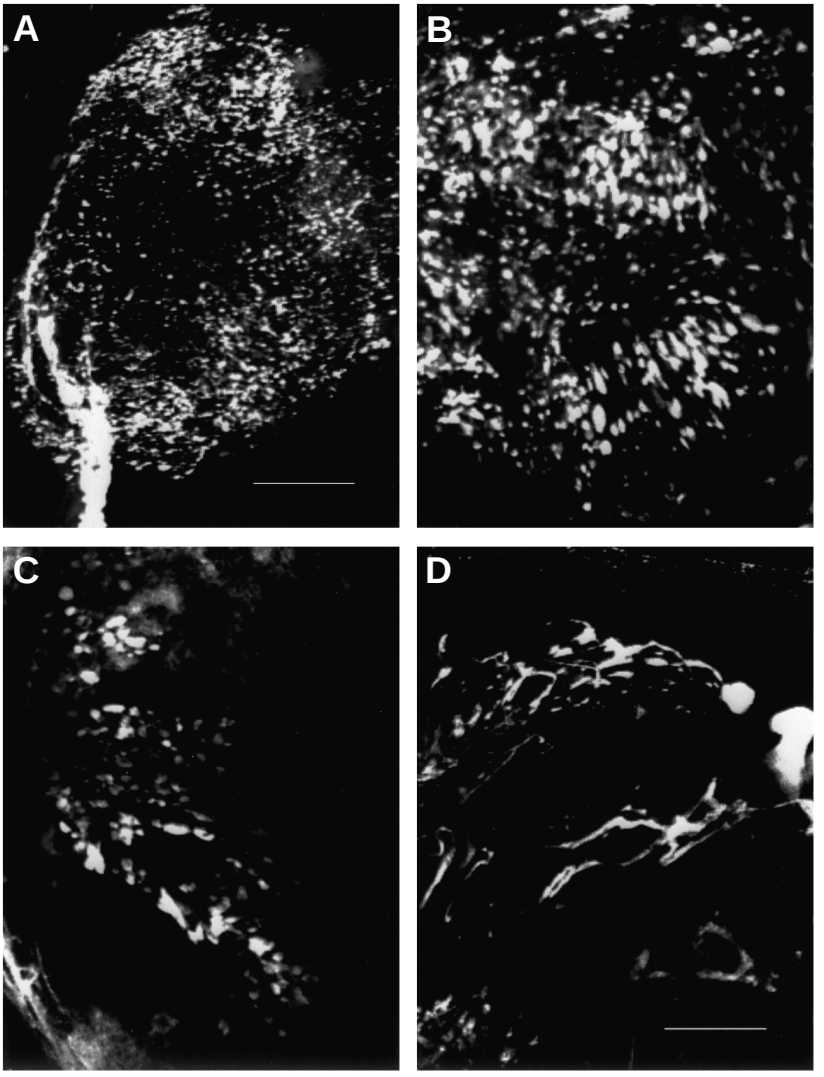

Fig. 2. Modulator immunolabeling in the sinus gland of the crab *Cancer borealis*. (A) Low-magnification view of red pigment concentrating hormone-like immunoreactivity in the sinus gland. This image, a maximum projection of 40 optical sections taken at 2.0 μm intervals, shows the entire sinus gland, which is composed of input fibers and their associated neurosecretory varicosities. (B) A high-magnification view of allatostatin-like immunoreactive profiles in the sinus gland. This image is a maximum projection of 15 optical sections taken at 1.0 μm intervals. (C) A high-magnification view of proctolin-like immunoreactive profiles in the sinus gland. This image is a maximum projection of five optical sections taken at 1.0 μm intervals. (D) A high-magnification view of β-pigment dispersing hormone-like immunoreactive profiles in the sinus gland. This image is a maximum projection of 20 optical sections taken at 1.0 μm intervals. Scale bar in A, 100 μm. B, C and D are presented at the same scale. Scale bar in D, 50 μm.

each case, these antibodies label both fibers and varicosities within the SG. Fibers labeled by anti-allatostatin, anti-proctolin and anti-RPCH are generally only faintly stained and average less than 1.0 μm in diameter. In contrast, the fibers stained by anti-β-PDH are more intensely labeled and are much larger, often exceeding 10 μm in diameter. The varicosities are brightly stained by all four labels, slightly ovoid and, on average, 15 μm in major cross-sectional diameter.

