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SHORT COMMUNICATION

BUCCALIN-LIKE AND MYOMODULIN-LIKE PEPTIDES IN THE STOMATOGASTRIC GANGLION OF THE CRAB *CANCER BOREALIS*

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The buccalins and myomodulins are families of peptides originally isolated and purified from the accessory radula closer (ARC) nerve–muscle system of the marine mollusc *Aplysia californica* (Cropper *et al.* 1987*a*,*b*, 1988). In *A. californica*, the buccalin family consists of 19 related peptides (buccalins A–S), each of which exhibits a homologous carboxyl terminal sequence, Phe-*X*-Gly-Gly-*X*-NH_2 (Cropper *et al.* 1988; Miller *et al.* 1993*a*). A cDNA clone encoding a buccalin precursor polypeptide has recently been isolated (Miller *et al.* 1993*a*). This buccalin gene appears to be present as a single copy in the *A. californica* genome. The precursor peptide predicted from this gene encodes all 19 buccalin-like molecules, several in multiple copies.

The myomodulin family consists of nine related peptides (myomodulins A–I), all possessing a common Met-Leu-Arg-Leu-NH_2 carboxyl terminal sequence (Cropper *et al.* 1987*a*; Miller *et al.* 1993*b*). As with buccalin, a cDNA clone encoding a myomodulin prohormone has been isolated from *A. californica* (Miller *et al.* 1993*b*). This cDNA clone encodes seven of the nine myomodulins (A, B, D and F–I), with multiple copies of myomodulin A. Because myomodulins C and E are not found in the prohormone, but have been biochemically isolated, additional myomodulin transcripts must be present.

Both the buccalins and myomodulins have pronounced physiological actions on nerve and muscle preparations in *A. californica*. For example, buccalin A decreases the size of contractions and excitatory junction potentials in several of the ARC muscles (Cropper *et al.* 1990) as well as increasing the excitability and duration of action potentials in a number of mechanosensory neurons (Raymond *et al.* 1989; Rosen *et al.* 1989). Myomodulin enhances the contractility of the ARC complex (Cropper *et al.* 1987*a*,*b*) in addition to producing varied effects in a number of peripherally located neurons (Cleary

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et al. 1987; Critz *et al.* 1989; Rosen *et al.* 1989). Because both peptide families are found in neurons that contain other small-molecule transmitters, it is believed that both the buccalins and the myomodulins function as cotransmitters or neuromodulators.

In the present paper, we show that buccalin-like and myomodulin-like immunoreactive fibers project into the neuropil of the stomatogastric ganglion (STG) of the crab *Cancer borealis*. Some of the likely sources of these projections include immunoreactive somata in the commissural ganglia (CGs). The anti-buccalin antibody used is a rabbit polyclonal antiserum raised against buccalin A (Gly-Met-Asp-Ser-Leu-Arg-Leu-NH_2; Miller *et al.* 1992). The myomodulin antibody is a rabbit polyclonal antiserum raised against myomodulin C (Gly-Trp-Ser-Met-Leu-Arg-Leu-NH_2; Miller *et al.* 1991).

