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Crustacean Hyperglycemic Hormone in the Lobster Nervous System: Localization and Release From Cells in the Subesophageal Ganglion and Thoracic Second Roots

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ABSTRACT

Crustacean hyperglycemic hormones (CHHs) are neuropeptides involved in the regulation of hemolymph glucose. The primary source of CHHs has been identified as the neurosecretory neurons of the eyestalk X-organ and its associated neurohemal organ, the sinus gland. We have identified another source of CHH-like peptides in the nervous system. With the use of immunocytochemistry, cells in the second roots of the thoracic ganglia have been observed to stain positively for CHH-reactive material. We also identified a pair of cells in the subesophageal ganglion that contain large amounts of CHH-reactive material. Depolarization of these cells with elevated potassium mediates a calcium-dependent release of CHH-like material from the ganglion as quantified with an enzyme-linked immunosorbent assay (ELISA). J. Comp. Neurol. 414:50–56, 1999.

Indexing terms: CHH; confocal microscopy; ELISA; *Homarus americanus*; neuropeptide

A recent flurry of research activity has been concerned with the isolation and characterization of members of the crustacean hyperglycemic hormone (CHH) family of neuropeptides (Keller, 1992; Chang, 1993; De Kleijn and Van Herp, 1995). The occurrence of these neuropeptides has so far been limited to the Arthropoda, and in particular, to the crustaceans. The functions attributed to members of this family are diverse, and include regulation of: hemolymph glucose concentration (from which the name CHH is derived; Keller, 1992); molting (molt-inhibiting hormone, MIH; Chang et al., 1990; Klein et al., 1993; Lee et al., 1995; Chung et al., 1996); reproduction (vitellogenesis-inhibiting hormone, VIH; Soyez et al., 1991); other glands (mandibular organ-inhibiting hormone; Wainright et al., 1996; Liu et al., 1997); and ion transport peptide in an insect (Meredith et al., 1996).

Among the fully characterized CHHs are those from the experimental animal of this study, the American lobster, *Homarus americanus* (Chang et al., 1990, 1998; Tensen et al., 1991; De Kleijn et al., 1995). Immunocytochemical studies, using antisera against CHHs from several species, consistently map the neurohormone to clusters of large neurosecretory perikarya in the X-organs on the surface of the medulla terminalis of crustacean eyestalks whose axons project to the sinus gland neurohemal organs where they terminate (c.f. Gorgels-Kallen et al., 1982; Leuven et al., 1982; Martin et al., 1984; Van Herp et al., 1984; Rotllant et al., 1993).

Limited evidence for the synthesis and release of CHH-related peptides from other parts of the nervous system has been presented (Keller et al., 1985; De Kleijn et al., 1995). In recent studies, we demonstrated the existence of CHH-immunoreactive materials in neural locations other than the eyestalk (Chang et al., 1999). In addition, we found small, but significant concentrations of CHH immu-

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noreactivity in the hemolymph of lobsters that had been eyestalk-ablated (removing the X-organs and sinus glands) for over 1 year (Chang et al., 1998). Stress-related increases in the levels of CHHs in the hemolymph also are seen in eyestalk-ablated animals, although the increases are smaller than those seen in normal animals (Chang et al., 1999). Because these results all point to the existence of extra-eyestalk sources of CHH-like neuropeptides, we examined the neurons-of-origin of these peptides.

Clusters of neurosecretory neurons are found along the thoracic second roots of lobsters (Wallace et al., 1974; Evans et al., 1976a). The cell bodies of these neurons are located in the proximal sections of these roots between the point in the ventral nerve cord where the roots emerge and a bifurcation of the root into medial and lateral branches. Originally suggested to be aminergic, the cells subsequently were found to be densely innervated by nerve terminals containing serotonin and octopamine, but not to contain the amines (Livingstone et al., 1981; Beltz and Kravitz, 1983; Schneider et al., 1993). Both serotonin and octopamine inhibit the firing of the cells (Konishi and Kravitz, 1978). Neurosecretory endings of these cells are found in the vicinity of their cell bodies and possibly also at remote central and peripheral sites (Konishi and Kravitz, 1978; Livingstone et al., 1981). The chemical nature of the neurosecretory material contained within the root cells has remained enigmatic.