The source of most of the allatostatin-like, proctolin-like and RPCH-like immunoreactivity appears to be the XO. In these preparations, fiber bundles can be seen to project from labeled somata in the XO to the SG. Anti-β-PDH does not label any

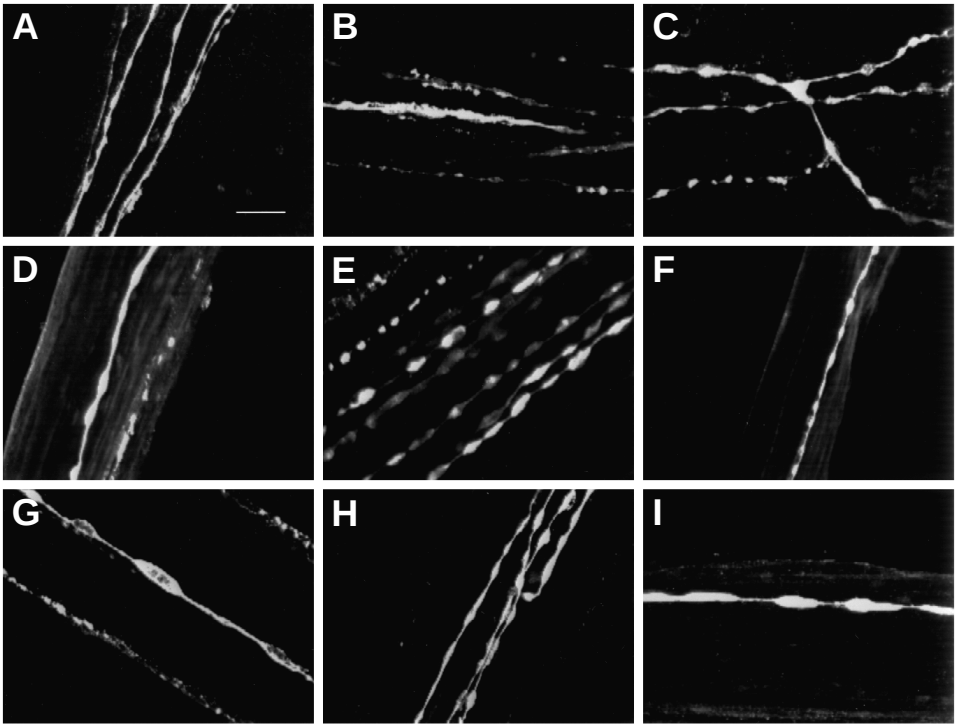

Fig. 3. Modulator immunolabeling in nerve trunks of the pericardial organ of the crab *Cancer borealis*. Immunocytochemical experiments show that (A) allatostatin-like, (B) buccalin-like, (C) crustacean cardioactive peptide-like, (D) $CCK_{243\text{-}4}$-like, (E) $CCK_{C36\text{-}9H}$-like, (F) FLRFamide-like, (G) GABA-like, (H) myomodulin-like and (I) 5-HT-like labeling are present in the POs. Images are maximum projections of (A) 33, (B) 16, (C) 28, (D) 10, (E) 20, (F) 20, (G) 10, (H) 15 and (I) 10 optical sections taken at 1.0 μm intervals. All images are presented at the same scale. Scale bar, 50 μm.

somata in the region of XO. In anti-β-PDH-labeled eyestalks, it is a fascicle of intensely immunoreactive fibers projecting from the optic nerve that is the source of the staining seen in the SG.

While not the focus of this paper, it is important to note that other structures in the eyestalk may also serve a neurohemal function (Cooke and Sullivan, 1982). Although not in the SG, buccalin-like (N=8), $CCK_{243\text{-}4}$-like (N=8), $CCK_{C36\text{-}9H}$-like (N=8), $CCK_{C37\text{-}4E}$-like (N=8), GABA-like (N=8), FLRFamide-like (N=8), locustatachykinin-like (N=8), myomodulin-like (N=8) and 5-HT-like (N=8) immunoreactivities are present in the eyestalk. Anti-CCAP (N=8) does not label any profiles in the eyestalk.

