The Journal of Experimental Biology 208, 3303-3319
Published by The Company of Biologists 2005
doi:10.1242/jeb.01787

Identification and characterization of a tachykinin-containing neuroendocrine organ in the commissural ganglion of the crab *Cancer productus*

Daniel I. Messinger[1,2], Kimberly K. Kutz[3], Thuc Le[4], Derek R. Verley[4], Yun-Wei A. Hsu[1], Christina T. Ngo[1,2], Shaun D. Cain[2], John T. Birmingham[4], Lingjun Li[3,5] and Andrew E. Christie[1,2,*]

[1]*Department of Biology, University of Washington, Box 351800, Seattle, WA 98195-1800, USA,* [2]*Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250, USA,* [3]*Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, WI 53706-1369, USA,* [4]*Department of Physics, Santa Clara University, 500 El Camino Real, Santa Clara, CA 95053-0315, USA* and [5]*School of Pharmacy, University of Wisconsin–Madison, 777 Highland Avenue, Madison, WI 53705-2222 USA*

*Author for correspondence (e-mail: crabman@u.washington.edu)

Accepted 7 July 2005

Summary

A club-shaped, tachykinin-immunopositive structure first described nearly two decades ago in the commissural ganglion (CoG) of three species of decapod crustaceans has remained enigmatic, as its function is unknown. Here, we use a combination of anatomical, mass spectrometric and electrophysiological techniques to address this issue in the crab *Cancer productus*. Immunohistochemistry using an antibody to the vertebrate tachykinin substance P shows that a homologous site exists in each CoG of this crab. Confocal microscopy reveals that its structure and organization are similar to those of known neuroendocrine organs. Based on its location in the anterior medial quadrant of the CoG, we have named this structure the anterior commissural organ (ACO). Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry shows that the ACO contains the peptide APSGFLGMRamide, commonly known as *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia). Using the same technique, we show that CabTRP Ia is also released into the hemolymph. As no tachykinin-like labeling is seen in any of the other known neuroendocrine sites of this species (i.e. the sinus gland, the pericardial organ and the anterior cardiac plexus), the ACO is a prime candidate to be the source of CabTRP Ia present in the circulatory system. Our electrophysiological studies indicate that one target of hemolymph-borne CabTRP Ia is the foregut musculature. Here, no direct CabTRP Ia innervation is present, yet several gastric mill and pyloric muscles are nonetheless modulated by hormonally relevant concentrations of the peptide. Collectively, our findings show that the *C. productus* ACO is a neuroendocrine organ providing hormonal CabTRP Ia modulation to the foregut musculature. Homologous structures in other decapods are hypothesized to function similarly.

Key words: stomatogastric nervous system, hormone, APSGFLGMRamide, *Cancer borealis* tachykinin-related peptide Ia, CabTRP Ia, anterior commissural organ, ACO, laser-scanning confocal microscopy, mass spectrometry, MALDI-FTMS.

Introduction

The tachykinins constitute one of the largest and most diverse groups of peptides in the animal kingdom. While the first sequenced family member was the molluscan peptide eledoisin (pEPSKDAFIGLMamide; Erspamer and Anastasi, 1962), perhaps the best known is the mammalian peptide substance P (RPKPQQFFGLMamide; Chang et al., 1971). Like all vertebrate tachykinins, and a few invertebrate isoforms sequenced from salivary tissues (e.g. eledoisin), these two peptides contain the carboxy (C)-terminal amino acid motif –F*X*GLMamide, where *X* represents a variable amino acid (Nachman et al., 1999; Nässel, 1999; Vanden Broeck et al., 1999; Severini et al., 2002). In invertebrates, a plethora of peptides containing the C-terminal amino acid motif –FX_{I}GX_{II}Ramide have been identified and collectively termed tachykinin-related peptides or TRPs (Nachman et al., 1999; Nässel, 1999; Vanden Broeck et al., 1999). Comparisons of sequence homology, tissue distribution, chemical/conformational requirements for receptor interaction and physiological function suggest a common evolutionary origin for both the vertebrate- and invertebrate-type peptides (Nachman et al., 1999; Nässel, 1999; Vanden Broeck et al., 1999; Severini et al., 2002).

In most invertebrates, particularly insects, large numbers of species-specific TRP isoforms are common (Nachman et al.,

1999; Nässel, 1999; Vanden Broeck et al., 1999; Severini et al., 2002). For example, 10 TRPs have been isolated from the cockroach *Leucophea maderae* (Muren and Nässel, 1996, 1997), seven from the honeybee *Apis mellifera* (Takeuchi et al., 2003), five from the locust *Locusta migratoria* (Schoofs et al., 1990a,b, 1993), five from the fruit fly *Drosophila melanogaster* (Siviter et al., 2000) and three from the mosquito *Culex salinarius* (Meola et al., 1998). In contrast to the diversity of species-specific TRPs present in insects, only a single isoform is thought to be present in decapod crustaceans (Christie et al., 1997a; Nieto et al., 1998; Li et al., 2002a; Huybrechts et al., 2003; Yasuda-Kamatani and Yasuda, 2004). This peptide, APSGFLGMRamide or *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia), has been isolated from or is predicted by cDNA to be present in the crab *Cancer borealis*, the shrimp *Panaeus vannamei*, the chelate marine lobster *Homarus americanus*, the freshwater crayfish *Procambarus clarkii* and the spiny lobster *Panulirus interruptus* (Christie et al., 1997a; Nieto et al., 1998; Li et al., 2002a; Huybrechts et al., 2003; Yasuda-Kamatani and Yasuda, 2004).

While no antibody has been generated directly against CabTRP Ia, the peptide has been shown to cross-react with a rat monoclonal antibody to substance P and with several antibodies generated against insect TRPs (Cuello et al., 1979; Nässel, 1993; Christie et al., 1997a; Winther and Nässel, 2001). Immunohistochemical studies using these antibodies have shown that CabTRP Ia is widely distributed within the nervous system of decapod crustaceans (Mancillas et al., 1981; Fingerman et al., 1985; Goldberg et al., 1988; Sandeman et al., 1990a,b; Schmidt and Ache, 1994, 1997; Blitz et al., 1995, 1999; Christie et al., 1995a, 1997a,b; Schmidt, 1997a,b; Langworthy et al., 1997; Fénelon et al., 1999; Glantz et al., 2000; Thirumalai and Marder, 2002; Pulver and Marder, 2002). In *C. borealis*, *P. interruptus* and *H. americanus*, one area of the nervous system that exhibits TRP immunoreactivity is the stomatogastric nervous system (STNS; Fig. 1), which controls the movement of the foregut musculature (Goldberg et al., 1988; Blitz et al., 1995, 1999; Christie et al., 1997a,b; Fénelon et al., 1999).

Within the STNS, one common and thus far unique feature of the CabTRP Ia labeling is a club-shaped plexus located in the anterior medial quadrant of each commissural ganglion (CoG; Goldberg et al., 1988; Fig. 1). Regardless of species, this structure originates from a fascicle of axons projecting from the circumoesophageal connective (*coc*) linking the CoG to the thoracic and abdominal nervous systems (Goldberg et al., 1988). The physiological role that this plexus plays in crustaceans is unknown. In the present report, we use a combination of anatomical, mass spectrometric and electrophysiological techniques to investigate the structural organization, co-transmitter complement and potential function(s) of this site in the Pacific red rock crab, *Cancer productus*. Collectively, our results show that this plexus is a neuroendocrine organ that provides hormonal CabTRP Ia modulation to the foregut musculature and, potentially, paracrine modulation to intrinsic CoG targets. Some of these data have appeared previously in abstract form (Messinger et al., 2004).

Materials and methods

Animals and tissue collection

Cancer productus Randall were collected by hand, ring-trap or trawl at multiple locations in the greater Puget Sound area and San Juan Archipelago of Washington State (USA). Animals were maintained in either flow-through natural seawater tanks (Friday Harbor Laboratories, Friday Harbor, Washington, USA; ambient water temperature 8–12°C) or aerated natural seawater aquaria chilled to 10°C (Department of Biology, University of Washington, Seattle, WA, USA).

For tissue collection, crabs were anesthetized by packing in ice for 30–60 min, after which the dorsal carapace was removed and the foregut isolated from the animal. Following extraction of the foregut, the CoGs, and in some cases the entire STNS, were dissected free in chilled (approximately 10°C) physiological saline [composition in mmol l^{-1}: 440 NaCl; 11 KCl; 13 $CaCl_2$; 26 $MgCl_2$; 10 Hepes acid, pH 7.4 (adjusted with NaOH)]. For some crabs, the eyestalks and dorsolateral walls of the pericardial chamber were also removed. From these structures, we isolated two well-known neuroendocrine organs: the sinus glands (SGs) and pericardial organs (POs).

Whole-mount immunohistochemistry

Immunohistochemistry was performed as whole mounts. Specifically, dissected tissue was pinned in a Sylgard 184 (World Precision Instruments, Inc., Sarasota, FL, USA; catalog #SYLG184)-lined Petri dish and fixed in a solution of either 4% paraformaldehyde (EM grade; Electron Microscopy Sciences, Hatfield, PA, USA; catalog #15710) in 0.1 mol l^{-1} sodium phosphate (P) buffer (pH 7.4), 4% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC; Sigma-Aldrich, St Louis, MO, USA; catalog #E-7750) in P, 4% paraformaldehyde and 1% EDAC in P or 100% methanol (HPLC grade; Sigma-Aldrich; catalog #27047-4), depending on the primary antibody being used (see below). All solutions containing paraformaldehyde or EDAC were prepared immediately prior to use, and tissue was fixed at 4°C. Methanol fixation was done at –20°C. Regardless of solution, tissues were fixed for 12–24 h, except as noted below (see Primary antibodies). Following fixation, tissue was rinsed five times over approximately five hours in a solution of P containing 0.3% Triton X-100 (P-Triton). Incubation in primary antibody (see below) was carried out in P-Triton, with 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; catalog #017-000-121) added to diminish nonspecific binding. Following incubation in primary antibody, tissue was again rinsed five times over approximately five hours in P-Triton and then incubated in a 1:300 dilution of secondary antibody (see below) for 12–24 h. As with the primary antibody, incubation with secondary antibody was

performed in P-Triton containing 10% NDS. After incubation in secondary antibody, preparations were rinsed five times over approximately five hours in P and mounted between a glass microscope slide and cover slip using Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA; catalog #H-1000). Incubations in primary and secondary antibodies were done using gentle agitation at 4°C. All tissues were rinsed at room temperature (18–24°C) without agitation. Secondary antibody incubation was conducted in the dark, as was all subsequent processing. Likewise, slides were stored in the dark at 4°C until examined.

Primary antibodies

Each of the primary antibodies employed in our study has been used previously to map the distribution of its respective antigen in crustacean/insect nervous systems and has been shown to be specific for its antigen. The references provided for each antibody describe its development/specificity and/or use in arthropod neural tissue.

For the detection of TRP, a rat monoclonal antibody to substance P (clone NC1/34 HL; Abcam Incorporated, Cambridge, MA, USA; catalog # ab6338; Cuello et al., 1979; Goldberg et al., 1988; Christie et al., 1997a) was used at a final dilution of 1:300. For the detection of the small molecule transmitter γ-aminobutryic acid (GABA), a rabbit polyclonal antibody (Sigma-Aldrich; catalog #A2052; Blitz et al., 1999; Swensen et al., 2000) was used at a final dilution of 1:500. For the detection of the amine dopamine, a mouse monoclonal antibody to its biosynthetic enzyme tyrosine hydroxylase (Immunostar Inc., Hudson, WI, USA; catalog # 22941; Tierney et al., 1999; Pulver and Marder, 2002) was used at a final dilution of 1:1000. For the detection of the amine histamine, a rabbit polyclonal antibody (Immunostar; catalog # 22939; Panula et al., 1988; Christie et al., 2004b) was used at a final dilution of 1:500. For the detection of the amine serotonin, a rabbit polyclonal antibody (Immunostar; catalog # 20080; Tierney et al., 1999; Pulver and Marder, 2002) was used at a final dilution of 1:500. To assay for the gas carbon monoxide, a rabbit polyclonal antibody to its biosynthetic enzyme heme oxygenase 2 (Stressgen Biotechnologies Corp, Victoria, BC, Canada; catalog # OSA-200; Christie et al., 2003) was used at a final dilution of 1:100. To assay for the gas nitric oxide, a rabbit polyclonal antibody to its biosynthetic enzyme nitric oxide synthase (Affinity Bioreagents, Golden, CO, USA; catalog # PA1-039; Christie et al., 2003) was used at a final dilution of 1:300. For the detection of allatostatin-like peptides, a mouse monoclonal antibody (Dr B. Stay, University of Iowa, Iowa City, IA, USA; Stay et al., 1992; Woodhead et al., 1992; Pulver and Marder, 2002) was used at a final dilution of 1:100. For the detection of buccalin-like peptides, a rabbit polyclonal antibody (Dr K. Weiss, Mount Sinai School of Medicine, New York, NY, USA; Miller et al., 1992; Christie et al., 1994) was used at a final dilution of 1:100. For the detection of cholecystokinin (CCK)-related peptides, a rabbit polyclonal antibody (Dr G. Turrigiano, Brandeis University, Waltham, MA, USA; Turrigiano and Selverston, 1991; Christie et al., 1995b) was used at a final dilution of 1:300. For the detection of corazonin-related peptides, a rabbit polyclonal antibody (Dr J. Veenstra, Université Bordeaux 1, Talence cedex, France; Veenstra, 1991; Christie and Nusbaum, 1995) was used at a final dilution of 1:500. For the detection of crustacean cardioactive peptide (CCAP), a rabbit polyclonal antibody (Dr H. Dircksen, Stockholm University, Stockholm, Sweden; Dircksen and Keller, 1988; Stangier et al., 1988; Christie et al., 1995a) was used at a final dilution of 1:500. For the detection of FLRFamide-related peptides, a rabbit polyclonal antibody (Immunostar; catalog # 20091; Christie et al., 2004a) was used at a final dilution of 1:300. For the detection of myomodulin-like peptides, a rabbit polyclonal antibody (Dr K. Weiss; Miller et al., 1991; Christie et al., 1994) was used at a final dilution of 1:300. For the detection of orcokinins, a rabbit polyclonal antibody (Dr H. Dircksen; Bungart et al., 1994; Li et al., 2002b; Skiebe et al., 2002) was used at a final dilution of 1:5000. For the detection of β-pigment dispersing hormone (β-PDH)-related peptides, a rabbit polyclonal antibody (Dr K. Rao, University of West Florida, Pensacola, FL, USA; Dircksen et al., 1987; Mortin and Marder, 1991) was used at a final dilution of 1:1000. For the detection of proctolin, a rabbit polyclonal antibody (Dr D. Nässel, Stockholm University; code K9832/13; Johnson et al., 2003) was used at a final dilution of 1:500. For the detection of red pigment concentrating hormone (RPCH), a rabbit polyclonal antibody (Dr R. Elde, University of Minnesota, Minneapolis, MN, USA; Madsen et al., 1985; Nusbaum and Marder, 1988) was used at a final dilution of 1:300.