In this paper, we present evidence that the thoracic second root neurosecretory cells and at least one distinct pair of cells located in the subesophageal ganglion contain CHH-like immunoreactivity. We also report that depolarization with elevated potassium mediates a calcium-dependent release of CHH-like material from the subesophageal ganglion.

MATERIALS AND METHODS

Animals

Adult lobsters were purchased from commercial sources in Boston, MA. Full-sibling juvenile lobsters were raised in our facility at Bodega Bay, CA, using methods previously described (Chang and Conklin, 1993; Conklin and Chang, 1993). Animals were fed a pelleted diet and frozen shrimp three times a week. The water temperature was 13 ± 2°C. The dates of molts were recorded on each animal for several consecutive molt cycles. The salinity of the circulating seawater was 33 parts per thousand and the photoperiod was 16:8 L:D. Lobsters were anesthetized by chilling on ice for 30 minutes prior to dissection. This work did not require any institutional experimental animal protocols because it involved invertebrates.

Enzyme-linked immunosorbent assay (ELISA) and release experiments

Dissections of the central nervous system (CNS), including the thoracic second roots, were performed as previously described (Evans et al., 1976a). For determination of CHH content, the CNS was divided into discrete sections, which were homogenized in 0.2 ml of ice-cold phosphate-buffered saline (136.9 mM NaCl, 10.1 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, pH 7.3). After centrifugation, the supernatant fluids were assayed using an ELISA for CHH (Chang et al., 1998).

Release experiments were modeled after those of Schwarz et al. (1984). Neural tissues were dissected and pinned onto Sylgard 184 (Dow-Corning, Midland, MI) filling the bottoms of wells in 48-well plates (Falcon, Franklin Lakes, NJ). The tissues were preincubated in lobster saline (462 mM NaCl, 16 mM KCl, 26 mM $CaCl_2$, 8 mM $MgCl_2$, 11 mM glucose, 10 mM HEPES, pH 7.4 with NaOH; modified from Evans et al., 1976b). The lobster saline was then removed for assay via ELISA and replaced with 0.5 ml of either normal saline, saline without added calcium (containing 34 mM $MgCl_2$, otherwise as above), high potassium saline without added calcium (362 mM NaCl, 116 mM KCl, 34 mM $MgCl_2$, otherwise as above), or high potassium saline (362 mM NaCl, 116 mM KCl, otherwise as above). After a 120-minute preincubation in normal saline at 14°C, the experiment was started. At 10-minute intervals, the above solutions were removed and assayed. At the end of the incubations, tissues were homogenized and assayed by ELISA to measure their remaining CHH.

Immunocytochemistry

For immunocytochemistry, neural tissues were fixed in 4% paraformaldehyde for several hours or overnight, depending on the thickness of the tissue, and washed in a modified phosphate buffer (0.1 M sodium phosphate, pH 7.4, with 0.45 M NaCl, and 0.4% Triton X-100). A primary rabbit antiserum made against lobster CHH-A (dilution of 1:500), that had been characterized previously (Chang et al., 1998), was added in the modified buffer, and tissues were incubated for 2 days. Further processing was by our previously established procedures (Beltz and Kravitz, 1983; Schneider et al., 1993). Tissues were incubated in the secondary antiserum (biotinylated goat anti-rabbit IgG) overnight, and all further steps (addition of avidin, biotinylated horseradish peroxidase, and diaminobenzidine) were made according to the manufacturers instructions (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA).

For confocal laser scanning microscopy, tissues were prepared as above except that the secondary antiserum was cyanine dye-conjugated (Cy-3) goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). The stained tissues were visualized with a confocal microscope (MRC 600; Bio-Rad, Hercules, CA) equipped with an argon/krypton laser. Optical sections (15- to 20-μm-thick) were collected and examined individually or in stacks. Figure 3 was stored digitally and manipulated to optimize contrast. The projection was captured with CoMOS software (Bio-Rad; v 7.0) and printed with Adobe Photoshop (v 5.0.2). Controls consisted of incubating tissues with either preimmune sera or with antisera that had first been mixed with either lobster sinus gland extracts or purified CHH.