Neurohormones in the pericardial organs

In brachyurans, the paired POs are located in the lateral pericardial cavity and consist of two or more longitudinal nerve trunks that are connected to one another by vertical nerve bars (Maynard, 1961*a*,*b*). The nerve trunks and bars that form the POs are elaborations of the segmental nerves, often referred to as the second roots, which project from the thoracic ganglionic mass to the pericardial cavity (Maynard, 1961*a*). Somata that give rise to the neurosecretory terminals in brachyuran POs are distributed throughout the nervous system of these species (Cooke and Sullivan, 1982). In *C. borealis*, the nerve trunks of the POs commonly exceed 1 cm in length.

In the nerve trunks of the POs, anti-allatostatin (N=8), anti-proctolin (N=8), anti-RPCH (N=8), anti-buccalin (N=8), anti-$CCK_{243\text{-}4}$ (N=8), anti-$CCK_{C36\text{-}9H}$ (N=8), anti-substance P (N=8), anti-GABA (N=8), anti-FLRFamide (N=8), anti-myomodulin (N=8), anti-5-HT (N=8) and anti-CCAP (N=8) all give rise to staining in a variety of profiles (Fig. 3). Only anti-β-PDH (N=8) and anti-$CCK_{C37\text{-}4E}$ (N=8) failed to label structures in the POs of *C. borealis*.

As was seen in the SGs, PO profiles labeled by all of the antibodies include both fibers and varicosities. The labeled fibers are generally 0.5–6.0 μm in diameter, although fibers in excess of 10 μm were occasionally seen in preparations labeled with anti-buccalin. The varicosities labeled by each of the antibodies ranged from less than 5 to over 20 μm in major cross-sectional diameter. Generally, the smaller varicosities, those less than 10 μm in major cross-sectional diameter, are present in the sheath region of the nerve trunks while the larger varicosities stud the immunoreactive fibers in such a way that they appear like ‘beads on a string’.

While we made no attempt to document the source(s) of immunolabeling seen in the POs, it is likely that somata present in the thoracic ganglionic mass and/or the commissural ganglia (CGs) contribute to the labeling, as neurons present in both areas are known to project to the POs (Cooke and Sullivan, 1982; Dircksen, 1994; Keller, 1992). Likewise, some of the immunoreactivity we report may originate from somata located in the segmental nerves (Cooke and Sullivan, 1982).

Patterns of co-localization in the pericardial organs

Because many of the modulators present in the STG are also present in the POs, we conducted a series of double-labeling experiments (Fig. 4) to determine whether the patterns of modulator co-localization present in the STG were preserved in the POs.

The gastropyloric receptor (GPR; Katz *et al.* 1989) neurons co-localize 5-HT and allatostatin (Skiebe and Schneider, 1994). Processes of the GPR neurons showing co-localization of allatostatin and 5-HT ramify within the STG neuropil. To