The fixative used for all antibodies except for those generated against tyrosine hydroxylase, histamine and CCAP was 4% paraformaldehyde (tissue for GABA immunoprocessing fixed for 2–3 h only). Fixation for tyrosine hydroxylase utilized 100% methanol, while 4% EDAC was employed for histamine, and a combination of 4% paraformaldehyde and 1% EDAC was used for CCAP.

Secondary antibodies

The secondary antibodies used in our experiments were donkey anti-rat immunoglobulin G (IgG) conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA; catalog #A-21208) or Alexa Fluor 594 (Molecular Probes; catalog #A-21209), donkey anti-rabbit IgG conjugated to Alexa Fluor 488 (Molecular Probes; catalog #A-21206) or Alexa Fluor 594 (Molecular Probes; catalog #A-21207) and donkey anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes; catalog #A-21202) or Alexa Fluor 594 (Molecular Probes; catalog #A-21203).

Confocal and epifluorescence microscopy

Fluorescently labeled tissue was viewed and data collected using one of two Bio-Rad MRC 600 laser scanning confocal microscopes (Bio-Rad Microscience Limited, Hemel Hempstead, UK), a Bio-Rad Radiance 2000 laser scanning confocal microscope or a Nikon Eclipse E600 epifluorescence microscope (Tokyo, Japan). The Bio-Rad MRC 600 system at

Friday Harbor Laboratories is equipped with a Nikon Diaphot inverted microscope and a krypton/argon mixed gas laser. Nikon Fluor 10× 0.5NA dry, PlanApo 20× 0.75NA dry, Nikon Fluor 40× 0.85NA dry and PlanApo 60× 1.4NA oil immersion objective lenses and Bio-Rad-supplied BHS, YHS and K1/K2 filter sets and Comos software were used for imaging preparations on this system (filter specifications are as described in Christie et al., 1997b). The Bio-Rad MRC 600 system located at the University of Washington (Department of Biology) is equipped with a Nikon Optiphot upright microscope and, with the exception of the Nikon Fluor 40× 0.85NA dry lens, uses the same laser, filters, software and objective lenses as the MRC 600 system located at Friday Harbor Laboratories. The Bio-Rad Radiance 2000 system is equipped with a modified Nikon Eclipse E600FN microscope and a krypton/argon mixed gas laser (568 nm excitation line used). For imaging on this system, Nikon PlanApo 10× 0.45NA DIC dry, PlanApo 20× 0.75NA DIC dry and PlanApo 60× 1.4NA DIC oil immersion objective lenses as well as a Bio-Rad-supplied E600LP emission filter and Bio-Rad LaserSharp 2000 software were used. The Nikon Eclipse E600 epifluorescence microscope is equipped with Nikon PlanFluor 10× 0.30NA, PlanFluor 20× 0.50NA and PlanFluor 40× 0.75NA dry objective lenses and ENDOW GFP HYQ (EX, 450–490 nm; DM, 495 nm; BA, 500–550 nm) and G-2E/C TRITC (EX, 528–553 nm; DM, 565 nm; BA, 600–660 nm) filter sets.

India ink mapping of the circulatory system

India ink injection into the circulatory system has long been used to map the distribution of blood vessels in neural tissue (Lane et al., 1981; Renkin et al., 1981; Renkin, 1985; Farley, 1990; Hogers et al., 1995; Murray and Wilson, 1997; Grivas et al., 2003; Sasaki et al., 2003; Cerri et al., 2004; Hutchinson and Savitzky, 2004; Marinkovic et al., 2004). Here, we developed a method using this reagent to visualize the distribution of hemolymph vessels and lacunae in the stomatogastric neuromuscular system. Specifically, Higgins Fountain Pen India ink (Eberhard Faber Inc., Lewisberg, TN, USA; catalog #723) or Koh-I-Noor Fount India drawing ink (Koh-I-Noor, Bloomsbury, NJ, USA; catalog #9150-D) was dried down and then reconstituted in a similar amount of physiological saline. The resulting saline/ink solution was injected into the pericardial chamber using a 22-gauge needle attached to a 1-ml plastic syringe *via* penetration through the junction of the thorax and abdomen. For small animals (<120 g), 200 μl of saline/ink solution was injected into each individual. Intermediate-sized animals (120–250 g) were injected with 500 μl and large animals (>250 g) received a 1 ml injection of the saline/ink solution. Following the injection, animals were returned to their tanks for 1–4 h, then anesthetized and dissected as described earlier. Nervous system tissue was then pinned flat in a Sylgard-lined Petri dish containing physiological saline, and ink infiltration into lacunae was visualized using either a Nikon SMZ800 or Nikon SMZ1000 stereomicroscope with incident illumination provided by a Fiber-Lite Model 190 fiber optic illuminator (Dolan-Jenner Industries, Inc., Woburn, MA, USA). Micrographs of ink infiltration were taken using either a Nikon CoolPix 4500 digital camera mounted on the SMZ800 microscope or a Nikon CoolSNAP digital camera mounted on the SMZ1000 microscope.

Coincidence of ink filling and tachykinin-like immunoreactivity

Following ink infiltration, some CoGs were fixed and immunoprocessed with the substance P antibody to assess the coincidence of the hemolymph lacunae and the TRP immunoreactivity. These ink-filled/substance P immunoprocessed ganglia were imaged using the Bio-Rad Radiance 2000 confocal system, with ink-filling visualized *via* its transmitted light detector.

Matrix-assisted laser desorption/ionization Fourier transform mass spectrometry

Anterior medial quadrant of the CoG

For the mass spectrometric identification of TRP isoforms in the tachykininergic plexus of the CoG, ganglia were dissected and pinned in a Sylgard-lined Petri dish containing chilled physiological saline. Incident illumination was used to identify the location of the plexus in each ganglion, then the site was subsequently isolated and placed in acidified methanol [90% methanol (HPLC grade; Sigma-Aldrich), 9% glacial acetic acid (sequencing grade; Fisher Scientific, Fair Lawn, NJ, USA; catalog #BP1185-500) and 1% water (HPLC grade; Sigma-Aldrich; catalog #27073-3)]. Tissue from 20 ganglia was pooled in approximately 200 μl of acidified methanol and stored at –80°C until utilized for analysis.

Direct tissue analysis of individual tissue pieces was performed as previously described (Kutz et al., 2004). Briefly, a tissue fragment was desalted in 10 mg ml^{-1} 2,5-dihydroxybenzoic acid (DHB; ICN Biomedicals Incorporated, Costa Mesa, CA, USA; catalog #190209). Next, 0.2 μl of saturated DHB solution (in 50:50 methanol:water, v/v) was added to one facet of a matrix-assisted laser desorption/ionization (MALDI) target plate, and the desalted tissue fragment quickly placed into the matrix. The tissue-containing matrix was then crystallized at room temperature.

Mass spectrometric analysis of peptides was carried out using an IonSpec HighRES MALDI Fourier transform mass spectrometer (FTMS; IonSpec Corporation, Lake Forest, CA, USA). In order to increase the signal-to-noise ratio, in-cell accumulation was performed to allow multiple packets of ions resulting from the tissue sample to accumulate in the analyzer cell prior to detection. For the detection of low-mass peptides (i.e. approximately 1000 *m*/*z*), a pulse sequence was used to more efficiently transport ions into the analyzer cell (Kutz et al., 2004).

Hemolymph

To determine if CabTRP Ia is a circulating hormone, we used MALDI-FTMS to assay the hemolymph. Hemolymph

was collected by inserting a 22-gauge needle attached to a 3-ml plastic syringe through the junction of the thorax and abdomen into the pericardial chamber. A fresh needle and syringe were used for removal of hemolymph from each animal. Approximately 2 ml of hemolymph was withdrawn from each animal and immediately placed in twice its volume of acidified methanol and vortexed for 3 min at 10°C using a Thermolyne Maxi Mix II tabletop vortexer (Barnstead/Thermolyne, Dubuque, IA, USA). Following vortexing, the hemolymph/acidified methanol mixture was centrifuged for 5 min at 15 800 ***g*** using an Eppendorf 5415C tabletop centrifuge (Eppendorf AG, Hamburg, Germany), also at 10°C. After centrifugation, the resulting supernatant was removed and stored at –80°C until utilized for analysis.

Prior to mass spectrometric analysis, large proteins and salts were removed from the extracted hemolymph. Large proteins were removed by placing 500 μl of crude extract in a 10 000 Da molecular mass cutoff tube (Argos Technologies, catalog #VS0101) and centrifuging it at 16 100 ***g*** for 10 min at room temperature. The resulting low-molecular-mass filtrate was concentrated using a Savant SC 110 SpeedVac concentrator (Thermo Electron Corporation, West Palm Beach, FL, USA) and then resuspended in 10 μl of 0.1% formic acid (puriss grade; Sigma-Aldrich; catalog #94318). The acidified sample was desalted by passing it through a $ZipTip_{C18}$ pipette tip (Millipore, Billerica, MA, USA; catalog #ZTC18S096) and eluting the bound peptides with 4 μl of 50% acetonitrile. Desalted extract was mixed 1:1 with DHB matrix on a MALDI plate and allowed to crystallize at room temperature, after which MALDI-FTMS analysis was performed as per the CoG fragments.

Muscle physiology

Neuromuscular preparations were dissected from the *C. productus* foregut and pinned flat in 5-ml Sylgard-lined Petri dishes. Nerves and muscles were identified according to the nomenclature of Maynard and Dando (1974). During recording sessions, the bath volume was maintained at approximately 3 ml and preparations were continuously superfused (4–5 ml min^{-1}) with physiological saline. (It should be noted that the saline used in the physiological experiments was buffered using 11.2 mmol l^{-1} Trizma base and 5.1 mmol l^{-1} maleic acid rather than the 10 mmol l^{-1} Hepes acid used in the saline employed for our anatomical and mass spectrometric experiments.) CabTRP Ia was bath-applied by means of a switching port at the inflow of the superfusion system. This peptide was synthesized and purified using standard techniques (Christie et al., 1997a) by the Cancer Research Center of the University of Pennsylvania School of Medicine (Philadelphia, PA, USA) and was a gift from Dr Michael P. Nusbaum (Department of Neuroscience, University of Pennsylvania School of Medicine). Synthetic CabTRP Ia was dissolved in distilled water at a concentration of 10^{-3} mol l^{-1} and stored at –20°C. Immediately before use, samples of dissolved peptide were thawed and diluted to final bath concentrations (10^{-9}–10^{-7} mol l^{-1}) in physiological saline. During all experiments, the saline temperature was cooled with an ice bath and regulated to within a few tenths of a degree at a temperature of approximately 10°C.

Excitatory junctional potential recordings

Measurements of excitatory junctional potentials (EJPs) were made from the gastric mill 4 (gm4), gastric mill 5a (gm5a), gastric mill 6a (gm6a), gastric mill 8a (gm8a), pyloric 1 (p1) and pyloric 2 (p2) muscles using conventional 2 mol l^{-1} potassium acetate-filled microelectrodes with resistances of 7–10 MΩ. The gm4 muscle is innervated by the dorsal gastric (DG) neuron *via* the dorsal gastric nerve (*dgn*). The gm5a muscle is innervated by the inferior cardiac (IC) neuron *via* the medial ventricular nerve (*mvn*). The other four muscles are innervated *via* the lateral ventricular nerve (*lvn*); gm6a and gm8a by the lateral gastric (LG) neuron, p1 by the lateral pyloric (LP) neuron, and p2 by the pyloric (PY) neurons (Maynard and Dando, 1974; Selverston and Moulins, 1987; Weimann et al., 1991; Harris-Warrick et al., 1992). Innervating nerves were stimulated extracellularly *via* stainless steel pin electrodes driven by an A-M systems Model 2100 isolated pulse stimulator (A-M Systems, Carlsborg, WA, USA). EJPs were measured with an Axoclamp 2-B intracellular amplifier (Axon Instruments, Union City, CA, USA), amplified 10-fold using a Model 440 instrumentation amplifier (Brownlee Precision, San Jose, CA, USA) and recorded using a Digidata 1322A acquisition system (Axon Instruments). EJP amplitude was defined as the peak membrane potential of the EJP relative to the baseline potential prior to stimulation. EJP amplitudes were analyzed using routines written in Matlab (The MathWorks, Natick, MA, USA).