Linking neurons to terminals: Filling second root neurons with horseradish peroxidase (HRP)

Thoracic ganglia and their second roots were dissected and pinned tautly in Sylgard-coated dishes through which fresh, oxygenated lobster saline was perfused. Low resistance (~20 megohm) electrodes were filled with 5% HRP in 0.5 M KAc. After penetrating a second root neuron and establishing a stable resting potential, depolarizing current (10–50 nannoamps) was passed at 2–5 Hz for 1 hour. Preparations then were moved to vials containing fresh oxygenated saline and stored at 4°C for 20 hours to allow diffusion of the HRP. Preparations were fixed for 1 hour in 1% glutaraldehyde, 1% paraformaldehyde, 10% sucrose, 0.1 picric acid, 1% potasssium dichromate, 0.5% calcium

TABLE 1. Content of Crustacean Hyperglycemic Hormone (CHH) in Various Portions of the Lobster Thoracic Nervous System (Mean ± S.D.)

Tissue	CHH total fmol	CHH fmol/mg tissue	n
Subesophageal ganglion	29.2 ± 9.5	1.3 ± 1.0	4
Subesophageal ganglion second roots	15.2 ± 6.1	43.3 ± 43.1	7
First thoracic ganglion	10.0 ± 10.3	0.6 ± 0.6	4
First thoracic ganglion first roots	16.3 ± 19.8	1.5 ± 1.7	4
First thoracic ganglion second roots	14.2 ± 4.7	41.5 ± 23.7	5
Second thoracic ganglion	2.9 ± 2.1	0.2 ± 0.1	4
Second thoracic ganglion second roots	22.9 ± 20.5	33.9 ± 13.0	5
Third thoracic ganglion	5.6 ± 5.4	0.3 ± 0.2	4
Third thoracic ganglion second roots	28.5 ± 17.0	16.3 ± 15.3	5
Fourth thoracic ganglion	11.0 ± 12.5	0.1 ± 0.1	4
Fourth thoracic ganglion second roots	44.8 ± 33.9	200.2 ± 405.6	5
Fifth thoracic ganglion	14.4 ± 6.0	0.9 ± 0.4	3
Fifth thoracic ganglion first roots	12.0 ± 21.9	1.5 ± 2.0	6
Fifth thoracic ganglion second roots	9.7 ± 1.8	11.5 ± 1.5	3
Interganglion connectives	9.6 ± 8.3	0.8 ± 0.8	6

chloride, and 0.1 M sodium cacodylate buffer at pH 7.4. This fixation was followed by rinses first in 0.1 M cacodylate and then 0.1 M phosphate buffer (PB). Tissues were incubated in 3,3′-diaminobenzidine (DAB; 0.5 mg/ml PB) for 30 minutes; the peroxidase label was visualized by treatment with DAB and 0.02% peroxide. Preparations were rinsed several times in PB, then in sodium cacodylate (0.1 M), and finally immersed in paraformaldehyde/glutaraldehyde fixative (see above) for 10–24 hours. During this time, preparations were photographed and sketches were made of fiber patterns and varicosities, which provided spatial guidance for later serial sectioning for electron microscopy. Following rinses in cacodylate buffer, tissues were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. After rinsing in buffer, tissues were placed in acetate buffer containing uranyl acetate for en bloc staining (2–24 hours), and finally dehydrated and placed in propylene oxide. Tissues were infiltrated with Epon (Polysciences, Warrington, PA) and embedded in flat blocks. Blocks were serially sectioned with a Sorvall Porter-Blum (Newtown, CT) MT2-B ultramicrotome, and sections were counterstained with uranyl acetate and lead citrate. Sections were examined with a JEOL (Peabody, MA) 100C electron microscope.