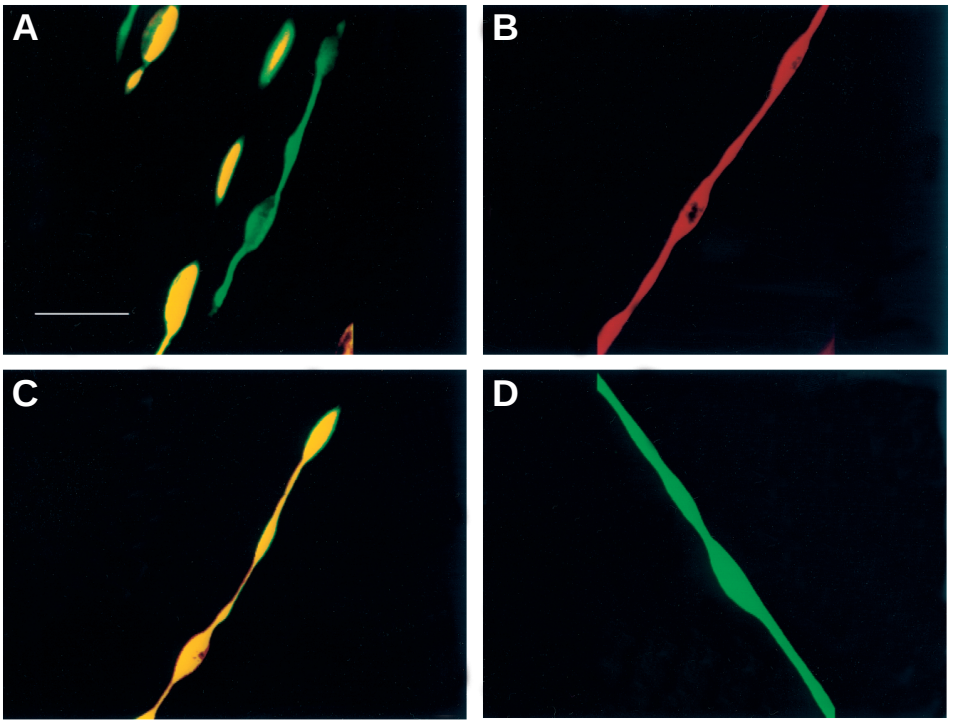

Fig. 4. Co-localization of modulator immunoreactivities in the pericardial organ. (A–D) This series of micrographs are merged pseudocolor composites of simultaneously collected fluorescein and rhodamine images from single focal planes. (A,B) In this set of micrographs, taken from the same pericardial organ, green codes for profiles showing only RPCH-like immunoreactivity, red codes for profiles exhibiting only $CCK_{C36\text{-}9H}$-like immunoreactivity and yellow codes for structures showing immunoreactivity for both antibodies. As is evident in these images, this antibody combination gives rise to both uniquely labeled and co-labeled profiles. (C,D) In these micrographs, taken from the same pericardial organ, green codes for profiles showing only substance P-like immunoreactivity, red codes for profiles exhibiting only proctolin-like immunoreactivity and yellow codes for structures showing both substance P-like and proctolin-like immunoreactivity. Again, this antibody combination gives rise to both uniquely labeled (proctolin-like single-labeled profiles, not shown) and co-labeled profiles. All images are presented at the same scale. Scale bar, 25 μm.

determine whether any of the 5-HT-like immunoreactive profiles in the POs also contain allatostatin-like immunoreactivity, we subjected eight pericardial organs to simultaneous immunoprocessing for both 5-HT-like and allatostatin-like labeling. None of the eight POs showed co-localization of allatostatin-like immunoreactivity and 5-HT-like immunoreactivity.

Another pair of projection neurons that contribute to the STG neuropil are the large varicosity fibers (LVFs), a pair of axons that projects to the STG from somata located in the commissural ganglia (CGs; Christie, 1995; Christie *et al.* 1995; A. E. Christie, D. Baldwin, E. Marder and K. Graubard, in preparation). In the *C. borealis* STG, the LVF arborization has been shown to contain $CCK_{C36\text{-}9H}$-like, $CCK_{243\text{-}4}$-like, FLRFamide-like and RPCH-like immunoreactivities (Christie *et al.* 1995; A. E. Christie, D. Baldwin, E. Marder and K. Graubard, in preparation). To determine whether any PO processes contained this cotransmitter complement, we paired anti-$CCK_{C36\text{-}9H}$ with antibodies specific for each of the remaining LVF cotransmitters (N=8 for each combination of antibodies). None of the antibody pairings gave rise to a complete overlap of labeled profiles, but each of the anti-$CCK_{C36\text{-}9H}$/anti-FLRFamide and anti-$CCK_{C36\text{-}9H}$/anti-RPCH immunoprocessed POs showed some profiles that were co-labeled (Fig. 4A,B). No overlap in immunoreactive structures was seen in the POs immunoprocessed for $CCK_{C36\text{-}9H}$-like and $CCK_{243\text{-}4}$-like immunoreactivities. Thus, while it is possible that some PO processes could contain $CCK_{C36\text{-}9H}$-like, FLRFamide-like and RPCH-like immunoreactivities, these processes do not contain $CCK_{243\text{-}4}$-like labeling and therefore do not have a cotransmitter complement identical to that of the LVFs.