Rock crabs, *Cancer borealis* (*N*=51), were obtained from local fishermen (Boston, MA, USA) and maintained in artificial seawater aquaria at 10 °C. In all experiments, tissues were processed for immunocytochemistry as whole mounts using the indirect immunofluorescence methods of Beltz and Kravitz (1983). Specifically, the entire stomatogastric nervous system (STNS) [the two CGs, the esophageal ganglion (OG) and the STG] was dissected in physiological saline chilled to approximately 4 °C, fixed overnight with 4 % paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.3–7.4), and rinsed six times over approximately 6 h in a solution of 0.1 mol l^{-1} sodium phosphate (pH 7.2), 0.3 % Triton X-100, 0.1 % sodium azide (PTA). Following the rinse series, tissue was incubated in a solution of PTA, primary antibody and 10 % goat normal serum for 18–24 h. Anti-buccalin A antibody was used at final dilutions ranging from 1:100 to 1:300. Anti-myomodulin antibody was used at final dilutions ranging from 1:200 to 1:750. After incubation in primary antibody, tissues were rinsed as before in PTA and subsequently incubated with a 1:25 dilution of fluorescein- or rhodamine-labeled secondary antibody (goat anti-rabbit affinity-purified IgG, Boehringer-Mannheim) in PTA plus 10 % goat normal serum for 12–24 h. Following incubation with secondary antibody, each preparation was rinsed six times in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.2) over approximately 6 h, then mounted between glass coverslips in 80 % glycerin and 20 % 20 mmol l^{-1} sodium carbonate, pH 9.5. Some preparations (*N*=38) were viewed with a Zeiss epifluorescence microscope (IM35) equipped with filter sets optimized for either fluorescein or rhodamine. Other preparations (*N*=13) were viewed with a Biorad MRC 600 laser-scanning confocal microscope equipped with a krypton/argon mixed-gas laser and the standard YHS (for rhodamine) or BHS (for fluorescein) filter blocks provided by the manufacturer. Cell counts and measurements were made only from preparations viewed on the confocal microscope.Values are presented as mean ± S.D.

Within the STG, buccalin-like immunoreactivity is confined to the centrally located neuropil (*N*=5 ganglia; Fig. 1). None of the approximately 30 neurons of the STG displays any buccalin-like staining. Within the neuropil, the antibody stains both fine neurites and small (less than 10 μm) varicosities. As with the patterns of immunoreactivity seen using antibodies that recognize other modulators (Baldwin *et al.* 1992; Christie *et al.* 1992), the majority of labeled profiles are found in the peripheral portion of the neuropil, with the central core of the neuropil being devoid of staining.

All buccalin-like labeling within the STG originates from a bundle of 2–4 brightly