RESULTS

Distribution of CHH-like immunoreactivity

Various portions of the lobster ventral nerve cord were assayed for the presence of CHH-like immunoreactive substances. Although our antisera cannot distinguish between CHH-A and CHH-B (nor their isoforms; Chang et al., 1990), it does not recognize any other sinus gland peptides in *H. americanus*. We found significant levels of CHH immunoreactivity in all parts of the nervous system assayed (Table 1). There was a wide range of variability, however, in the contents. The lowest levels were found in thoracic ganglia 1–5 (T1–T5, <1 fmol/mg tissue), whereas the highest levels were found in thoracic second roots emerging from those ganglia (up to 200 fmol/mg tissue). Even examining total amounts per tissue fragment, the second roots contained up to 10 times the amounts of CHH-like material found in the ganglia. The subesophageal ganglion also contained higher levels of CHH-like material than the T1–T5 ganglia, ranging from 2 to 10 times higher than what was found in the thoracic ganglia.

Immunocytochemical localization of the active material

Next we examined the thoracic portion of the ventral nerve cord by using immunocytochemistry and found many fibers and varicosities that stained positively, particularly in the thoracic second roots (Fig. 1). Of particular interest, however, was the observation that small clusters of neuronal somata in the vicinity of the bifurcation of these roots stained as well (Fig. 1). The only cell bodies known to be present along these roots were sets of neurosecretory cells originally described over 20 years ago (Fig. 2A; Wallace et al., 1974; Evans et al., 1976a,b). These cells sent out long processes that could be traced directly to varicosities along the roots in the vicinity of the cell bodies that also stained positively for CHH. Tissues incubated with either preimmune sera or antisera preincubated with sinus gland extracts or purified CHH did not display any staining above background levels (data not shown).

In earlier studies, injections of fluorescent dyes into the root cell bodies also had demonstrated their linkage to nearby varicosities (Evans et al., 1976a). The earlier studies, however, had not provided detailed information on the morphological features of these nearby endings. To establish the morphological and ultrastructural properties of the neurons and their endings, 48 cells were filled with HRP by microinjection (Fig. 2B); of these, 15 were sectioned and examined with the electron microscope (Fig. 2C,D). The HRP-filled varicosities found (Fig. 2C,D), contained large (150–250 nm) round, dense granules, most similar to the type 1 terminals defined by Livingstone et al. (1981). These granules have an electron-dense, homogeneous matrix, typical of granules seen in identified lobster peptidergic neurons (cf. Kobierski et al., 1987). In addition, Livingstone et al. (1981) noted that type 1 terminals also contain clusters of small (45–55 nm) round, clear vesicles. In the terminals seen in HRP-filled second root cells, we did not see any of these vesicles. These vesicles, however, could easily have been obscured by the presence of the HRP reaction product. Ultrastructural characteristics of the other terminal types observed in our preparations matched those described by Livingstone et al. (1981) and therefore the tissues appear to have been well-preserved by our method.

In all preparations (n = 4), we also observed a pair of laterally positioned cells in the subesophageal ganglion that stained positively for CHH (Fig. 3). In one experiment, a second pair of immunostaining cells was also seen in the subesophageal ganglion. We saw no other cells in the ventral nerve cord that stained positively for CHH.

Release experiments

We then placed subesophageal ganglia and isolated thoracic second roots individually into small chambers and sequentially added the following solutions: normal saline; saline without added calcium; saline with high potassium without added calcium; and saline with high potassium. In the first 120 minutes after dissection, relatively large amounts of CHH were detected in the normal saline. This was likely due to release from the severed roots. The amount of release was low and relatively constant after 120 minutes. Subsequently, after each 10-minute incubation, the salines were withdrawn and assayed by ELISA for CHH immunoreactivity. We observed elevated levels of immunoreactive material released with high potassium