The modulatory commissural neuron 1 (MCN1) contains locustatachykinin-like peptide, GABA and proctolin (Christie *et al.* 1993; Blitz *et al.* 1995; A. E. Christie, B. J. Norris, M. J. Coleman, I. Cournil, M. P. Nusbaum and E. Marder, in preparation). To determine whether the locustatachykinin-like peptide-containing profiles of the POs also contain GABA and/or proctolin, double-labeling experiments pairing anti-substance P, which recognizes locustatachykinin-like peptides in the crab (Blitz *et al.* 1995), with either anti-proctolin (N=8 POs) or anti-GABA (N=8 POs) were conducted. In anti-substance P/anti-proctolin labeling experiments, although no preparation gave rise to complete overlap of stained profiles, all preparations showed some profiles that clearly contained both immunoreactivities (Fig. 4C,D). In none of the POs labeled with both anti-substance P and anti-GABA was any co-localization of immunostaining seen. On the basis of the results of our anti-substance P/anti-proctolin and anti-substance P/anti-GABA double-labeling experiments, it is clear that the combination of locustatachykinin-like peptide, proctolin and GABA seen in the STG is not present in the POs.

One pair of neurons that is known to project to the POs in several brachyuran species are the L-cells of the CGs (Cooke and Sullivan, 1982). The L-cell somata are the largest somata found in the CGs. In *C. borealis*, these neurons commonly exhibit soma diameters greater than 150 μm (A. E. Christie, V. L. Kilman and E. Marder, unpublished results). As the L-cell somata are readily identifiable by their size, it is easy to document the modulators contained within them using

immunocytochemical techniques. Immunocytochemical studies performed on *C. borealis* indicate that the L-cells contain at least 5-HT, an extended FLRFamide-like peptide and proctolin (Harris-Warrick *et al.* 1992). Anti-5-HT/anti-proctolin and anti-5-HT/anti-FLRFamide double-labeling of the POs in *C. borealis* show that each antibody pair gives rise to both singly labeled and co-labeled profiles (*N*=8 POs for each pairing). Although we have not directly shown that 5-HT/proctolin and 5-HT/FLRFamide co-labeled profiles are projections from the L-cells and anti-proctolin/anti-FLRFamide double-labels have not been undertaken, it is likely that some of the co-localized processes originate from the L-cell.

Discussion

The stomatogastric ganglion (STG) is located within the ophthalmic artery and is therefore likely to be modulated by neuroactive substances released into the hemolymph. There is an extensive literature characterizing the substances found in the pericardial organs (POs) and sinus glands (SGs) of many crustacean species and describing many important hormonal actions of these substances (Cooke and Sullivan, 1982; Stangier *et al.* 1988; Dircksen *et al.* 1987, 1988; Dircksen, 1992, 1994; Keller, 1992). However, little is known about the substances present in the POs and SGs of *C. borealis*, the species of crab on which many studies of STG neuromodulation have focused. In the present paper, we have shown that many neuromodulatory substances found in the input axons that project to the *C. borealis* STG are also present in the POs and/or SGs, as is summarized in Fig. 5.

We did not attempt to locate the somata that give rise to the immunoreactivities seen in the POs in *Cancer borealis*, although much elegant anatomical work in other species has identified the positions of some of these somata (Dircksen, 1994; Keller, 1992). Until recordings from the neurons that give rise to the PO terminals are made, it will be difficult to determine the physiological pathways that control release from these terminals. It is interesting that we found GABA-like immunoreactivity in preparations of the POs, although GABA is usually not thought to function as a hormone. This suggests the intriguing possibility that there might be local control by GABAergic terminals of peptide release in the PO.

The localization of a neuroactive substance to both local and hormonal release sites suggests that the substance in question may serve a dual function as both an intrinsic modulator and a neurohormone (Keller, 1992). This dual function is significant physiologically as the effects of many modulators are highly concentration-dependent, and the concentration of intrinsic *versus* hormonally released modulator is likely to be quite different at a given target. As we have shown, in *C. borealis*, proctolin-like immunoreactivity is present in both the STG neuropil and the neurohemal organs. In this animal, the effects of proctolin show concentration-dependence. The threshold for proctolin action on the STG is quite low (Marder *et al.* 1986), well within the concentrations that could result from hormonal release. However, higher concentrations, that almost certainly would require local release within the STG neuropil, produce different effects. At 10^{-7} mol l^{-1} proctolin or less, the pyloric motor pattern is activated or enhanced (Marder *et al.* 1986). As the concentration of proctolin is raised to 10^{-6} mol l^{-1}, the gastric rhythm is also enhanced. At concentrations of proctolin greater than 10^{-5} mol l^{-1}, a novel rhythm, resulting from a fusion of the pyloric and gastric motor outputs, is elicited (M. P. Nusbaum, personal communication). It is likely therefore that hormonally delivered proctolin might produce qualitatively different effects from those that occur when proctolin is released from individual modulatory projection neurons.