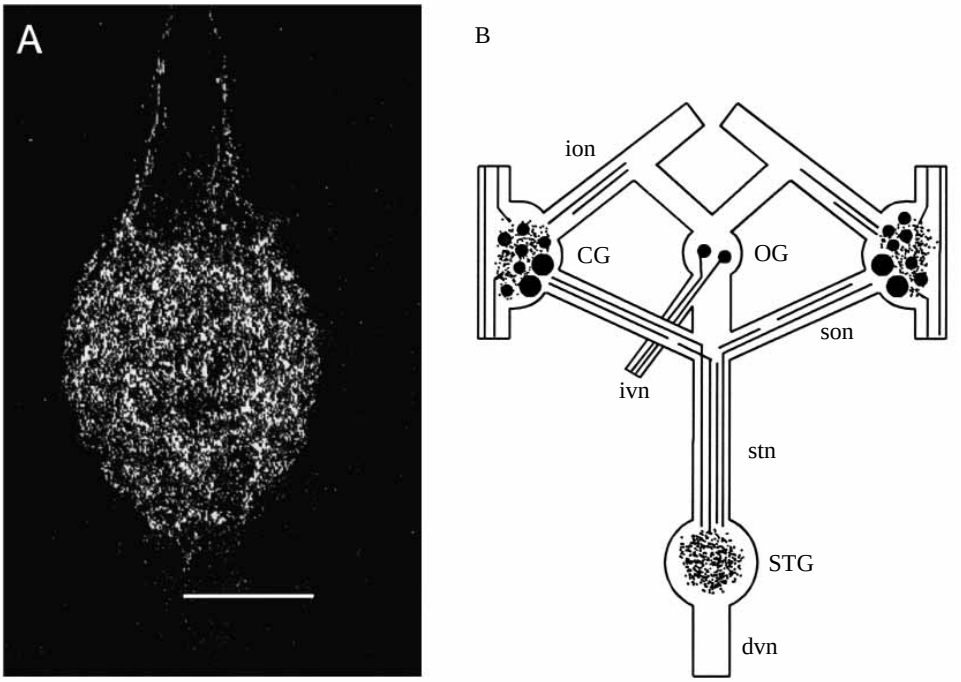

Fig. 1. Distribution of buccalin-like immunoreactivity in the stomatogastric ganglion (STG) and other regions of the stomatogastric nervous system. (A) Laser-scanning confocal micrograph of rhodamine-labeled buccalin-like immunoreactivity in the STG. This image is the summation of 40 optical sections taken at 2 μm intervals. Scale bar, 100 μm. (B) Schematic representation of buccalin-like immunoreactivity in the ganglia and nerves of the stomatogastric nervous system. CG, commissural ganglion; OG esophagael ganglion; ion, inferior esophageal nerve; ivn, inferior ventricular nerve; son, superior esophageal nerve; stn, stomatogastric nerve; dvn, dorsal ventricular nerve.

labeled fibers that enter the ganglion through the stomatogastric nerve (stn) (Fig. 1B). These fibers can be traced back from the stn to its junction with the paired superior oesophageal nerves (sons). At the son/stn junction these fibers separate, with 1–2 buccalin-like immunoreactive axons projecting into each son.

The buccalin-like immunoreactivity in the STG probably represents projections from somata present in the CGs. Within each CG (N=8 ganglia), the buccalin antibody stains 1–2 large (76±5 μm, N=15 somata) and 5–8 small (29±6 μm, N=51 somata) neurons in addition to neuropil and several fibers (Fig. 1B). The two large buccalin-immunopositive somata are located in the area of the CG immediately adjacent to the son and, in one preparation, appeared to give rise to the labeled son fibers. The majority of processes in the neuropil of each CG are derived from fibers traveling in the commissure and originating in both the supraesophageal ganglion (‘the brain’) and the thoracic nervous system. Several fibers can also be seen to exit each CG through the inferior esophageal nerves (ions). The source and destination of these projections remain unknown. Within the OG (Fig. 1B), two neurons approximately in 50 μm in diameter (50±4 μm, N=10