Contraction measurements

Recordings of contractions from the gm8 muscle were obtained using a FT03 force displacement transducer (Astro-Med, West Warwick, RI, USA). The neuromuscular preparation consisted of fibers of both the gm8a and gm8b muscles, which were not separated in order to minimize damage to the muscle fibers and innervating nerves. One of the muscle insertions was pinned down to the Sylgard in the recording dish, while the other insertion was tied to the transducer with a short piece (~3 cm) of size 6/0 silk suture thread (Fine Science Tools, Foster City, CA, USA). The transducer was positioned so that the muscle was stretched just past its relaxed length. The innervating nerve was stimulated with a train of pulses applied through the pin electrode. This resulted in muscle shortening, and the force transducer measured the increased tension. The transducer signal was amplified by a factor of 10 000 using the Brownlee Precision Model 440 amplifier and recorded using the Digidata interface board. Contraction amplitudes were analyzed using the Clampfit program (Axon Instruments).

Statistics

In the experiments in which the effect of a single concentration (10^{-7} mol l^{-1}) of CabTRP Ia on the EJP

amplitude or contraction of a muscle was tested, a paired *t*-test was used to test for statistical significance. Error bars on plots correspond to standard errors.

Figure production

Anatomy figures were produced using a combination of Photoshop (version 7.0; Adobe Systems Inc., San Jose, CA, USA) and Canvas (version 8.0; Deneba Systems Inc., Miami, FL, USA) software. Contrast and brightness were adjusted as needed to optimize the clarity of the printed images. Mass spectra were collected using IonSpec99 version 7.0 software (IonSpec Corp.). Boston University Data Analysis (BUDA) version 1.4 was used to export the spectra as a bitmap into Macromedia Fireworks MX 2004 Version 7.0 (Macromedia Incorporated, San Francisco, CA, USA). Resolution was increased and peaks were labeled with mass and peptide identity in Fireworks. Physiology figures were produced using Sigma Plot (version 8.0; Systat Software, Point Richmond, CA, USA).

Results

Tachykinin-like immunoreactivity in the stomatogastric nervous system and neuroendocrine organs

Stomatogastric nervous system

To determine the distribution of TRPs in the STNS of *C. productus*, we immunoprocessed this portion of the nervous system with a rat monoclonal antibody generated against the vertebrate tachykinin substance P (Cuello et al., 1979). This antibody was used previously for mapping the distribution of TRPs in the STNS of the crab *C. borealis*, the spiny lobster *P. interruptus* and the chelate lobster *H. americanus* (Goldberg et al., 1988; Blitz et al., 1995; Christie et al., 1997a) and is known to cross-react with the peptide CabTRP Ia, the only TRP thus far isolated from crustaceans and probably the sole isoform of this peptide family present in this group of arthropods (Christie et al., 1997a; Nieto et al., 1998; Li et al., 2002a; Huybrechts et al., 2003; Yasuda-Kamatani and Yasuda, 2004).

Within the STNS of *C. productus*, numerous TRP-immunopositive profiles were identified (Fig. 1). As with the previously mapped crab and lobster species (Goldberg et al., 1988), the most striking of these structures is a club-shaped plexus located in the anterior medial portion of each CoG (Figs 1, 2; *N*=148 ganglia). Labeling in the plexus consisted of a dense aggregation of nerve terminals that originate from a fascicle of small (<1 μm)-diameter axons projecting from the *coc* connecting the CoG to the thoracic nervous system (Fig. 2A,B). On average, the individual terminals that comprise the plexus were rather elongate, with major cross-sectional diameters of <1 to >15 μm. In the posterior portion of the plexus, the terminal aggregates were often extensively fenestrated by unstained tubular areas (Fig. 2C).

In addition to the plexus just described, a number of other TRP-immunopositive structures were also seen in the STNS (*N*=41 intact nervous systems examined). The distribution of this immunoreactivity is summarized schematically in Fig. 1. Seven

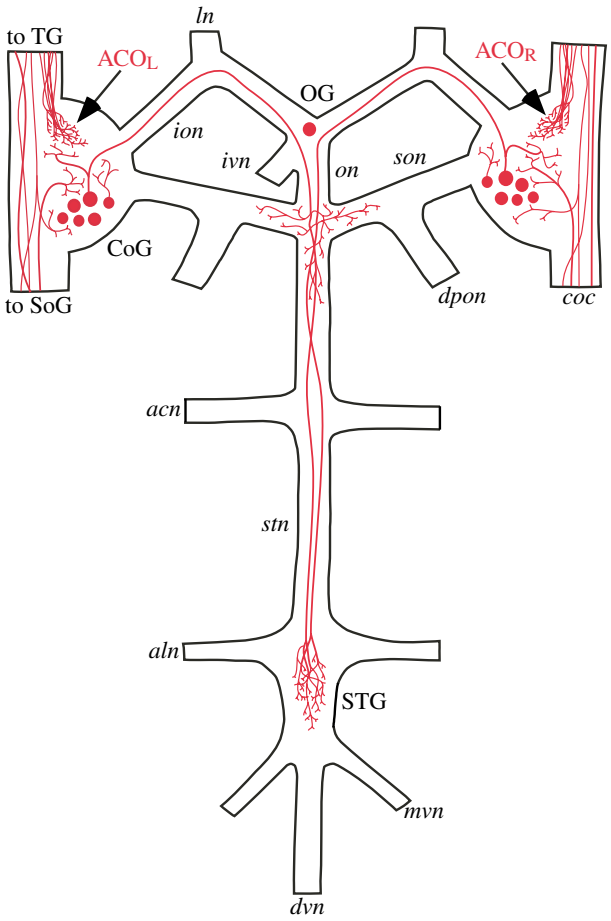

Fig. 1. Schematic representation of the *Cancer productus* stomatogastric nervous system (STNS), showing the distribution of tachykinin-related peptide (TRP)-immunopositive structures, including the anterior commissural organs (ACOs). The STNS of the crab *C. productus* consists of four ganglia as well as a number of interconnecting and motor nerves. The four ganglia are the paired commissural ganglia (CoGs), the single oesophageal ganglion (OG) and the single stomatogastric ganglion (STG). The inferior oesophageal (*ion*), oesophageal (*on*), superior oesophageal (*son*) and stomatogastric (*stn*) nerves link these ganglia, while motor nerves, including the labral (*ln*), dorsal posterior oesophageal (*dpon*), anterior cardiac (*acn*), anterior lateral (*aln*), medial ventricular (*mvn*) and dorsal ventricular (*dvn*) nerves, provide innervation to the foregut musculature. The inferior ventricular nerve (*ivn*) and the circumoesophageal connectives (*cocs*) link the STNS with the supraoesophageal (SoG) and fused thoracic ganglia (TG), respectively. The distribution of TRP immunoreactivity in the STNS of *C. productus* is shown in red. Here, immunopositive somata are schematized with filled circles, while axons within nerves are represented by thick lines, and immunopositive neuropil by tangles of thinner lines. The locations of the left and right anterior commissural organs (ACO$_L$ and ACO$_R$) are indicated with arrows. The nomenclature of ganglia and nerves is per Maynard and Dando (1974).

immunopositive somata were present in each CoG (Fig. 2A; three somata shown, with a single labeled soma present in the oesophageal ganglion). Immunopositive axons were present in

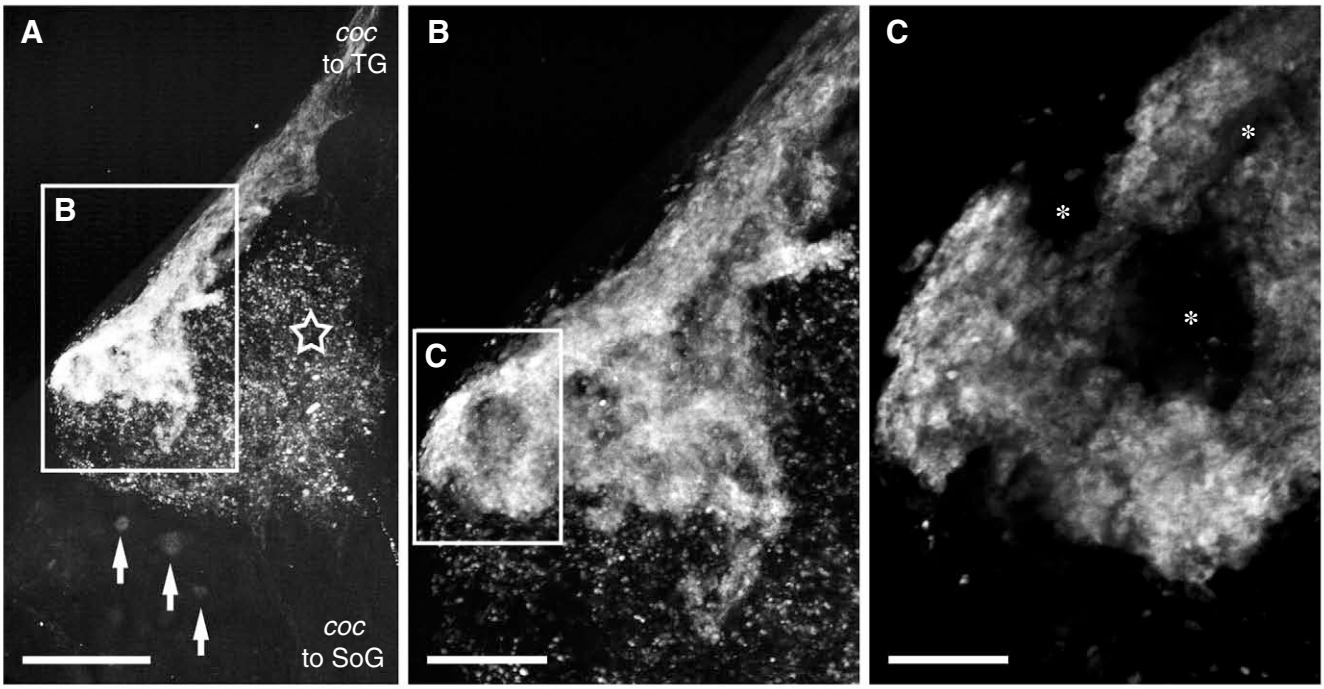

Fig. 2. Distribution of substance P-like labeling in the commissural ganglion (CoG) of the crab *Cancer productus*. (A) Within each CoG, substance P-like immunoreactivity is present in seven neuronal somata (three denoted by arrows) as well as in neuropilar processes (star) and a large club-shaped plexus (boxed). This plexus is located in the anterior medial quadrant of the ganglion and originates from a fascicle of small-diameter axons that project from the circumoesophageal connective (*coc*) connecting the supraoesophageal (SoG) and thoracic ganglia (TG) to the CoG. This micrograph is a brightest pixel projection of 24 optical sections taken at 2.0 μm intervals. (B) A higher magnification view of the plexus boxed in A. As can be seen from this micrograph, the plexus is composed of tightly aggregated, flocculent varicosities. The aggregated varicosities appear to cluster around unlabeled tubular structures, particularly in the posterior portion of the plexus. This micrograph is a brightest pixel projection of 37 optical sections taken at 1.0 μm intervals. (C) A projection of three optical sections taken at 1.0 μm intervals from the boxed region in B, showing aggregated substance P-immunopositive terminals enveloping several unlabeled tubular structures (indicated by asterisks). Scale bars, 200 μm in A, 75 μm in B and 25 μm in C.

the inferior oesophageal (*ion*; one axon per *ion*), oesophageal (*on*; two axons) and stomatogastric (*stn*; two axons) nerves, and neuropil was present in each CoG (Fig. 2A), at the junction of the superior oesophageal nerves (*sons*) with the *on* and the *stn* and also in the stomatogastric ganglion (STG). No TRP labeling was seen in any of the motor nerves posterior to the STG nor was any immunoreactivity seen in the anterior cardiac plexus (ACP), a neuroendocrine structure located on each of the paired anterior cardiac nerves (Christie et al., 2004a).

Neuroendocrine organs

In addition to examining the STNS for the presence of TRP-immunopositive profiles, we also examined two well-known neuroendocrine organs located outside this system, namely the SG and PO, for TRP-like labeling. In brachyuran crabs, the SG is recognizable as a superficial, intensely iridescent structure located between the medulla interna and medulla externa of the eyestalk ganglia (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992). The PO is located on the dorsolateral wall of the pericardial chamber and, in brachyurans, consists of two or more longitudinal nerve trunks that are interconnected by vertical nerve bars (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992). In neither the SG (N=6) nor the PO (N=6) was any TRP immunoreactivity evident (data not shown).

Distribution of hemolymph lacunae in the CoG

The existence of hemolymph sinuses and lacunae penetrating deep into the ganglia of crustaceans is well documented (Abbott, 1971, 1972; Skinner, 1985; McGaw and Reiber, 2002). In the blue crab, *Callinectes sapidus*, mapping by radiography and corrosion casting showed that the CoGs are among the ganglia fenestrated by hemolymph lacunae (McGaw and Reiber, 2002). As just described, the TRP-like labeling in the posterior portion of the *C. productus* CoG plexus is fenestrated by tubular unlabeled areas. One explanation for these regions of immunolabel avoidance is that they are hemolymph lacunae. To investigate this hypothesis, we injected physiological saline containing India ink into the pericardial chamber surrounding the heart and allowed it to be pumped throughout the vascular system, thereby filling hemolymph vessels with an easily visualized substrate.