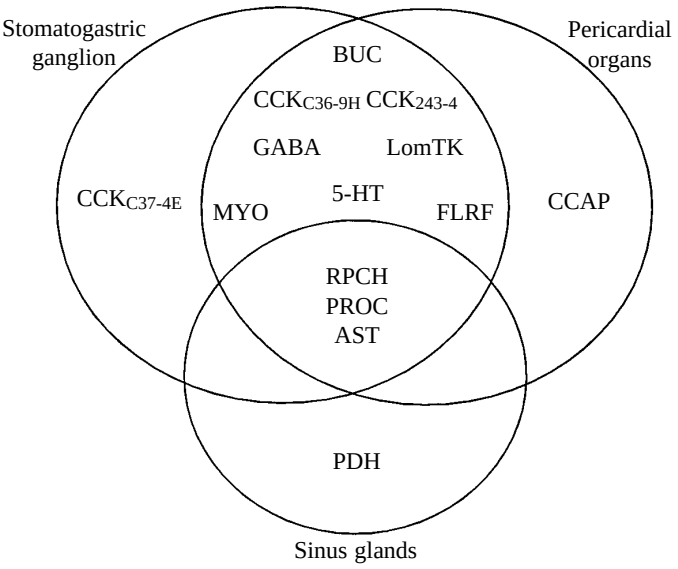

Fig. 5. Venn diagram showing the differential distribution of neuromodulatory substances in the stomatogastric ganglion, the pericardial organs and the sinus glands of *Cancer borealis*. AST, allatostatin (Skiebe and Schneider, 1994); BUC, buccalin (Christie *et al.* 1994*a*); CCAP, crustacean cardioactive peptide; CCK, cholecystokinin (243-4, C36-9H and C37-4E refer to three different antibodies, each of which recognizes a different set of CCK-like peptides in *C. borealis*; Christie *et al.* 1995); FLRF, FLRFamide (Marder *et al.* 1987; Weimann *et al.* 1993); GABA, γ-aminobutyric acid (Nusbaum *et al.* 1989); LomTK, locustatachykinin (Blitz *et al.* 1995); MYO, myomodulin (Christie *et al.* 1994*a*); PDH, β-pigment dispersing hormone (Mortin and Marder, 1991); PROC, proctolin (Marder *et al.* 1986); RPCH, red pigment concentrating hormone (Nusbaum and Marder, 1988); 5-HT, serotonin (Beltz *et al.* 1984; Katz *et al.* 1989).

Only allatostatin-like, proctolin-like and RPCH-like peptides are present in the STG, POs and SGs (Fig. 5). All but one of the immunoreactivities found in the STG was also found in the POs, indicating that almost all of the neuromodulatory substances found in input fibers to the STG may also be circulating hormones and may reach the STG by two different routes. Only two substances were found in the neurosecretory structures that are not also present in input fibers to the STG. β-PDH is found in the sinus gland only, does not have any known actions on the networks of the STG, and may therefore

play no role in the modulation of the STG networks. However, the identification of CCAP-like immunoreactivities in the POs of *C. borealis* helps to explain the modulatory effects of CCAP on the STG neural circuit. CCAP has strong excitatory actions on the STG (Weimann *et al.*1992; Marder and Weimann, 1992; J. M. Weimann, P. Skiebe, H. G. Heinzel and E. Marder, unpublished observations) but is not found in terminals in the STG, suggesting that the actions of CCAP are hormonally mediated. Likewise, many muscles of the gastric mill and pylorus are modulated by a variety of bioactive agents that are not present in the fibers that innervate them (Jorge-Rivera and Marder, 1995), suggesting that hormonal modulation may account for these actions as well.