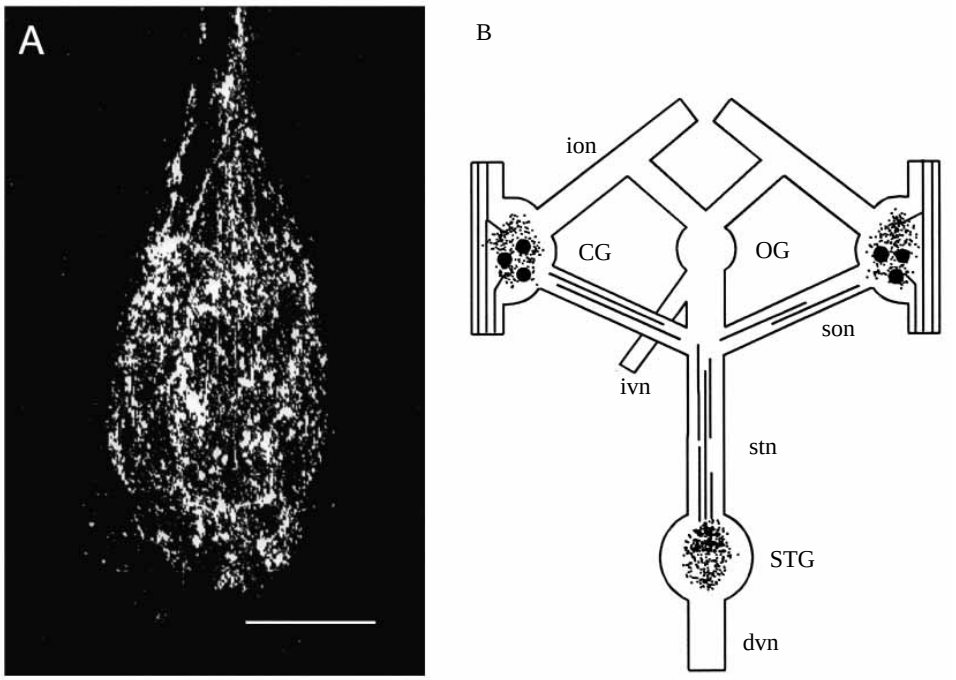

Fig. 2. Distribution of myomodulin-like immunoreactivity in the STG and other regions of the stomatogastric nervous system. (A) Laser-scanning confocal micrograph of fluorescein-labeled myomodulin-like immunoreactivity in the STG. This image is the summation of 36 optical sections taken at 2 μm intervals. Scale bar, 100 μm. (B) Schematic representation of myomodulin-like immunoreactivity in the ganglia and nerves of the stomatogastric nervous system. Abbreviations as in Fig. 1.

somata) were labeled. These somata project into the inferior ventricular nerve (ivn), which connects the OG with the brain, but do not project to the STG.

As with the buccalin antibody, the antiserum generated against myomodulin C labels a wide variety of structures within the STNS (Fig. 2). Like anti-buccalin, anti-myomodulin stains an extensive region of neuropil within the STG but does not stain any STG somata (N=8 ganglia). This neuropil, like that labeled by the buccalin antiserum, is composed of stained neurites and varicosities (Fig. 2). Although the majority of varicosities stained with anti-myomodulin are in the same size range as those stained by anti-buccalin, a small but distinct population of larger profiles (greater than 10 μm in largest cross-sectional diameter) is also present. As with the buccalin-like immunoreactivity, the myomodulin-like label is confined to the peripheral portion of the neuropil, with the central core of the neuropil being devoid of staining.

The myomodulin-like immunoreactivity present in the STG originates from a bundle of fibers that enter the ganglion from the stn (Fig. 2). Because the axons within this bundle are often tightly clustered, it is difficult to count accurately the number of fibers entering the ganglion, but the bundle contains at least two distinct fibers, and in two preparations, three and five axons were distinguished. Although staining in these fibers is not

continuous, it seems likely that they enter the stn from the sons, as immunoreactive fibers are found in these nerves but not in the esophageal nerve, the only other nerve joining the stn.

Within each CG (*N*=8 ganglia), myomodulin-like immunoreactivity is present in 2–3 small (29±6 μm, *N*=30 somata) neurons, as well as in a region of dense neuropil and several fibers (Fig. 2B). As with the buccalin-like immunopositive axons, myomodulin-like immunoreactive fibers enter the CG through the commissures from the brain and the thoracic nervous system. No myomodulin-like immunoreactivity is present in the OG (*N*=8 ganglia).

To confirm that the buccalin-like and myomodulin-like immunoreactivity seen in the stomatogastric nervous system was due to the presence of peptides similar to the species found in *A. californica*, and not to cross reactivity for unrelated molecules, we conducted preabsorption controls of each antiserum. Each primary antibody was preincubated with various peptides for 3 h at room temperature, prior to incubation with tissue. Preincubation of anti-buccalin with 10^{-7} mol l^{-1} buccalin (*N*=4 preparations) completely abolished staining, whereas preincubation with the same or higher concentrations (10^{-7} to 10^{-5} mol l^{-1}) of myomodulin (*N*=3 preparations), proctolin (*N*=3 preparations), Ser-Asp-Arg-Asn-Phe-Leu-Arg-Phe-NH_2 (SDRNFLRFamide) (*N*=3 preparations), Thr-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2 (TNRNFLRFamide) (*N*=3 preparations) or red pigment concentrating hormone (RPCH) (*N*=3 preparations) had no effect. Preincubation of anti-myomodulin with 10^{-7} mol l^{-1} myomodulin (*N*=4 preparations) blocked myomodulin staining, but preincubation with buccalin (*N*=3 preparations), proctolin (*N*=3 preparations), SDRNFLRFamide (*N*=3 preparations), TNRNFLRFamide (*N*=3 preparations) or RPCH (*N*=3 preparations) (10^{-7} to 10^{-5} mol l^{-1}) did not affect staining.