As can be seen in the ganglion shown in Fig. 3, numerous ink-filled lacunae are present in the CoG. While the location, size, shape and depth of invagination of the individual lacunae varied between ganglia, each CoG taken from an ink-injected

animal (*N*=14 ganglia) possessed some deeply invaginated channels in its anterior medial quadrant, particularly in the posterior portion of this region.

Coincidence of hemolymph lacunae and the tachykinin-immunopositive plexus

To confirm that the ink-filled lacunae just described are coincident with the areas of immunolabel avoidance seen in the CoG plexus, we fixed and immunoprocessed a number of ink-filled ganglia (*N*=6) with the substance P antibody. Upon fixation, ink-filling was often lost in some of the smaller lacunae, particularly the superficially located ones. Irrespectively, in each of the immunoprocessed ganglia, many of the unstained regions fenestrating the label in the posterior portion of the plexus were found to contain ink (Fig. 4), thereby confirming that these areas of immunolabel avoidance are hemolymph lacunae. This organization (i.e. nerve terminals in direct apposition to hemolymph lacunae/sinuses) is similar to that of the SG (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992). Thus, this finding suggests strongly that the TRP-immunopositive CoG plexus, like the SG, is a neuroendocrine release site. Based on its location within the nervous system, we have named this structure the anterior commissural organ or ACO.

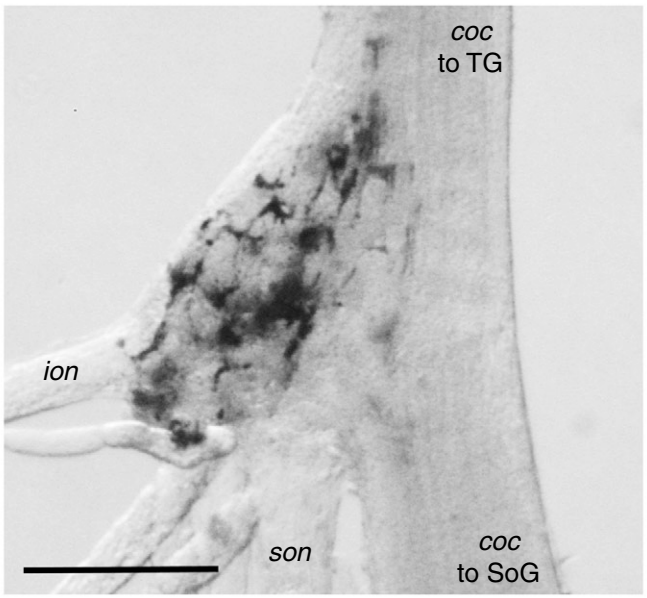

Fig. 3. Visualization of hemolymph sinuses/lacunae in the commissural ganglion (CoG) using India ink. India ink injected into the pericardial chamber is incorporated into the circulating fluid and fills blood vessels and sinuses with an easily visualized substrate. As this micrograph shows, the CoG is one of the region of the nervous system possessing vascularization. Here, India ink filling reveals an extensive array of hemolymph lacunae/sinuses within the CoG, including many in the anterior medial quadrant of the ganglion. This image is a digital micrograph of a living ganglion. Abbreviations: *coc*, circumoesophageal connective; *ion*, inferior oesophageal nerve; *son*, superior oesophageal nerve; SoG, supraoesophageal ganglia; TG, thoracic ganglia. Scale bar, 200 μm.

Immunohistochemical survey for co-transmitters in the ACO

Previous work has shown that most crustacean neuroendocrine sites contain large and often complex complements of signaling molecules (Christie et al., 1995a; Li

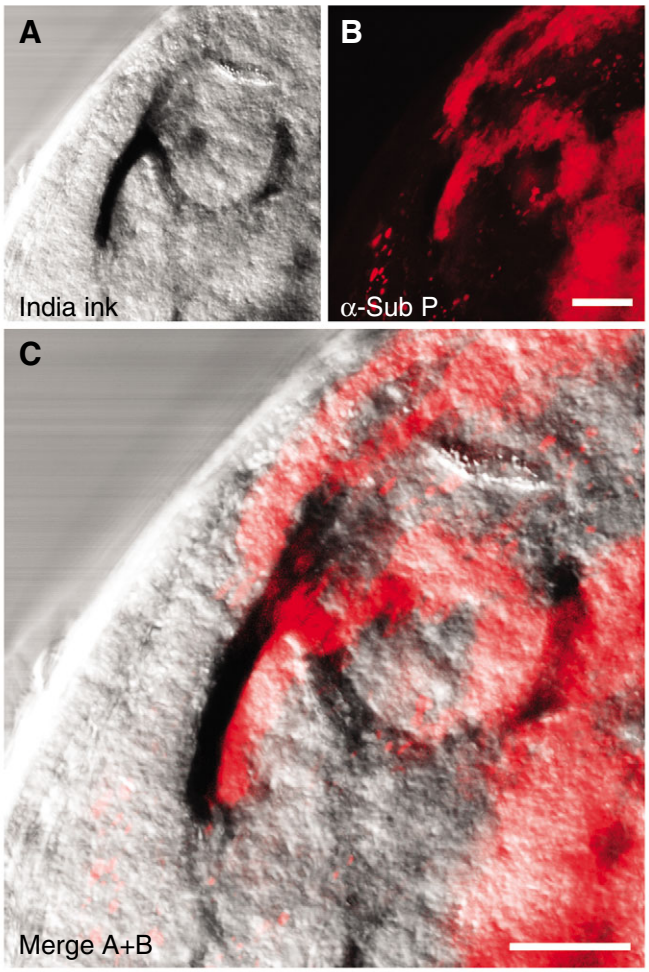

Fig. 4. Hemolymph lacunae are coincident with areas of immunolabel avoidance in the anterior commissural organ (ACO). In the commissural ganglion, the substance P-immunopositive plexus is fenestrated by what appears to be a network of branched tubes. To determine if these tubular structures are hemolymph lacunae, several India ink-filled ganglia were immunoprocessed with the substance P antibody, and the ink and immunolabel were simultaneously imaged *via* confocal microscopy. As can be seen in this set of micrographs, ink-filling (A) was evident in numerous lacunae in the portion of the ganglion containing the substance P-immunopositive plexus (B). When micrographs of the individual labels were merged (C), it became apparent that the plexus is fenestrated by hemolymph vessels. As the merged micrograph shows, the substance P-immunopositive nerve terminals that form the plexus envelop the hemolymph lacunae. This organization, with nerve terminals in direct apposition to the hemolymph space, is considered the defining characteristic of a crustacean neuroendocrine site. We hypothesize that this plexus is a neuroendocrine organ and we have named it the anterior commissural organ based on its location. Each micrograph is a brightest pixel projection of nine optical sections taken at 0.5 μm intervals. A and B are shown at the same scale. Scale bars, 20 μm in B and C.

et al., 2002a, 2003; Fu et al., 2005a,b). In an attempt to determine what other substances might be co-localized with TRP in the ACO, we immunolabeled CoGs with antibodies generated against a number of known crustacean hormones and/or neuromodulators and examined them for labeling of the plexus. In some preparations, double-labeling with substance P antibody was undertaken to unambiguously delimit the location of the ACO. The assayed substances were the small molecule transmitter GABA, the amines dopamine, histamine and serotonin, the gases carbon monoxide and nitric oxide as well as the peptides allatostatin, buccalin, CCK, corazonin, CCAP, FLRFamide, myomodulin, orcokinin, β-PDH, proctolin and RPCH. While each of the antibodies labeled neuronal profiles in the CoG and/or other regions of the *C. productus* nervous system (data not shown), none gave rise to labeling within the ACO ($N \geqslant 6$ ganglia for each antibody; Fig. 5).

Interestingly, while no co-transmitters were found in the ACO, our double-label experiments did show that this structure is surrounded in three dimensions by neuropil containing many other neuroactive compounds (Fig. 5). In many areas, these immunopositive neuropils directly abut ACO terminals. Thus, in addition to neuroendocrine release, the ACO may also function to modulate the CoG neuropil in a paracrine fashion.

Mass spectrometric detection of authentic CabTRP Ia in the CoG and hemolymph

Anterior medial quadrant of the CoG

As shown earlier, the ACO is immunolabeled by an antibody generated against the vertebrate tachykinin substance P. In crustaceans, the only TRP thus far identified is APSGFLGMRamide, commonly referred to as CabTRP Ia (Christie et al., 1997a; Nieto et al., 1998; Li et al., 2002a; Huybrechts et al., 2003; Yasuda-Kamatani and Yasuda, 2004). Previous studies have shown that the substance P antibody cross-reacts with this peptide (Christie et al., 1997a); thus, it is likely that the substance P immunoreactivity seen in the ACO results from the presence of authentic CabTRP Ia in this structure. To confirm that this is indeed the case, we isolated the quadrant of the CoG containing the ACO and subjected it to direct tissue MALDI-FTMS analysis.

As the mass spectrum in Fig. 6A shows, many peptides are present in the portion of the CoG containing the ACO. Among the detected peaks are ones corresponding to a number of previously identified peptides including several isoforms of orcokinins, corazonin, three FLRFamide-related peptides, Gly^1-

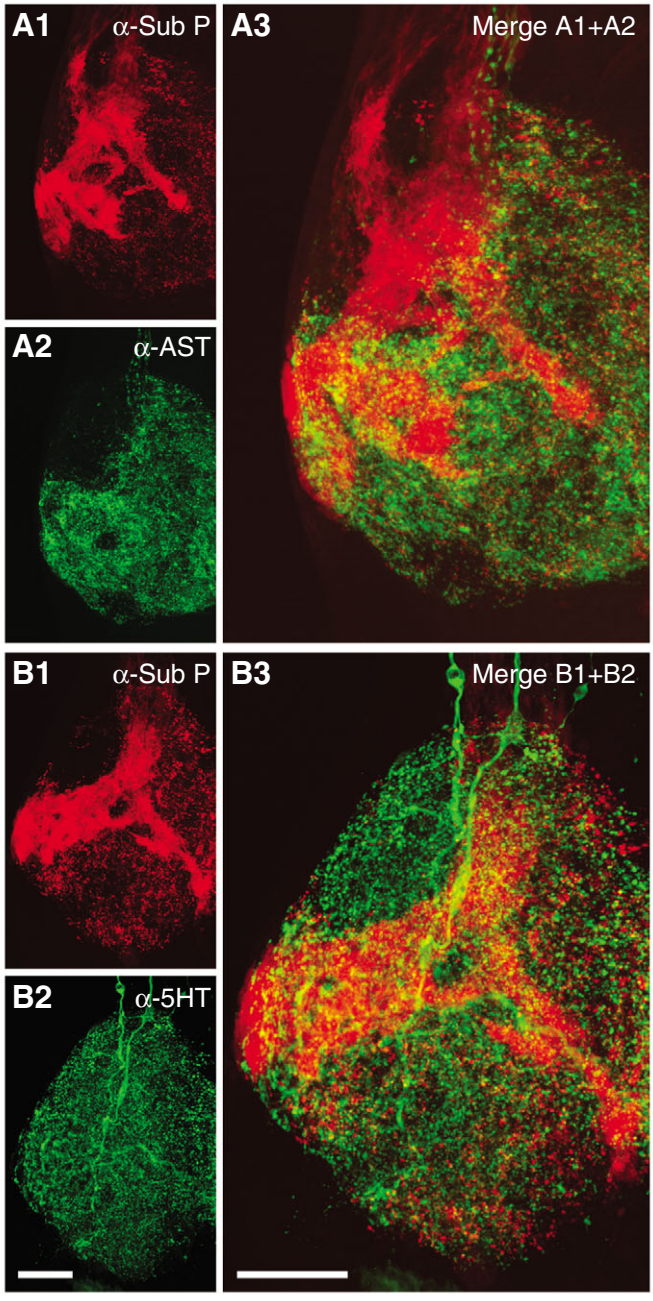

Fig. 5. Double-immunolabeling shows that the anterior commissural organ (ACO) does not possess allatostatin-like peptide or serotonin co-transmitters. (A) Co-labeling of the commissural ganglion (CoG) with antibodies to substance P (α-Sub P) and allatostatin (α-AST). Both the substance P (A1) and allatostatin (A2) antibodies give rise to extensive labeling within the CoG. However, superimposition of micrographs of the two labels (A3) shows that the ACO (as defined by the substance P label) is not allatostatin-immunopositive. All micrographs in A are brightest pixel projections of 25 optical sections taken at 2.0 μm intervals. (B) Co-labeling of the CoG with antibodies to substance P and serotonin (α-5HT). As in the previous pairing, both the substance P (B1) and serotonin (B2) antibodies give rise to extensive labeling within the CoG. Again, superimposition of micrographs of the two labels (B3) shows that the ACO (as defined by the substance P label) is not serotonin-immunopositive. All micrographs in B are brightest pixel projections of 30 optical sections taken at 2.0 μm intervals. A1, A2, B1 and B2 are shown at the same scale. Likewise, A3 and B3 are shown at the same scale. It should be noted that in A3 and B3, the apparent co-localization of the immunolabels (yellow coloration) is an artifact of the projection of multiple optical sections spanning the entire thickness of the ganglia shown rather than true co-localization of the immunoreactivities in a common structure. Scale bars, 100 μm.