Recent work on the intrinsic inputs to the STG has shown that many of these projection neurons contain unique constellations of cotransmitters (Katz *et al.* 1989; Nusbaum and Marder, 1989; Christie, 1995; Christie *et al.* 1993, 1995; Skiebe and Schneider, 1994; A. E. Christie, B. J. Norris, M. J. Coleman, I. Cournil, M. P. Nusbaum and E. Marder, in preparation; A. E. Christie, D. Baldwin, E. Marder and K. Graubard, in preparation). These unique cotransmitter complements have been postulated as one mechanism for segregating the modulatory actions of inputs sharing a common modulator (Marder *et al.* 1995). From our double-labeling experiments in the POs, it appears that none of the cotransmitter complements thus far identified in the STG are preserved in the neurohemal tissues. For example, in the STG, all locustatachykinin-like immunoreactivity is found co-localized with both proctolin-like and GABA-like immunoreactivity (Christie *et al.* 1993; A. E. Christie, unpublished results). In the PO, however, while some profiles show a co-localization of both locustatachykinin-like and proctolin-like immunoreactivity, additional profiles containing only locustatachykinin-like peptide are also present. Moreover, in the POs, no locustatachykinin-like profiles contain GABA-like immunolabeling. Thus, the patterns of cotransmitters found with locustatachykinin-like peptides in the POs are different from those in the STG. This reinforces the conclusion of Siwicki *et al.* (1987), who found different patterns of co-localization of small-molecule transmitters in the proctolin-containing neurons in *Homarus americanus*. As more knowledge of the co-localization patterns of neurotransmitters in these animals becomes available, it will be interesting to determine whether each identified neuron displays a unique complement of cotransmitters.

We thank Dr A. Stretton for the anti-CCK monoclonal antibodies C36-9H and C37-4E, Dr. G. Turrigiano for the polyclonal anti-CCK antibody, Drs K. Weiss and E. Cropper for the anti-buccalin and anti-myomodulin antibodies, Dr H. Dircksen for the anti-CCAP, Dr K. R. Rao for the anti-β-PDH, Dr N. Davis for the anti-proctolin antibody and Dr R. Elde for the anti-RPCH. We also thank V. Kilman and J. C. Jorge-Rivera for their helpful comments on early drafts of this manuscript. This research was supported by NS 17813, NIH Shared Instrument Grant RR05615 and a grant from the Human Frontiers Science Program Organization. We also gratefully acknowledge the support of the W. M. Keck Foundation.

References

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. (1995). Distribution and effects of tachykinin-like peptides in the stomatogastric nervous system of the crab, *Cancer borealis*. *J. comp. Neurol*. **354**, 282–294.

CHRISTIE, A. E. (1995). Chemical neuroanatomy of the crab stomatogastric ganglion: a study using immunocytochemistry and laser scanning confocal microscopy. PhD thesis, Department of Biology, Brandeis University.

CHRISTIE, A. E., BALDWIN, D., TURRIGIANO, G., GRAUBARD, K. AND MARDER, E. (1995). Immunocytochemical localization of multiple cholecystokinin-like peptides in the stomatogastric nervous system of the crab *Cancer borealis*. *J. exp. Biol.* **198**, 263–271.

CHRISTIE, A. E., HALL, C., OSHINSKY, M. AND MARDER, E. (1994*a*). 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 projection neuron. *Soc. Neurosci. Abstr*. **19**, 931.

CHRISTIE, A. E., SKIEBE, P. AND MARDER, E. (1994*b*). Distribution and colocalization of putative neurohormones in the neurohemal tissues of the crab, *Cancer borealis*. *Soc. Neurosci. Abstr*. **20**, 914.

COOKE, I. M. AND SULLIVAN, R. E. (1982). Hormones and neurosecretion. In *The Biology of Crustacea*, vol. 3, *Neurobiology: Structure and Function* (ed. H. L. Atwood and D. C. Sandeman), pp. 206–278. New York: Academic Press.

DAVIS, N. T., VELLEMAN, S. G., KINGAN, T. AND KESHISHIAN, H. (1989). Identification and distribution of a proctolin-like neuropeptide in the nervous system of the gypsy moth, *Lymantria dispar* and in other Lepidoptera. *J. comp Neurol*. **283**, 71–85.

DIRCKSEN, H. (1992). Fine structure of the neurohemal sinus gland of the shore crab *Carcinus maenas* L. and immuno-electron microscopic identification of neurosecretory endings according to their neuropeptide contents. *Cell Tissue Res.* **269**, 249–266.

DIRCKSEN, H. (1994). Distribution and physiology of crustacean cardioactive peptide in arthropods. *Perspectives in Comparative Endocrinology*, National Research Council of Canada, pp. 139–148.