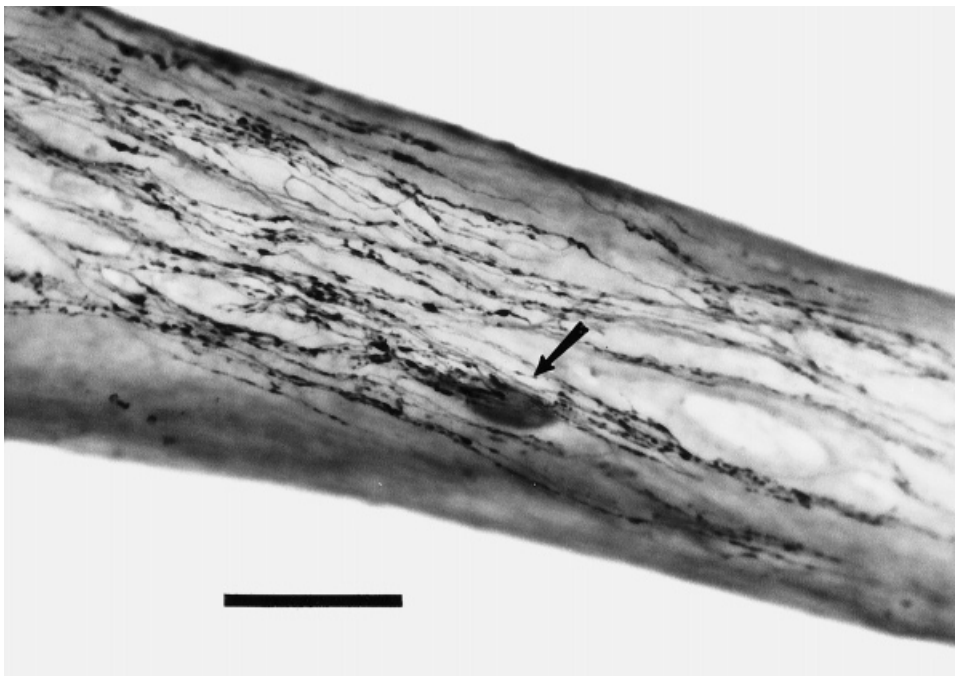

Fig. 1. Light micrograph of the second root of the third thoracic ganglion just proximal to the bifurcation. Arrow indicates a cell body surrounded by numerous fibers and varicosities that stained positively for crustacean hyperglycemic hormones (CHH). The tissue was fixed and stained with diaminobenzidine as described in Materials and Methods. Scale bar = 100 μm.

(in the presence of calcium). The most reproducible results were obtained in the subesophageal ganglion incubations. The data from one such series of experiments is shown in Figure 4. The preparations could be repeatedly stimulated with high levels of potassium to release CHH immunoreactivity; however, the effect was less robust and more variable over time.

Relatively large amounts of CHH immunoreactivity still remained in subesophageal ganglia following release experiments. For the preparations used in Figure 4, a mean of 14.7 fmol of immunoreactivity to CHH still remained in the tissues at the end of the release experiment. We are uncertain as to why the release experiments using the thoracic second roots were more variable.

DISCUSSION

Identification of peptides

The clusters of neurons located in the proximal portions of thoracic second roots were originally identified as neurosecretory on the basis of their morphological and physiological properties (Evans et al., 1976a,b; Konishi and Kravitz, 1978; Livingstone et al., 1981). These neurons show large overshooting action potentials with a prominent afterhyperpolarization, and their cell bodies, processes, and nerve terminals all show numerous large dense-cored granules, characteristic of peptidergic neurons (Fig. 3 and Evans et al., 1976a; Livingstone et al., 1981). The chemical nature of the contents of these root cells was previously unknown, although they were originally misidentified as having been aminergic (Evans et al., 1976b). Our present observations suggest that these cells likely contain one of the two forms of the 72-amino acid peptide CHH. The antiserum we used in these studies cannot distinguish between the two primary forms of this peptide: CHH-A (which also has molt-inhibiting activity) and CHH-B (Chang et al., 1990). We also cannot rule out the possibility that some peptide, either in addition to or other than CHH/MIH, may be contained within the root cells and the positively staining subesophageal ganglion cells. In earlier studies, however, we did not find any other peptides in either the hemolymph or sinus gland that cross-reacted with our antiserum (Chang et al., 1998).