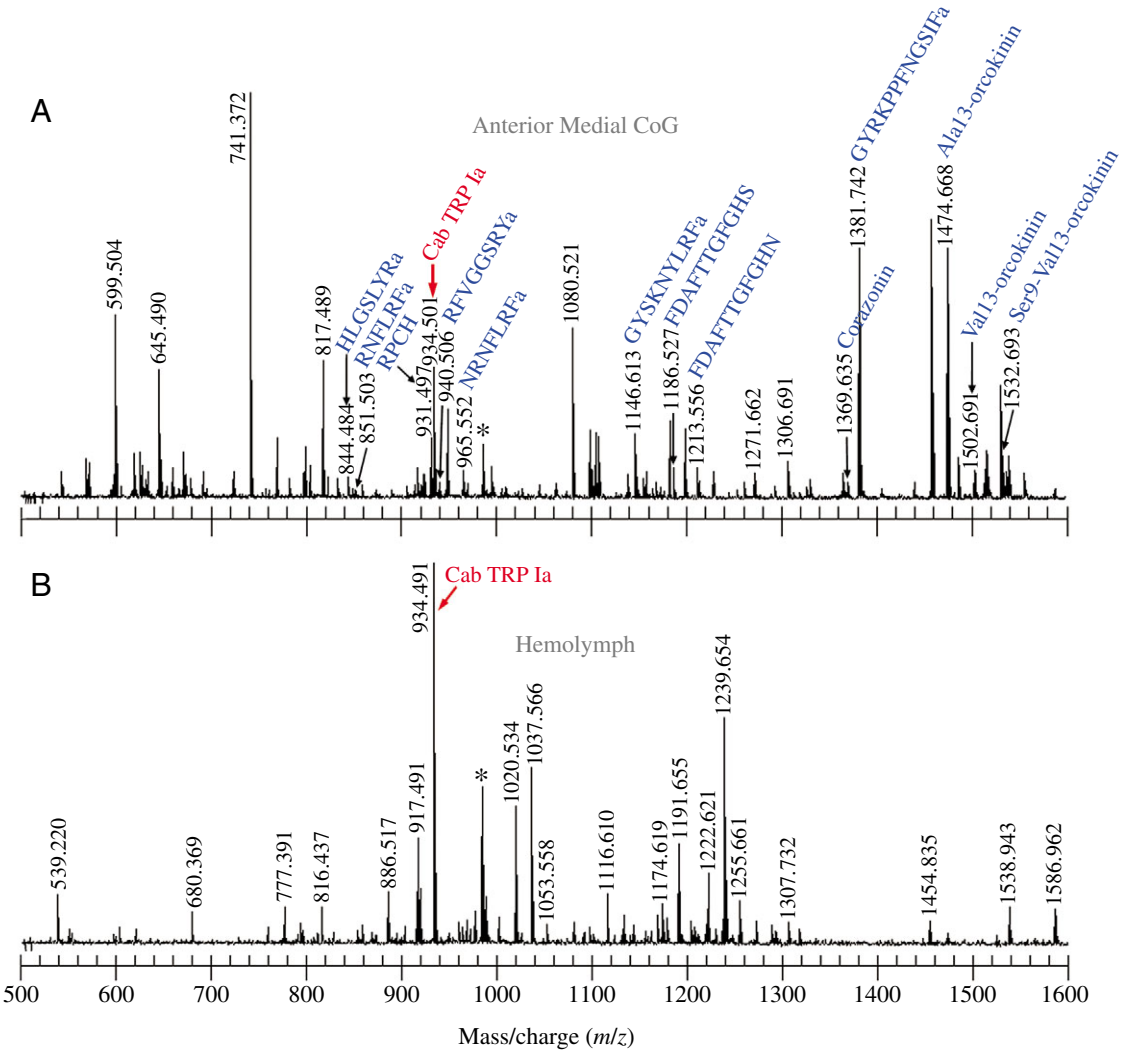

Fig. 6. Matrix-assisted laser desorption/ionization Fourier transform mass spectrometric (MALDI-FTMS) identification of APSGFLGMRamide (CabTRP Ia) in the anterior medial quadrant of the commissural ganglion (CoG) and hemolymph. (A) CabTRP Ia in the anterior medial quadrant of the CoG. As can be seen in this mass spectrum, taken from a tissue fragment from the quadrant of the CoG containing the anterior commissural organ, a number of peaks corresponding to individual peptides are evident. These include peaks corresponding to three isoforms of orcokinin, corazonin, three FLRFamide-related peptides, two orcomyotropin-related peptides (FDAFTTGFGHS and FDAFTTGFGHN), Gly^1-SIFamide, red pigment concentrating hormone (RPCH) and the recently identified peptide HLGSLYRamide (all labeled in blue). Of particular interest is the peak at *m/z* 934.501 (red arrow). This *m/z* is identical to that of authentic CabTRP Ia (a mass measurement error of 8.56 p.p.m. from its theoretical *m/z* of 934.493) and confirms the presence of this peptide in the portion of the CoG containing the ACO. (B) MALDI-FTMS detection of CabTRP Ia in the hemolymph. Hemolymph extract examined *via* MALDI-FTMS shows an *m/z* peak at 934.491 (red arrow). Again, this *m/z* peak is characteristic of authentic CabTRP Ia (mass measurement error 2.14 p.p.m.) and strongly suggests that this peptide is a circulating hormone in *C. productus*. It should be noted that in both A and B there is an *m/z* peak that corresponds to a known electrical noise artifact in the MALDI-FTMS system used. This peak is denoted *via* an asterisk in both spectra.

SIFamide and RPCH. Of particular interest is the mass/charge (*m/z*) peak at 934.501, which is essentially identical to the theoretical *m/z* of authentic CabTRP Ia (i.e. 934.493). This result is strongly suggestive of CabTRP Ia being present in authentic form in the anterior medial quadrant of the CoG and hence in the ACO.

Hemolymph

Since the ACO possesses the organization of a neuroendocrine organ, it is logical to expect that the CabTRP Ia contained within it would be released into the circulatory system. As none of the other known neuroendocrine organs in *C. productus* (i.e. the SG, the PO and the ACP) exhibits any evidence of TRP immunoreactivity, the ACO may well be the sole source of this peptide in the hemolymph. To determine if CabTRP Ia does circulate, we collected hemolymph samples from three crabs and subjected the extract to MALDI-FTMS analysis. In two of three animals,

the mass spectra of the hemolymph extract showed an *m/z* peak corresponding to that of authentic CabTRP Ia (i.e. *m/z* 934.491 in Fig. 6B). This finding strongly supports the notion that this peptide is a circulating hormone potentially released from the ACO.

Physiological effects of CabTRP Ia on the muscles of the gastric mill and pylorus

In the crab *C. borealis*, Jorge-Rivera and colleagues showed that many peptides that do not directly innervate the muscles of the gastric mill and pyloric regions of the foregut are nonetheless potent modulators of the musculature (Jorge-Rivera and Marder, 1996, 1997; Jorge-Rivera, 1997; Jorge-Rivera et al., 1998). As shown by our schematic diagram of substance P immunolabeling in the STNS, no direct TRP innervation of the gastric mill or pyloric muscles is present in *C. productus* (Fig. 1). To determine if circulating CabTRP Ia can similarly modulate stomatogastric muscles in *C. productus*, we studied its actions on EJP and contraction amplitude for a subset of the intrinsic muscles of the foregut.

Modulation of EJP amplitude

We tested the effect of 10^{-7} mol l^{-1} CabTRP Ia on the amplitude of nerve-evoked EJPs in six stomatogastric muscles (Fig. 7). Three of the muscles (gm4, gm6a and gm8a) are innervated by motor neurons (i.e. DG or LG) involved in generating the gastric mill motor pattern, while the other three muscles (gm5a, p1 and p2) are innervated by neurons (IC, LP or PY, respectively) that participate in the pyloric circuit (Maynard and Dando, 1974; Selverston and Moulins, 1987; Weimann et al., 1991; Harris-Warrick et al., 1992). For all muscles, a statistically significant and reversible increase in EJP amplitude was noted (gm4, *N*=5; gm5a, *N*=6; gm6a, *N*=8; gm8a, *N*=6; p1, *N*=8; p2, *N*=6). For all muscles, the maximum effect of CabTRP Ia occurred approximately 5–10 min after bath application was initiated and required at least 30 min of rinsing in physiological saline to return to baseline. No effect of CabTRP Ia on membrane potential was observed for any of the muscles tested.

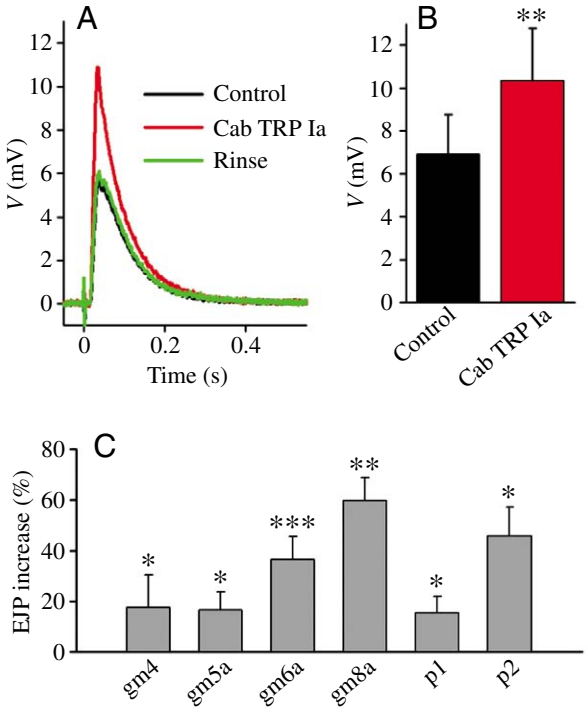

Fig. 7. The effects of CabTRP Ia on nerve-evoked excitatory junction potentials (EJPs) in six stomatogastric muscles. (A) Nerve-evoked EJP in a gastric mill 8a (gm8a) muscle fiber in control saline (black), after 10 min in 10^{-7} mol l^{-1} CabTRP Ia (red), and after rinsing the peptide (green). The membrane potential was –77 mV for all three conditions. Each trace in this panel is the average of four EJPs elicited at 20 s intervals. (B) A bar graph plot of average gm8a EJP amplitude in control saline and after 10 min in 10^{-7} mol l^{-1} CabTRP Ia. The increase in amplitude in CabTRP Ia was significant (*N*=6; paired *t*-test, $^{**}P<0.01$). (C) A bar graph showing the average increase in EJP amplitude in 10^{-7} mol l^{-1} CabTRP Ia for six stomatogastric muscles: gastric mill 4 (gm4; *N*=5), gastric mill 5a (gm5a; *N*=6), gastric mill 6a (gm6a; *N*=8), gm8a (*N*=6), pyloric 1 (p1; *N*=8) and pyloric 2 (p2; *N*=6). For each individual experiment used to construct this panel, the EJP amplitude was determined by averaging four EJPs, as in A. For each muscle, significance was computed using paired *t*-tests on the EJP amplitudes ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

Modulation of contraction amplitude

Since gm8a showed the largest increase in EJP amplitude in the presence of CabTRP Ia, we expected that nerve-evoked contractions of this muscle might also be potentiated due to application of the peptide. For these contraction experiments, the preparation consisted of fibers of both the gm8a and gm8b muscles, which were not separated so as not to damage the muscle fibers or innervating nerve. As Fig. 8 shows, bath application of 10^{-7} mol l^{-1} CabTRP Ia produced a statistically significant increase in the tension exerted by the gm8 muscle in response to a 3 s duration, 10 Hz stimulation of the *dgn* (a frequency chosen to be comparable with that of measured LG activity during a gastric mill rhythm; Beenhakker et al., 2004).

We had hoped to construct a dose–response curve characterizing the concentration dependence of the effect of CabTRP Ia on gm8 contraction but this was hindered by dramatic and consistent desensitization to the applied peptide. While the effects of a short application (5 min) of 10^{-7} mol l^{-1} CabTRP Ia could be reversed with a 10–15 min rinse of control saline, a second application of 10^{-7} mol l^{-1} CabTRP Ia an hour later resulted in a much smaller (~50%) increase in contraction. However, in measurements on individual preparations, we found that application of 10^{-8} mol l^{-1} CabTRP Ia increased contraction appreciably in each of three experiments (mean increase=35%), while the contraction increased in only one out of five experiments when 10^{-9} mol l^{-1} CabTRP Ia was applied. Hence, we conclude that the threshold concentration for a reproducible effect of CabTRP Ia on gm8 contraction is between 10^{-9} and 10^{-8} mol l^{-1}.