DIRCKSEN, H., WEBSTER, S. G. AND KELLER, R. (1988). Immunocytochemical demonstration of the neurosecretory systems containing putative moult-inhibiting and hyperglycemic hormone in the eyestalk of brachyuran crustaceans. *Cell Tissue Res.* **251**, 3–12.

DIRCKSEN, H., ZAHNOW, C. A., GAUS, G., KELLER, R., RAO, K. R. AND RIEHM, J. P. (1987). The ultrastructure of nerve endings containing pigment dispersing hormone (PDH) in the crustacean sinus glands:

identification by an antiserum against a synthetic PDH. *Cell Tissue Res*. **250**, 377–387.

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.

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

JORGE-RIVERA, J. C. AND MARDER, E. (1995). Convergent modulation of a neuromuscular junction by nine neurohormones. *Soc. Neurosci. Abst.* **21**, 2095.

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.

KELLER, R. (1992). Crustacean neuropeptides: Structures, functions and comparative aspects. *Experientia* **48**, 439–448.

MADSEN, A. J., HERMAN, W. S. AND ELDE, R. (1985). Differential distribution of two homologous neuropeptides (RPCH & AKH) in the crayfish nervous system. *Soc. Neurosci. Abstr*. **11**, 941.

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., CHRISTIE, A. AND KILMAN, V. L. (1995). Functional organization of cotransmission systems: lessons from small nervous systems. *Invert. Neurosci*. (in press).

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., SKIEBE, P. AND CHRISTIE, A. E. (1994). Multiple modes of network modulation. *Verh. dt. zool. Ges*. **87**, 177–184.

MARDER, E. AND WEIMANN, J. M. (1992). Modulatory control of multiple task processing in the stomatogastric nervous system. In *Neurobiology of Motor Programme Selection: New Approaches to Mechanisms of Behavioral Choice* (ed. J. Kien, C. McCrohan and W. Winlow), pp. 3–19. Oxford: Pergamon Press.

MAYNARD, D. M. (1961*a*). Thoracic neurosecretory structures in Brachyura. I. Gross anatomy. *Biol. Bull. mar. biol. Lab., Woods Hole* **121**, 316–329.

MAYNARD, D. M. (1961*b*). Thoracic neurosecretory structures in Brachyura. II. Secretory neurons. *Gen. comp. Endocr*. **1**, 237–263.

MERCIER, A. J., ORCHARD, I., TEBRUGGE, V. AND SKERRRETT, M. (1993). Isolation of two FMRFamide-related peptides from crayfish pericardial organs. *Peptides* **14**, 137–143.

MILLER, M. W., ALEVIZOS, A., CROPPER, E. C., KUPFERMANN, I. AND WEISS, K. R. (1992). Distribution of buccalin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica. J. comp. Neurol.* **320**, 182–195.

MILLER, M. W., ALEVIZOS, A., CROPPER, E. C., VILIM, F. S., KARAGOGEOS, D., KUPFERMANN, I. AND WEISS, K. R. (1991). Localization of myomodulin-like immunoreactivity in the central nervous system and peripheral tissues of *Aplysia californica. J. comp. Neurol.* **314**, 627–644.

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.

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), p. 228. Stuttgart: Georg Thieme Verlag.

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.

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

SIWICKI, K. K., BELTZ, B. S. AND KRAVITZ, E. A. (1987). Proctolin in identified serotonergic, dopaminergic and cholinergic neurons in the lobster, *Homarus americanus. J. Neurosci.* **7**, 522–532.

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.

STANGIER, J., HILBICH, C., DIRCKSEN, H. AND KELLER, H. (1988). Distribution of a novel cardioactive neuropeptide (CCAP) in the nervous system of the shore crab *Carcinus maenas* L. *Cell Tissue Res*. **254**, 347–360.

TRIMMER, B. A., KOBIERSKI, L. A. AND KRAVITZ, E. A. (1987). Purification and characterization of immunoreactive substances from the lobster nervous system: Isolation and sequence analysis of two closely related peptides. *J. comp. Neurol.* **266**, 16–26.

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.

WEIMANN, J. M., HEINZEL, H. G. AND MARDER, E. (1992). Crustacean cardioactive peptide activation of the pyloric network of the crab, *Cancer borealis*. *Soc. Neurosci. Abstr.* **18**, 1056.

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.