Extra-eyestalk location of CHH peptides

The demonstration of CHH localization in extra-eyestalk locations in lobsters is supported by reports of CHH gene expression in the ventral nerve cord of *H. americanus* (De Kleijn et al., 1995; Reddy et al., 1997) and by the finding of CHH immunoreactivity in the brain, thoracic ganglion, and pericardial organs of *C. maenas* (Keller et al., 1985; Dircksen and Heyn, 1998). The observations reported here continue our earlier studies in which we used ELISA measurements of distinct areas of the CNS from both intact and eyestalk-ablated animals (Chang et al., 1998, 1999). After more than 1 year, lobsters still have significant levels of CHH in their hemolymph after eyestalk ablation (about 10% of intact controls). Although we are uncertain whether the observed immunoreactivity represents simple storage or actual synthesis, we suspect that it reflects synthesis. Support for this hypothesis comes from RNA hybridization data (De Kleijn et al., 1995). In addition, from measurements of a short half-life of CHH (about 30 minutes; R. Keller, personal communication), the CHH immunoreactivity observed in eyestalk-ablated lobsters could not be due to peptide synthesized over 1 year earlier.

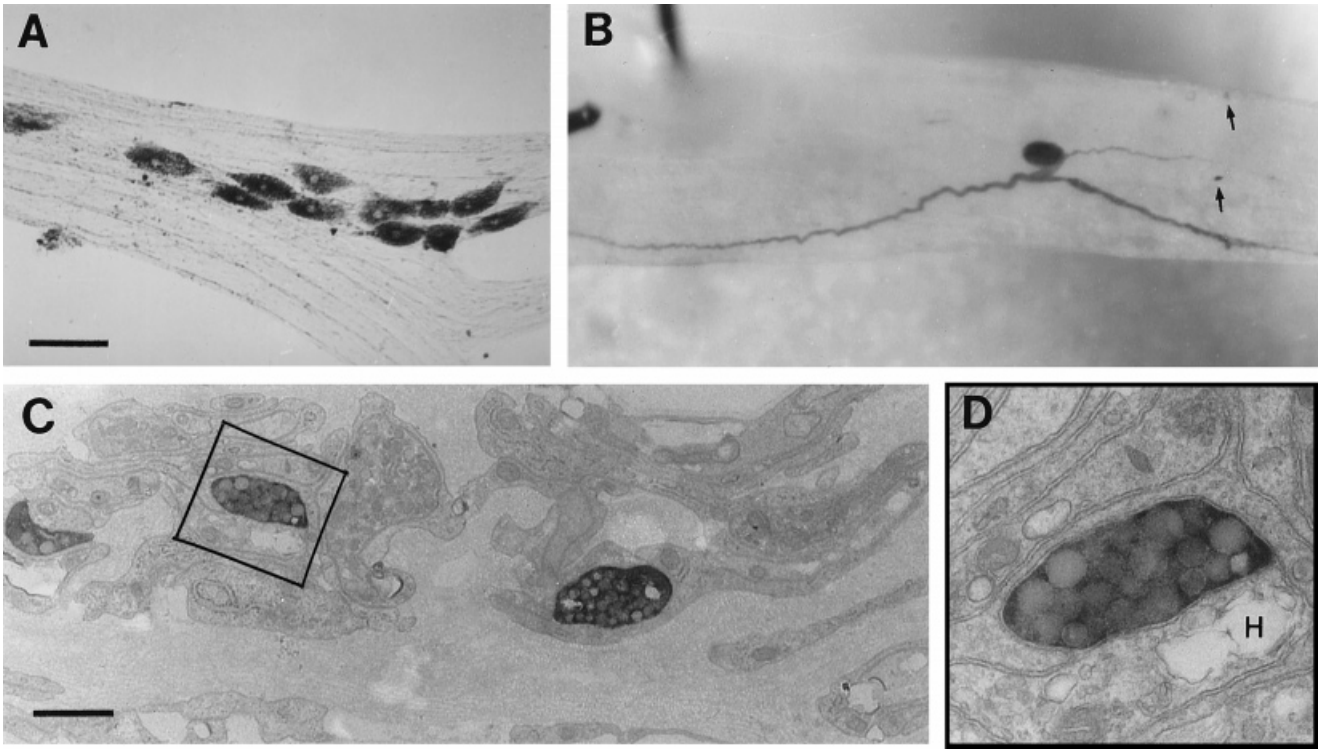

Fig. 2. **A:** A cluster of neurosecretory neurons in the second root of a thoracic ganglion in the lobster *Homarus americanus* is stained with the dye neutral red. **B:** Horseradish peroxidase (HRP) has been iontophoretically injected into the cell body of one of the thoracic second root neurons. The cell body (~40 μm diameter), its projections, and a few terminals (arrows) have been filled with HRP. **C:** Second root preparations were prepared for electron microscopy and thin-sectioned to observe the features of the filled terminals. Three varicose terminals from the same injected cell are shown in this micrograph of a region at the surface of the nerve root. **D:** A higher magnification of one of the HRP-filled terminals (see box in C) illustrates the morphology of the dense granules. H, hemolymph sinus. Scale bars = 100 μm for A and B; 150 nm for C; 420 nm for D.

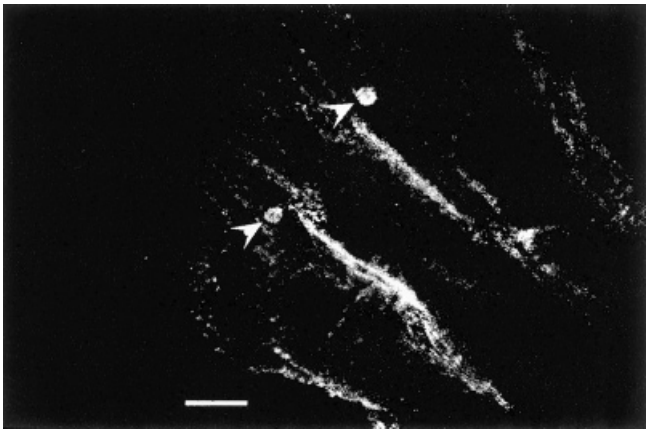