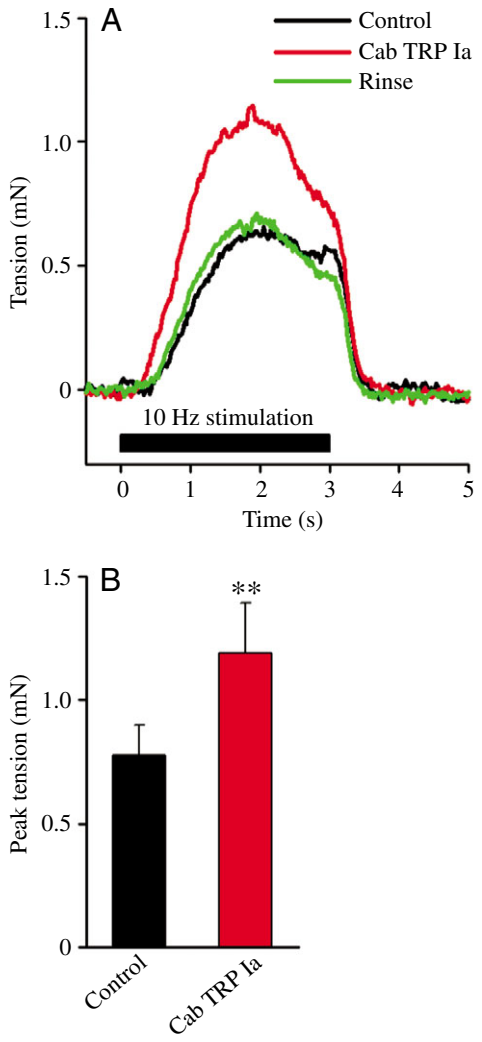

Fig. 8. CabTRP Ia increases contraction of the gastric mill 8 (gm8) muscle. (A) The lateral ventricular nerve (*lvn*) was stimulated at 10 Hz for 3 s, and the resulting contractions of a gm8 muscle in control saline (black), after 10 min in 10^{-7} mol l^{-1} CabTRP Ia (red), and after rinsing the peptide (green) are shown. (B) A bar graph of average gm8 peak contraction amplitude in control saline and after 10 min in 10^{-7} mol l^{-1} CabTRP Ia. The increase in amplitude in peptide was significant (N=7; paired t-test, $^{**}P$<0.01).

Discussion

The organization of the ACO suggests it is a neuroendocrine release site

To date, a number of well-delimited neuroendocrine organs have been identified in the nervous systems of decapod crustaceans (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992; Christie et al., 2004a). Included among these structures are the SG, the PO, the ACP and the post-commissural organ (PCO). In terms of gross organization, each of these structures is composed of aggregations of nerve terminals that are in direct apposition to the circulatory system. For some sites (i.e. the PO, the ACP and the PCO), proximity to the circulatory system is achieved by possessing a superficial location just below the sheath of the nerves containing/comprising them (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992; Christie et al., 2004a). For neuroendocrine sites whose terminals are not superficially located (i.e. the SG), invaginating hemolymph lacunae/sinuses provide released hormones with access to the hemolymph (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992).

In the present study, we have shown that the ACO of *C. productus* consists of aggregations of tachykinin-immunopositive nerve terminals that directly abut/surround hemolymph lacunae. Thus, the general organization of this structure is homologous to those of other crustacean neuroendocrine sites, and to the SG in particular. Based on this homology, we believe that, like the SG, the ACO is a neuroendocrine release organ. Our identification of the ACO as a neuroendocrine center now brings the number of characterized neuroendocrine organs in *C. productus* to four (i.e. the SG, PO, ACP and ACO; Christie et al., 2004a; Christie and Messinger, 2005; Fu et al., 2005a,b; Messinger et al., 2005).

Given that structures apparently homologous to the *C. productus* ACO have been identified in species from six infraorders of decapods [i.e. Brachyura (true crabs), Anomura (hermit crabs, porcelain crabs and squat lobsters), Palinura (spiny lobsters), Thalassinidea (ghost shrimp), Astacidea (chelate lobsters and freshwater crayfish) and Caridea (caridean shrimp); Goldberg et al., 1988; Messinger et al., 2004], the ACO appears to be a highly conserved structure and we hypothesize that its function as a neuroendocrine center is conserved in other decapod species.

The hormone complement of the ACO appears to be limited in comparison to those of other neuroendocrine sites

Recently, much work has focused on the identification of hormone complements in the neuroendocrine organs (i.e. the SG, PO and ACP) of *C. productus* (Hsu et al., 2004, 2005a,b; de la Iglesia et al., 2005; Fu et al., 2005a,b; Messinger et al., 2005). Like those of other crustacean species (Christie et al., 1995a; Li et al., 2002a, 2003), each of these sites has been shown to contain multiple neuroactive compounds. For example, using a combination of anatomical, mass spectrometric and molecular techniques, over 50 peptide hormones have been identified from the *C. productus* SG (Hsu et al., 2004, 2005a,b; de la Iglesia et al., 2005; Fu et al., 2005a,b). Similarly, over 40 putative hormones (peptides, amines and small molecules) have been identified in the *C. productus* PO (Hsu et al., 2004; Fu et al., 2005b). Even in the ACP, which is innervated by just four neurons, multiple hormones have been identified (Christie et al., 2004a; Hsu et al., 2004; Christie and Messinger, 2005; Messinger et al., 2005).

In contrast to the chemical complexity seen in the hormone complements of the other neuroendocrine organs of *C. productus*, only CabTRP Ia has been identified as a putative hormone in the ACO. Immunohistochemistry using antibodies to other known crustacean hormones/neuromodulators failed to

identify any co-transmitters in this site. However, in our mass spectrometric characterization of the anterior medial quadrant of the CoG that contains the ACO, we detected a number of peptides for which no antibodies are currently available (e.g. Gly^1-SIFamide and HLGSLYRamide) as well as a number of currently unidentified ones, and it is certainly possible that some of these peptides are contained within and utilized as signaling agents by the ACO.

The ACO as a neurohemal source of circulating CabTRP Ia

Thus far, the only neuroactive compound identified in the *C. productus* ACO is CabTRP Ia. In our study, we show *via* mass spectrometry that this peptide is also present in the hemolymph of this crab. Using immunohistochemistry, we screened the other known neuroendocrine organs of *C. productus* (i.e. the SG, PO and ACP) for the presence of CabTRP Ia and found that each tissue lacked labeling. Moreover, mass spectrometric analysis also failed to detect this peptide in these sites (Christie and Messinger, 2005; Fu et al., 2005b; Messinger et al., 2005). Collectively, these findings suggest that if the CabTRP Ia seen in the hemolymph is derived from a neuroendocrine organ, the ACO is a likely source of the peptide.

Hormonally delivered CabTRP Ia is capable of modulating the contractile properties of the musculature of the foregut

As just described, CabTRP Ia is detectable in the hemolymph of *C. productus*, and the ACO is one potential source of this circulating peptide. Given a hormonal mode of delivery, multiple target tissues are likely to exist for this peptide in the crab. As we have shown here, one target of hemolymph-borne CabTRP Ia is the musculature of the foregut. In this species, none of the muscles of the gastric mill or pyloric regions of the foregut is directly innervated by TRP-immunopositive axons. Nevertheless, a hormonally relevant concentration (10^{-7} mol l^{-1}) of CabTRP Ia increased EJPs in three of three gastric mill and three of three pyloric muscles tested. Moreover, concentrations as low as 10^{-8} mol l^{-1} were observed to consistently increase the contraction amplitude of at least one muscle, with the enhancement presumably due in part to the CabTRP Ia-evoked increase in EJP amplitude.

The most thoroughly studied peptide modulators of stomatogastric musculature are TNRNFLRFamide and SDRNFLRFamide in the crab *C. borealis* (Jorge-Rivera and Marder, 1996; Jorge-Rivera et al., 1998). Our investigation of the effects of CabTRP Ia in *C. productus*, while not nearly as extensive as the earlier work, has produced some similar observations. Application of either FLRFamide peptide increased the nerve-evoked contraction in 15 out of 17 muscles tested, with a threshold concentration of 10^{-10} mol l^{-1} (TNRNFLRFamide) or 10^{-9}–10^{-8} mol l^{-1} (SDRNFLRFamide) (Jorge-Rivera and Marder, 1996). We measured the threshold concentration of CabTRP Ia for an increase on gm8 contraction to be between 10^{-9} mol l^{-1} and 10^{-8} mol l^{-1} and observed effects on EJP amplitude in all six muscles tested.

Although we have not explored the biophysical mechanisms responsible for the increase in EJP amplitude, we note that no shift in muscle membrane potential was observed that would clearly indicate a change in muscle input resistance. In the crab *C. borealis*, CabTRP Ia and four other peptides (including TNRNFLRFamide) have been observed to activate an inward current (Swensen and Marder, 2000) in subsets of STG motor neurons (Swensen and Marder, 2001). Interestingly, the other four peptides, all presumably acting as hormones, also increase muscle contractions in *C. borealis* (Jorge-Rivera et al., 1998). Given the low-frequency motor discharge that drives many of the foregut muscles, we hypothesize that circulating CabTRP Ia may be crucial for maintaining appreciable muscle contractions in the gastric mill and pylorus, as has been proposed for other peptide hormones, including the extended FLRFamides (Jorge-Rivera and Marder, 1996).

Paracrine signaling from the ACO to intrinsic targets in the CoG

Our results show that many of the nerve terminals constituting the ACO envelop and/or directly abut hemolymph lacunae. This organization should allow released hormone direct access to the circulatory system and is widely recognized as the defining characteristic of a crustacean neuroendocrine release site (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992; Christie et al., 2004a). Intriguingly, there are some areas of the ACO that do not directly abut the circulatory system, but instead are in apposition to putative synaptic neuropil. Here, peptide released from the ACO may also function as a paracrine modulator of intrinsic CoG targets.

One possible role for paracrine signaling by the ACO is the coordination of multiple neuroendocrine systems (Fig. 9). The soma of the large (L)-cell, which provides both peptidergic and aminergic innervation to the PO (Cooke and Sullivan, 1982; Beltz, 1988; Fingerman, 1992), is located within the CoG and, in *C. productus*, extends processes to the general vicinity of the ACO (A.E.C., unpublished observations). Likewise, the somata innervating the ACP [i.e. anterior commissural neurons 1 and 2 (ACN1/2); Christie et al., 2004a; Christie and Messinger, 2005; Messinger et al., 2005] are contained within the CoG and arborize in the vicinity of the plexus. If the L-cell and/or ACN1/2 are modulated by CabTRP Ia (or some other unidentified co-transmitter within the ACO), then elements of at least two other neuroendocrine centers (i.e. the PO and ACP) could be simultaneously modulated/synchronized locally within the CoG, with concurrent release of hormones to the circulatory system.

Non-endocrine neurons within the CoG may also be targets of paracrine signals emanating from the ACO (Fig. 9). In *Cancer* species, the area of the CoG containing this plexus is known to be the location of the intrinsic arbors of many projection neurons that innervate and modulate the STG (Coleman and Nusbaum, 1994; Norris et al., 1994, 1996; Coleman et al., 1995; Bartos and Nusbaum, 1997; Blitz et al., 1999; Nusbaum, 2002; Wood et al., 2004). If these neurons are influenced by CabTRP Ia (or another compound) released from the ACO in paracrine fashion, then a profound reorganization

of the motor patterns produced by the stomatogastric circuit could occur, in addition to the effects that hemolymph-borne hormones have on the foregut musculature.

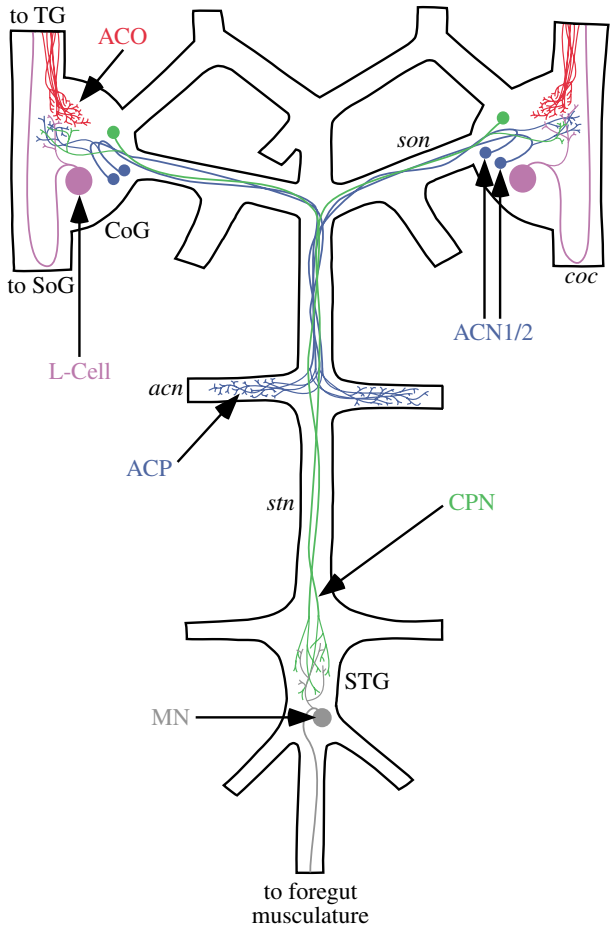

Fig. 9. Schematic representation of hypothetical paracrine actions of the anterior commissural organ (ACO) in the commissural ganglion (CoG). In addition to serving as a source of CabTRP Ia to the hemolymph, some portions of the ACO terminate directly on areas of synaptic neuropil, and this structure may thus also function as a paracrine modulator of intrinsic CoG targets. One potential role for this paracrine signaling is the coordination of multiple neuroendocrine systems. The soma of the large (L)-cell, which projects to and innervates the pericardial organ (PO), is located within the CoG and is known to arborize in the vicinity of the ACO. Likewise, anterior commissural neurons 1 and 2 (ACN1/2), which are the sole source of innervation to the anterior cardiac plexus (ACP), are also located in the CoG and arborize near the ACO. If these neurons are modulated by CabTRP Ia, then elements of at least two other neuroendocrine centers could be modulated/synchronized locally within the CoG, concurrent with the release of CabTRP Ia from the ACO into the circulatory system. Non-endocrine neurons within the CoG [specifically CoG projection neurons (CPN) that innervate and modulate the stomatogastric neural circuit] may also be targets of paracrine signals from the ACO. If these neurons are influenced by CabTRP Ia released from the ACO, then a profound reorganization of the motor patterns produced by the motor neurons (MN) of the circuits contained within the stomatogastric ganglion could occur. Abbreviations not defined in this legend are as per Fig. 1.