Several studies have reported the presence of buccalin-like and myomodulin-like peptides in molluscan species other than *A. californica*, demonstrating that the buccalins and myomodulins represent families of peptides that are conserved in this phylum (Kobayashi and Muneoka, 1990). Our finding of buccalin-like and myomodulin-like immunoreactivity in the crustacean species *C. borealis* shows that the distribution of these peptide families is not confined to molluscs and suggests that they may be widespread.

In molluscs, the buccalins and myomodulins show not only structural conservation, but also exhibit a great degree of similarity in their role as neuromodulators within the ARC nerve–muscle system (Kobayashi and Muneoka, 1990). Although the presence of buccalin-like and myomodulin-like immunoreactivities within the *C. borealis* STNS does not necessarily imply that biologically active molecules are present, many of the neuromodulators, particularly the neuropeptides, known to exist in the STNS were originally detected in this fashion. For example, the presence of TNRNFLRFamide and SDRNFLRFamide in the STNS was initially described using antibodies generated against the molluscan peptide FMRFamide (Marder *et al.* 1987; Weimann *et al.* 1993). Likewise, the existence of cholecystokinin-like peptides was detected using antibody generated against the mammalian octapeptide (Turrigiano and Selverston, 1989). Future work employing bath-applications of buccalin-like and myomodulin-like molecules should shed more light on the roles these families of peptides play in the STG.

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References

BALDWIN, D., CHRISTIE, A. E., MARDER, E. AND GRAUBARD, K. (1992). The distribution and microstructure of peptidergic immunoreactivities in the stomatogastric ganglion of the crab, *Cancer borealis*. *Soc. Neurosci. Abstr.* **18**, 417.

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

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

CLEARY, L. J., BAXTER, D. A. AND BRYNE, J. H. (1987). Myomodulin, a novel neuropeptide, modulates action potentials in pleural sensory neurons in *Aplysia*. *Soc. Neurosci. Abstr.* **13**, 1440.

CRITZ, S. D., BAXTER, D. A. AND BYRNE, J. H. (1989). Modulatory effects of serotonin, FMRFamide and myomodulin on the duration of action potentials, excitability and membrane currents in tail sensory neurons of *Aplysia*. *J. Neurophysiol.* **66**, 1912–1926.

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

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

CROPPER, E. C., MILLER, M. W., VILIM, F. S., TENENBAUM, R., KUPFERMANN, I. AND WEISS, K. R. (1990). Buccalin is present in the cholinergic motor neuron B16 of *Aplysia* and it depresses accessory radula closer muscle contractions evoked by stimulation of B16. *Brain Res.* **512**, 175–179.

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

KOBAYASHI, M. AND MUNEOKA, Y. (1990). Structure and action of molluscan neuropeptides. *Zool. Sci.* **7**, 801–814.

MARDER, E., CALABRESE, R. L., NUSBAUM, M. P. AND TRIMMER, B. (1987). Distribution and partial characterization of FMRFamide-like peptides in the stomatogastric nervous systems of the rock crab, *Cancer borealis* and the spiny lobster, *Panulirus interruptus*. *J. comp. Neurol.* **259**, 150–163.

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

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

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

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

RAYMOND, J. L., SHULMAN, E. E., BAXTER, D. A., CLEARY, L. J. AND BYRNE, J. H. (1989). Differential effects of the peptide buccalin and serotonin on membrane currents, action potential duration and excitability in pleural sensory neurons of *Aplysia*. *Soc. Neurosci. Abstr.* **15**, 1284.

ROSEN, S. C., SUSSWEIN, A. J., CROPPER, E. C., WEISS, K. R. AND KUPFERMANN, I. (1989). Selective modulation of spike duration by serotonin and the neuropeptides FMRFamide, SCP_B, buccalin and myomodulin in different classes of mechanoafferent neurons in the cerebral ganglion of *Aplysia*. *J. Neurosci.* **9**, 390–402.

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

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