Fig. 3. A confocal projection of 12 collected (0.2-μm sections) images of the subesophageal ganglion. Anterior is towards the upper left. The tissue was fixed and stained with cyanine dye-conjugated IgG (Cy-3) as described in Materials and Methods. Note the 2 fluorescent cells (arrowheads) and the posteriorly projecting axons that may belong to them. Scale bar = 100 μm.

The function of the CHH produced from extra-eyestalk locations is unknown. Based upon measurements of hemolymph CHH (Chang et al., 1998), the amount of extra-eyestalk CHH measured in the neural tissue reported in this study is unlikely to contribute significantly to glucose regulation at the organismal level. However, it could mediate a localized regulation of cellular glucose metabolism. For example, under periods of stress, localized release of CHH could be important in meeting elevated metabolic requirements of neural cells. Thus far, little is known about the action of CHH in target cells other than the hepatopancreas and muscle (Keller, 1992).

It is also possible that the extra-eyestalk material could serve neuromodulatory roles in addition to endocrine roles. In vertebrate systems, for example, hypothalamic peptides that were originally thought only to serve roles in the secretion of pituitary hormones, have since been found to have multiple roles within the CNS as well (cf. Dunn and Berridge, 1990). Other crustacean neuropeptides found in the hemolymph that serve endocrine functions also function as neuromodulators. To illustrate, red pigment-concentrating hormone (RPCH) was first characterized as a hormone from the X-organ-sinus gland complex that mediates chromatophore contraction (Fernlund and Josefsson, 1968). By bioassay, RPCH was also found in cell bodies of the abdominal nerve cord (Fingerman and Couch, 1967), where receptors for the hormone were subsequently localized (Prestwich et al., 1991). Finally, in several crustacean preparations, RPCH was found to be a modulator of synaptic transmission (Nusbaum and Marder, 1988; Sarojini et al., 1995). Release caused by depolarization of the second root cells and subesophageal ganglion with high potassium in the presence of calcium further suggests a