We thank all members of the Friday Harbor Laboratories' Spring 2004 Research Apprenticeship 'From Neurons to Behavior: Comparative Neurobiology of Marine Invertebrates' for their support. Likewise, we thank Jackson ImmunoResearch Inc., Leica Microsystems, Molecular Probes and Vector Laboratories for their contributions of equipment and reagents to the above class. A. Countryman, T. Larson and T. Halatchev are thanked for their help with immunohistochemistry. Drs H. Dircksen, R. Elde, D. Nässel, K. Rao, B. Stay and J. Veenstra are thanked for their generous contributions of antibodies and Dr M. Nusbaum is thanked for his contribution of synthetic CabTRP Ia. Drs B. Swalla and D. Tauck are thanked for allowing us use of their epifluorescence microscope system and microelectrode puller, respectively. A.E.C., D.I.M. and C.T.N. acknowledge financial support from the University of Washington Department of Biology's Casey Fund for Undergraduate Research, the Mary Gates Endowment for Students, the Howard Hughes Medical Institute (HHMI), the Ellis B. Ridgeway Fellowship Fund and the University of Washington Office of the Provost. L.L. acknowledges support from the University of Wisconsin, Wisconsin Alumni Research Foundation, a research award from the American Society for Mass Spectrometry and a National Science Foundation CAREER Award (CHE-0449991). K.K.K. acknowledges support from NIH Chemistry–Biology Interface Training Grant (T32 GM008505). J.T.B. and D.R.V. acknowledge support from the Research Corporation, the Grass Foundation, Santa Clara University and an award to SCU under the Undergraduate Biological Sciences Education Program of the HHMI.

References

Abbott, N. J. (1971). The organization of the cerebral ganglion in the shore crab, *Carcinus maenas*. II. The relation of intracerebral blood vessels to other brain elements. *Z. Zellforsch. Mikrosk. Anat*. **120**, 401-419.

Abbott, N. J. (1972). Access of ferritin to the interstitial space of *Carcinus* brain from intracerebral blood vessels. *Tissue Cell*. **4**, 99-104.

Bartos, M. and Nusbaum, M. P. (1997). Intercircuit control of motor pattern modulation by presynaptic inhibition. *J. Neurosci*. **17**, 2247-2256.

Beenhakker, M. P., Blitz, D. M. and Nusbaum, M. P. (2004). Long-lasting activation of rhythmic neuronal activity by a novel mechanosensory system in the crustacean stomatogastric nervous system. *J. Neurophysiol*. **91**, 78-91.

Beltz, B. S. (1988). Crustacean neurohormones. In *Invertebrate Endocrinology,* Vol. 2, *Endocrinology of Selected Invertebrate Types* (ed. H. Laufer, R. G. H. Downer), pp. 235-258. New York: Alan R. Liss, Inc.

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.

Blitz, D. M., Christie, A. E., Coleman, M. J., Norris, B. J., Marder, E. and Nusbaum, M. P. (1999). Different proctolin neurons elicit distinct motor patterns from a multifunctional neuronal network. *J. Neurosci.* **19**, 5449-5463.

Bungart, D., Dircksen, H. and Keller, R. (1994). Quantitative determination and distribution of the myotropic neuropeptide orcokinin in the nervous system of astacidean crustaceans. *Peptides* **15**, 393-400.

Cerri, P. S., de Faria, F. P., Villa, R. G. and Katchburian, E. (2004). Light microscopy and computer three-dimensional reconstruction of the blood capillaries of the enamel organ of rat molar tooth germs. *J. Anat*. **204**, 191-195.

Chang, M. M., Leeman, S. E. and Niall, H. D. (1971). Amino-acid sequence of substance P. *Nat. New. Biol.* **232**, 86-87.

Christie, A. E. and Messinger, D. I. (2005). Hormonal signaling from the crustacean stomatogastric nervous system. *Comp. Biochem. Physiol.* **144A**, S157.

Christie, A. E. and Nusbaum, M. P. (1995). Distribution and effects of corazonin-like and allatotropin-like peptides in the crab stomatogastric nervous system. *Soc. Neurosci. Abstr.* **21**, 629.

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., Skiebe, P. and Marder, E. (1995a). Matrix of neuromodulators in neurosecretory structures of the crab *Cancer borealis*. *J. Exp. Biol*. **198**, 2431-2439.

Christie, A. E., Baldwin, D., Turrigiano, G., Graubard, K. and Marder, E. (1995b). 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., Lundquist, C. T., Nässel, D. R. and Nusbaum, M. P. (1997a). Two novel tachykinin-related peptides from the nervous system of the crab *Cancer borealis*. *J. Exp. Biol*. **200**, 2279-2294.

Christie, A. E., Baldwin, D. H., Marder, E. and Graubard, K. (1997b). Organization of the stomatogastric neuropil of the crab, *Cancer borealis*, as revealed by modulator immunocytochemistry. *Cell Tissue Res*. **288**, 135-148.

Christie, A. E., Edwards, J. M., Cherny, E., Clason, T. A. and Graubard, K. (2003). Immunocytochemical evidence for nitric oxide- and carbon monoxide-producing neurons in the stomatogastric nervous system of the crayfish *Cherax quadricarinatus*. *J. Comp. Neurol*. **467**, 293-306.

Christie, A. E., Cain, S. D., Edwards, J. M., Clason, T. A., Cherny, E., Lin, M., Manhas, A. S., Sellereit, K. L., Cowan, N. G., Nold, K. A. et al. (2004a). The anterior cardiac plexus: an intrinsic neurosecretory site within the stomatogastric nervous system of the crab *Cancer productus*. *J. Exp. Biol*. **207**, 1163-1182.

Christie, A. E., Stein, W., Quinlan, J. E., Beenhakker, M. P., Marder, E. and Nusbaum, M. P. (2004b). Actions of a histaminergic/peptidergic projection neuron on rhythmic motor patterns in the stomatogastric nervous system of the crab *Cancer borealis*. *J. Comp. Neurol*. **469**, 153-169.

Coleman, M. J. and Nusbaum, M. P. (1994). Functional consequences of compartmentalization of synaptic input. *J. Neurosci.* **14**, 6544-6552.

Coleman, M. J., Meyrand, P. and Nusbaum, M. P. (1995). A switch between two modes of synaptic transmission mediated by presynaptic inhibition. *Nature* **378**, 502-505.

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.

Cuello, A. C., Galfre, G. and Milstein, C. (1979). Detection of substance P in the central nervous system by a monoclonal antibody. *Proc. Natl. Acad. Sci. U.S.A*. **76**, 3532-3536.

de la Iglesia, H. O., Hsu, Y. A., Weller, J. R. and Christie, A. E. (2005). Molecular cloning of cDNAs encoding crustacean hyperglycemic hormone (CHH) and its precursor related peptide (CPRP) from the sinus gland of the crab, *Cancer productus.* Program No. 30.6. *2005 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience 2005 (Online).

Dircksen, H. and Keller, H. (1988). Immunocytochemical localization of CCAP, a novel crustacean cardioactive peptide, in the nervous system of the shore crab *Carcinus maenas* L. *Cell Tissue Res.* **254**, 347-360.

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.

Erspamer, V. and Anastasi, A. (1962). Structure and pharmacological actions of eledoisin, the active endecapeptide of the posterior salivary glands of *Eledone*. *Experientia* **18**, 58-59.

Farley, R. D. (1990). Regulation of air and blood flow through the book lungs of the desert scorpion *Paruroctonus mesaensis*. *Tissue Cell* **22**, 547-569.

Fénelon, V. S., Kilman, V., Meyrand, P. and Marder, E. (1999). Sequential developmental acquisition of neuromodulatory inputs to a central pattern-generating network. *J. Comp. Neurol*. **408**, 335-351.

Fingerman, M. (1992). Decapod crustacean glands. In *Microscopic Anatomy of Invertebrates,* Vol. 10, *Decapod Crustacea* (ed. F. W. Harrison and A. G. Humes), pp. 345-394. New York: Wiley-Liss Inc.

Fingerman, M., Hanumante, M. M., Kulkarni, G. K., Ikeda, R. and Vacca, L. L. (1985). Localization of substance P-like, leucine-enkephalin-like, methionine-enkephalin-like, and FMRFamide-like immunoreactivity in the eyestalk of the fiddler crab, *Uca pugilator*. *Cell Tissue Res*. **241**, 473-477.

Fu, Q., Christie, A. E. and Li, L. (2005a). Mass spectrometric characterization of crustacean hyperglycemic hormone precursor-related peptides (CPRPs) from the sinus gland of the crab, *Cancer productus*. *Peptides* In press.

Fu, Q., Kutz, K. K., Schmidt, J. J., Hsu, Y. A., Messinger, D. I., Cain, S. D., de la Iglesia, H. O., Christie, A. E. and Li, L. (2005b). Hormone complement of the *Cancer productus* sinus gland and pericardial organ: an anatomical and mass spectrometric investigation. *J. Comp. Neurol*. In press.

Glantz. R. M., Miller, C. S. and Nässel, D. R. (2000). Tachykinin-related peptide and GABA-mediated presynaptic inhibition of crayfish photoreceptors. *J. Neurosci*. **20**, 1780-1790.

Goldberg, D., Nusbaum, M. P. and Marder, E. (1988). Substance P-like immunoreactivity in the stomatogastric nervous systems of the crab *Cancer borealis* and the lobsters *Panulirus interruptus* and *Homarus americanus*. *Cell Tissue Res*. **252**, 515-522.

Grivas, I., Michaloudi, H., Batzios, C., Chiotelli, M., Papatheodoropoulos, C., Kostopoulos, G. and Papadopoulos, G. C. (2003). Vascular network of the rat hippocampus is not homogeneous along the septotemporal axis. *Brain Res*. **971**, 245-249.

Harris-Warrick, R. M., Marder, E., Selverston, A. I. and Moulins, M. (ed.) (1992). *Dynamic Biological Networks: The Stomatogastric Nervous System.* Cambridge: MIT Press.

Hogers, B., DeRuiter, M. C., Baasten, A. M., Gittenberger-de Groot, A. C. and Poelmann, R. E. (1995). Intracardiac blood flow patterns related to the yolk sac circulation of the chick embryo. *Circ. Res*. **76**, 871-877.

Hsu, Y. A., Weller, J. R., de la Iglesia, H. O. and Christie, A. E. (2004). Immunocytochemical evidence for crustacean hyperglycemic hormone-related peptides in the neuroendocrine organs of *Cancer* crabs. Program No. 274.4. *2004 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience 2004 (Online).

Hsu, Y. A., de la Iglesia, H. O. and Christie, A. E. (2005a). Crustacean hyperglycemic hormone-related peptides in *Cancer productus*. *Comp. Biochem. Physiol.* **141A**, S157.

Hsu, Y. A., Messinger, D. I., Christie, A. E. and de la Iglesia, H. O. (2005b). Molecular characterization and putative circadian cycling of β-pigment dispersing hormone (β-PDH) in the sinus gland of the crab *Cancer productus*. Program No. 30.3. *2005 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience 2005 (Online).

Hutchinson, D. A. and Savitzky, A. H. (2004). Vasculature of the parotoid glands of four species of toads (bufonidae: bufo). *J. Morphol*. **260**, 247-254.

Huybrechts, J., Nusbaum, M. P., Bosch, L. V., Baggerman, G., De Loof, A. and Schoofs, L. (2003). Neuropeptidomic analysis of the brain and thoracic ganglion from the Jonah crab, *Cancer borealis*. *Biochem. Biophys. Res. Commun*. **308**, 535-544.

Johnson, E. C., Garczynski, S. F., Park, D., Crim, J. W., Nässel, D. R. and Taghert, P. H. (2003). Identification and characterization of a G protein-coupled receptor for the neuropeptide proctolin in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **100**, 6198-6203.

Jorge-Rivera, J. C. (1997). *Modulation of stomatogastric musculature in the crab,* Cancer borealis. PhD dissertation, Brandeis University, Waltham.

Jorge-Rivera, J. C. and Marder, E. (1996). TNRNFLRFamide and SDRNFLRFamide modulate muscles of the stomatogastric system of the crab *Cancer borealis*. *J. Comp. Physiol. A.* **179**, 741-751.

Jorge-Rivera, J. C. and Marder, E. (1997). Allatostatin decreases stomatogastric neuromuscular transmission in the crab *Cancer borealis*. *J. Exp. Biol.* **200**, 2937-2946.

Jorge-Rivera, J. C., Sen, K., Birmingham, J. T., Abbott, L. F. and Marder, E. (1998). Temporal dynamics of convergent modulation at a crustacean neuromuscular junction. *J. Neurophysiol.* **80**, 2559-2570.

Kutz, K. K., Schmidt, J. J. and Li, L. (2004). *In situ* tissue analysis of neuropeptides by MALDI FTMS in-cell accumulation. *Anal. Chem.* **76**, 5630-5640.

Lane, N. J., Harrison, J. B. and Bowerman, R. F. (1981). A vertebrate-like blood–brain barrier, with intraganglionic blood channels and occluding junctions, in the scorpion. *Tissue Cell* **13**, 557-576.