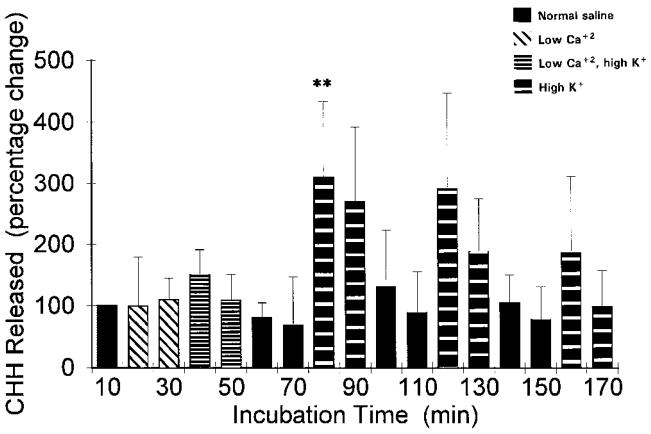

Fig. 4. Calcium-dependent release of crustacean hyperglycemic hormones (CHH) immunoreactivity in response to depolarization of the subesophageal ganglion. Ganglia were incubated in 0.5 ml of saline. Saline was replaced after 120 minutes (start of the experiment) and then at 10-minute intervals thereafter. Following the incubations, the fractions were assayed for CHH immunoreactivity with enzyme-linked immunosorbent assay (ELISA). The saline was replaced with either normal saline (black bars), saline without added calcium (diagonal stripes), saline containing high potassium without added calcium (thin horizontal stripes), or saline containing high potassium (thick horizontal stripes). See Materials and Methods for the ionic compositions of the saline solutions. Although no additional calcium was added to the incubations labeled "low Ca^{+2}," there likely were residual amounts of calcium from the incubations in normal saline; hence, the "low" designation. Means (± S.D.) of the percentage change from the first 10-minute incubation are shown (n = 4). The first high potassium incubation (after 80 minutes) resulted in a significant increase in CHH release compared to the prior 10-minute interval (**$P < 0.01$, t-test). Subsequent high potassium incubations (after 120 and 160 minutes) continued to show this trend, but due to the high interanimal variability, the data did not display statistical significance.

physiological role of the extra-eyestalk CHH as either a neurohormone or neuromodulator (Schwarz et al., 1984).

Synaptic physiology of the root cells

The physiological properties of the thoracic second root neurosecretory neurons have been extensively studied (Konishi and Kravitz, 1978). The neurons are temperature-sensitive, varying in activity from silent to firing continuously, over the normal range of temperature that lobsters live within. The root neurons are surrounded by terminals containing serotonin and octopamine, both of which inhibit the firing of the cells (Livingstone et al., 1981). The probable origins of these nerve terminals are sets of serotonin and octopamine neurosecretory neurons found within ganglia of the ventral nerve cord (Beltz and Kravitz, 1983; Schneider et al., 1993). These latter cells, which are important in postural regulation in lobsters, have central and peripheral sets of endings and form neurosecretory plexuses in the vicinity of the CHH-immunopositive cells along the thoracic second roots (Beltz and Kravitz, 1983, 1987). Moreover, CHH-related peptides selectively turn on cyclic guanosine monophosphate metabolism in lobsters, with exoskeletal muscles and the nervous system as particularly responsive targets (Goy et al., 1987). Explorations of possible linkages between the putative CHH-releasing thoracic second root neurosecretory neurons described in this publication, the serotonin- and octopamine-containing neurosecretory neurons of the ventral nerve cord, and stress and other endocrine functions in the lobster should be an exciting area of future investigation.

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NOTE ADDED IN PROOF

At the meeting on "Frontiers in Crustacean Neurobiology," held July 8–11, 1999, Dr. Heinrich Dircksen (University of Bonn, Germany), presented results identifying the sequences of CHH peptides isolated from lobster pericardial organs, and showed immunocytochemical evidence of the existence of neurosecretory neurons containing CHH-like immunoreactivity. His results were obtained independently of the data reported in our publication.

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