Langworthy, K., Helluy, S., Benton, J. and Beltz, B. (1997). Amines and peptides in the brain of the American lobster: immunocytochemical localization patterns and implications for brain function. *Cell Tissue Res*. **288**, 191-206.

Li, L., Pulver, S. R., Kelley, W. P., Sweedler, J. V. and Marder, E. (2002a). Towards revealing the full complement of neurohormones in crustacean pericardial organs and hemolymph by mass spectrometry. Program No. 609.13. *2002 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2002 (Online).

Li, L., Pulver, S. R., Kelley, W. P., Thirumalai, V., Sweedler, J. V. and Marder, E. (2002b). Orcokinin peptides in developing and adult crustacean stomatogastric nervous systems and pericardial organs. *J. Comp. Neurol.* **444**, 227-244.

Li, L., Kelley, W. P., Billimoria, C. P., Christie, A. E., Pulver, S. R., Sweedler, J. V. and Marder, E. (2003). Mass spectrometric investigation of the neuropeptide complement and release in the pericardial organs of the crab, *Cancer borealis*. *J. Neurochem.* **87**, 642-656.

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.

Mancillas, J. R., McGinty, J. F., Selverston, A. I., Karten, H. and Bloom, F. E. (1981). Immunocytochemical localization of enkephalin and substance P in retina and eyestalk neurones of lobster. *Nature* **293**, 576-578.

Marinkovic, S., Milisavljevic, M., Gibo, H., Malikovic, A. and Djulejic, V. (2004). Microsurgical anatomy of the perforating branches of the vertebral artery. *Surg. Neurol.* **61**, 190-197.

Maynard, D. M. and Dando, M. R. (1974). The structure of the stomatogastric neuromuscular system in *Callinectes sapidus*, *Homarus americanus* and *Panulirus argus* (Decapoda Crustacea). *Phil. Trans. R. Soc. Lond. B* **268**, 161-220.

McGaw, I. J. and Reiber, C. L. (2002). Cardiovascular system of the blue crab *Callinectes sapidus*. *J. Morphol.* **251**, 1-21.

Meola, S. M., Clottens, F. L., Holman, G. M., Nachman, R. J., Nichols, R., Schoofs, L., Wright, M. S., Olson, J. K., Hayes, T. K. and Pendleton, M. W. (1998). Isolation and immunocytochemical characterization of three tachykinin-related peptides from the mosquito, *Culex salinarius*. *Neurochem. Res.* **23**, 189-202.

Messinger, D. I., Ngo, C. T., Cain, S. D. and Christie, A. E. (2004). Phylogenetic conservation of a tachykinin-containing neuroendocrine organ in the commissural ganglia of decapod crustaceans. Program No. 274.3. *2004 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, 2004 (Online).

Messinger, D. I., Verley, D. R., Fu, Q., Kutz, K. K., Hsu, Y. A., Li, L., Birmingham, J. T. and Christie, A. E. (2005). Structural and functional characterization of the anterior cardiac neuron 1/2-anterior cardiac plexus (ACN1/2-ACP) neuroendocrine system of the crab *Cancer productus*. *Soc. Neurosci. Abstr.* Program No. 30.8. *2005 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience 2005 (Online).

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.

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.

Mortin, L. I. and Marder, E. (1991). Differential distribution of beta-pigment-dispersing hormone (beta-PDH)-like immunoreactivity in the stomatogastric nervous system of five species of decapod crustaceans. *Cell Tissue Res.* **265**, 19-33.

Muren, J. E. and Nässel, D. R. (1996). Radioimmunoassay determination of tachykinin-related peptide in different portions of the central nervous system and intestine of the cockroach *Leucophaea maderae*. *Brain Res.* **739**, 314-321.

Muren, J. E. and Nässel, D. R. (1997). Seven tachykinin-related peptides isolated from the brain of the Madeira cockroach: evidence for tissue-specific expression of isoforms. *Peptides* **18**, 7-15.

Murray, B. and Wilson, D. J. (1997). Muscle patterning, differentiation and vascularisation in the chick wing bud. *J. Anat.* **190**, 261-273.

Nachman, R. J., Moyna, G., Williams, H. J., Zabrocki, J., Zadina, J. E., Coast, G. M. and Vanden Broeck, J. (1999). Comparison of active conformations of the insectatachykinin/tachykinin and insect kinin/Tyr-W-MIF-1 neuropeptide family pairs. *Ann. N.Y. Acad. Sci.* **897**, 388-400.

Nässel, D. R. (1993). Insect myotropic peptides: differential distribution of locustatachykinin- and leucokinin-like immunoreactive neurons in the locust brain. *Cell Tissue Res.* **274**, 27-40.

Nässel, D. R. (1999). Tachykinin-related peptides in invertebrates: a review. *Peptides* **20**, 141-158.

Nieto, J., Veelaert, D., Derua, R., Waelkens, E., Cerstiaens, A., Coast, G., Devreese, B., Van Beeumen, J., Calderon, J., De Loof, A. et al. (1998). Identification of one tachykinin- and two kinin-related peptides in the brain of the white shrimp, *Penaeus vannamei*. *Biochem. Biophys. Res. Commun.* **248**, 406-411.

Norris, B. J., Coleman, M. J. and Nusbaum, M. P. (1994). Recruitment of a projection neuron determines gastric mill motor pattern selection in the stomatogastric nervous system of the crab, *Cancer borealis*. *J. Neurophysiol.* **72**, 1451-1463.

Norris, B. J., Coleman, M. J. and Nusbaum, M. P. (1996). Pyloric motor pattern modification by a newly identified projection neuron in the crab stomatogastric nervous system. *J. Neurophysiol.* **75**, 97-108.

Nusbaum, M. P. (2002). Regulating peptidergic modulation of rhythmically active neural circuits. *Brain. Behav. Evol.* **60**, 378-387.

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.

Panula, P., Happola, O., Airaksinen, M. S., Auvinen, S. and Virkamaki, A. (1988). Carbodiimide as a tissue fixative in histamine immunohistochemistry and its application in developmental neurobiology. *J. Histochem. Cytochem.* **36**, 259-269.

Pulver, S. R. and Marder, E. (2002). Neuromodulatory complement of the pericardial organs in the embryonic lobster, *Homarus americanus*. *J. Comp. Neurol.* **451**, 79-90.

Renkin, E. M. (1985). Flow and distribution of India ink in microvessels of the frog. *Microvasc. Res.* **29**, 32-44.

Renkin, E. M., Gray, S. D. and Dodd, L. R. (1981). Filling of microcirculation in skeletal muscles during timed India ink perfusion. *Am. J. Physiol.* **241**, 174-186.

Sandeman, D. C., Sandeman, R. E. and de Couet, H. G. (1990a). Extraretinal photoreceptors in the brain of the crayfish *Cherax destructor*. *J. Neurobiol.* **21**, 619-629.

Sandeman, R. E., Sandeman, D. C. and Watson, A. H. (1990b). Substance P antibody reveals homologous neurons with axon terminals among somata in the crayfish and crab brain. *J. Comp. Neurol.* **294**, 569-582.

Sasaki, F., Doshita, A., Matsumoto, Y., Kuwahara, S., Tsukamoto, Y. and Ogawa, K. (2003). Embryonic development of the pituitary gland in the chick. *Cells Tissues Organs* **173**, 65-74.

Schmidt, M. (1997a). Distribution of presumptive chemosensory afferents with FMRFamide- or substance P-like immunoreactivity in decapod crustaceans. *Brain Res.* **746**, 71-84.

Schmidt, M. (1997b). Distribution of centrifugal neurons targeting the soma clusters of the olfactory midbrain among decapod crustaceans. *Brain Res.* **752**, 15-25.

Schmidt, M. and Ache, B. W. (1994). Descending neurons with dopamine-like or with substance P/FMRFamide-like immunoreactivity target the somata of olfactory interneurons in the brain of the spiny lobster, *Panulirus argus*. *Cell Tissue Res.* **278**, 337-352.

Schmidt, M. and Ache, B. W. (1997). Immunocytochemical analysis of glomerular regionalization and neuronal diversity in the olfactory deutocerebrum of the spiny lobster *Cell Tissue Res.* **287**, 541-563.

Schoofs, L., Holman, G. M., Hayes, T. K., Kochansky, J. P., Nachman, R. J. and De Loof, A. (1990a). Locustatachykinin III and IV: two additional insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *Regul. Pept.* **31**, 199-212.

Schoofs, L., Holman, G. M., Hayes, T. K., Nachman, R. J. and De Loof, A. (1990b). Locustatachykinin I and II, two novel insect neuropeptides with homology to peptides of the vertebrate tachykinin family. *FEBS Lett.* **261**, 397-401.

Schoofs, L., Vanden Broeck, J. and De Loof, A. (1993). The myotropic peptides of *Locusta migratoria*: structures, distribution, functions and receptors. *Insect Biochem. Mol. Biol.* **23**, 859-881.

Selverston, A. I. and Moulins. M. (ed.) (1987). *The Crustacean Stomatogastric System.* Berlin: Springer.

Severini, C., Improta, G., Falconieri-Erspamer, G., Salvadori, S. and Erspamer, V. (2002). The tachykinin peptide family. *Pharmacol. Rev.* **54**, 285-322.

Siviter, R. J., Coast, G. M., Winther, A. M., Nachman, R. J., Taylor, C. A., Shirras, A. D., Coates, D., Isaac, R. E. and Nässel, D. R. (2000). Expression and functional characterization of a *Drosophila* neuropeptide precursor with homology to mammalian preprotachykinin A. *J. Biol. Chem.* **275**, 23273-23280.

Skiebe, P., Dreger, M., Meseke, M., Evers, J. F. and Hucho, F. (2002). Identification of orcokinins in single neurons in the stomatogastric nervous system of the crayfish, *Cherax destructor*. *J. Comp. Neurol.* **444**, 245-259.

Skinner, K. (1985). The structure of the fourth abdominal ganglion of the crayfish, *Procambarus. clarki* (Girard). I. Tracts in the ganglionic core. *J. Comp. Neurol.* **234**, 168-181.

Stangier, J., Hilbich, C., Dircksen, H. and Keller, R. (1988). Distribution

of a novel cardioactive neuropeptide (CCAP) in the nervous system of the shore crab *Carcinus maenas*. *Peptides* **9**, 795-800.

Stay, B., Chan, K. K. and Woodhead, A. P. (1992). Allatostatin-immunoreactive neurons projecting to the corpora allata of adult *Diploptera punctata*. *Cell Tissue Res.* **270**, 15-23.

Swensen, A. M. and Marder, E. (2000). Multiple peptides converge to activate the same voltage-dependent current in a central pattern-generating circuit. *J. Neurosci*. **20**, 6752-6759.

Swensen, A. M. and Marder, E. (2001). Modulators with convergent cellular actions elicit distinct circuit outputs. *J. Neurosci*. **21**, 4050-4058.

Swensen, A. M., Golowasch, J., Christie, A. E., Coleman, M. J., Nusbaum, M. P. and Marder, E. (2000). GABA and responses to GABA in the stomatogastric ganglion of the crab *Cancer borealis*. *J. Exp. Biol*. **203**, 2075-2092.

Takeuchi, H., Yasuda, A., Yasuda-Kamatani, Y., Kubo, T. and Nakajima, T. (2003). Identification of a tachykinin-related neuropeptide from the honeybee brain using direct MALDI-TOF MS and its gene expression in worker, queen and drone heads. *Insect Mol. Biol.* **12**, 291-298.

Thirumalai, V. and Marder, E. (2002). Colocalized neuropeptides activate a central pattern generator by acting on different circuit targets. *J. Neurosci.* **22**, 1874-1882.

Tierney, A. J., Godleski, M. S. and Rattananont, P. (1999). Serotonin-like immunoreactivity in the stomatogastric nervous systems of crayfishes from four genera. *Cell Tissue Res*. **295**, 537-551.

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.

Vanden Broeck, J., Torfs, H., Poels, J., Van Poyer, W., Swinnen, E., Ferket, K. and De Loof, A. (1999). Tachykinin-like peptides and their receptors. A review. *Ann. N.Y. Acad. Sci.* **897**, 374-387.

Veenstra, J. A. (1991). Presence of corazonin in three insect species, and isolation and identification of [His7]corazonin from *Schistocerca americana*. *Peptides* **12**, 1285-1289.

Weimann, J. M., Meyrand, P. and Marder, E. (1991). Neurons that form multiple pattern generators: identification and multiple activity patterns of gastric/pyloric neurons in the crab stomatogastric system. *J. Neurophysiol*. **65**, 111-122.

Winther, A. M. and Nässel, D. R. (2001). Intestinal peptides as circulating hormones: release of tachykinin-related peptide from the locust and cockroach midgut. *J. Exp. Biol*. **204**, 1269-1280.

Wood, D. E., Manor, Y., Nadim, F. and Nusbaum, M. P. (2004). Intercircuit control *via* rhythmic regulation of projection neuron activity. *J. Neurosci*. **24**, 7455-7463.

Woodhead, A. P., Stoltzman, C. A. and Stay, B. (1992). Allatostatins in the nerves of the antennal pulsatile organ muscle of the cockroach *Diploptera punctata*. *Arch. Insect. Biochem. Physiol*. **20**, 253-263.

Yasuda-Kamatani, Y. and Yasuda, A. (2004). APSGFLGMRamide is a unique tachykinin-related peptide in crustaceans. *Eur. J. Biochem*. **271**, 1546-1556.