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Short Communication

Snail peptide expression pattern in the nervous system of the medicinal leech

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Abstract

Distribution of neurons immunopositive to antibodies against the "command neuron peptides" (CNPs) encoded by the snail Helix Command-Specific 2 (HCS2) gene was investigated in the nervous system of medicinal leech *Hirudo*. Immunopositive neurons were found in the leech segmental ganglia, brain and tail ganglionic masses, and peripheral ganglia. The CNPs immunopositive fibers were observed in neuropils of all ganglia and in some nerves. The role of CNPs immunopositive cells in animal behavior and the putative functions of the CNPs neuropeptide family are discussed.

Theme: Neurotransmitters, modulators, transporters, and receptors
Topic: Peptides: anatomy and physiology

Keywords: Hirudo; Neuropeptide; Neuron; Immunohistochemistry

A novel gene named Helix Command-Specific 2 (HCS2, accession number X92111) was shown to be expressed predominantly in four giant parietal interneurons involved in withdrawal behavior of the terrestrial snail *Helix lucorum* L. and several single neurons in other ganglia [1]. The predicted amino acid sequence of the HCS2-encoded protein contains at the N-terminus a hydrophobic leader sequence followed by four putative neuropeptides, and in the C-terminal part a sequence matching the consensus motif of the EF-hand family of the Ca^{2+}-binding proteins. All four predicted neuropeptides (CNPs, Command Neuron Peptides) bear a C-terminal signature sequence Tyr-Pro-Arg-*X* (where *X* is Pro, Leu, Ile or Val), and three of them are likely to be amidated [1]. Therefore, it is likely that the HCS2 gene encodes the neuropeptides belonging to a novel neuropeptide subfamily. The HCS2 gene pattern of expression can be down-regulated by a decrease in synaptic activity in the nervous system, and up-regulated by the external noxious inputs, as well as by the application of neurotransmitters and second messengers known to be involved in the withdrawal behavior. When up-regulated, the HCS2 expression appears mostly in neurons involved in the withdrawal behavior [1].

It is well known that homologous peptides can be found in various invertebrates or even mammals (e.g., opioids, myomodulin, or FMRF amide-related peptides). Moreover, the structure of most molluscan peptides is closely related to annelidan ones, as if annelids and mollusks were members of the same phylum [9].

The medicinal leech is a model animal widely used in neurobiology [10]. Avoidance behavior in the leech is exhibited as the whole-body shortening reflex. The neural circuit for shortening of the leech, that includes the command-like neurons, is known [5,13].

In the present report, we describe a distribution pattern of the neurons immunopositive to antibodies to a peptide CNP4 [1] encoded by the HCS2 gene in the nervous system of medicinal leech *Hirudo medicinalis*.

Abbreviations: ARG, anterior root ganglion; SG, segmental ganglion; CNS, central nervous system; HCS2, *Helix* Command-Specific 2 gene; CNPs, command neuron peptides

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1. Animals and saline

Adult *H. medicinalis* weighing 1–5 g were obtained from the International Leech Center, Udelnaya, Russia. The dissection was performed in ice-cold Ringer saline (115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES; pH 7.4) to minimize pain to the animal.

2. Fixation

Isolated CNS was fixed in fresh 4% paraformaldehyde in 0.1 PBS. The duration of fixation was 2 h at room temperature, and was followed by washes in PTA (0.1 M PBS, 0.5% Triton X-100, 0.01% sodium azide) for more than eight times during 12 h.

3. Immunochemistry

Immunochemical procedures of visualizing the CNP-IR somata were similar to those performed in *Aplysia* [6].

3.1. Antibodies

The antigen was prepared by coupling 14 aa. CNP4 (GVFTQGAHGSYPRV-amide) or 5 aa. CNP2 (DYPRL-amide) peptide to BSA using 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDC, Sigma). The coupling was performed in a 0.5-ml volume of 50 mM NaH_2PO_4, pH 7.2, containing 10 mg of BSA, 1 mg of peptide, and 10 mg of EDC. Two female rabbits were immunized by intradermal (into back) injection with 1 mg of antigen in an emulsion of 0.5 ml of PBS and 0.5 ml of Freund's complete adjuvant. At 30 days after initial injection, the rabbits were boosted by intramuscular (injection into a paw) injection with 1 mg of antigen in an emulsion of 0.5 ml of PBS and 0.5 ml of Freund's incomplete adjuvant. Serum was checked with immunoblotting. The stock antibody solution was purified by means of preabsorption. The stock antibody solution contained 1.6×10^{-12} M of the antibody and was used at dilution 1:25 x 1:100.

Primary polyclonal antibodies for CNP2 and CNP4 were used. Secondary antibodies were AlexaFluor™ 488 or FITC conjugated goat anti-rabbit IgG (Molecular Probes, Sigma) used at 1:200 dilution. As a control for specificity of the antibody, we incubated the leech ganglia with the secondary antibody alone. No signal was observed. Specificity of binding with antigens was shown for used lot of antibody in snail nervous system.

3.2. Double-labeling

To visualize the long-projection neurons in the brain ganglionic mass, we used the neurobiotin back-filling technique. Neurobiotin (SIGMA, USA) 5% solution in dH_2O was loaded via M4–M5 connectives for a duration more than 3 days at a temperature 4 °C. Later, the CNS was desheathed, fixed, and treated for CNPs immunohistochemical procedures as in ordinary preparations. After washing in PBS, the brains were stained for neurobiotin visualization with Avidin-Texas Red antibodies (1: 200 in PBS) 12 or more hours at room temperature. The ganglia were washed 5 times with PBS, cleared, and mounted in glycerol.

For archive storage, the whole-mount preparations were washed with PBS and embedded without dehydration series in Aqua Poly-Mount media (PolySciences).

3.3. Visualization

The preparations were examined using the AxioPlan (Zeiss, Germany) fluorescence microscope connected to the digital camera Camedia C-4000 (Olympus, USA) for acquiring images.

4. Distribution of the CNPs immunoreactive neurons in the leech nervous system

We found no detectable difference in patterns of the immunopositive neurons in leech nervous system stained with antibodies to CNP2 (n = 12) or to CNP4 (n = 26). It suggests that these antibodies are staining the same peptide epitope. Averaged data from all preparations are presented.

The somata of the neurons immunoreactive (IR) to the CNPs antibodies were observed in all ganglia of the *Hirudo* nervous system (Fig. 1; a complete list of all immunopositive neurons is given in Table 1).

On the ventral surface of each segmental ganglion, 3 pairs of IR cells were found: one pair in the caudal ventromedial packet, another one in the rostral anteromedial packet, and two cells on the medial border of anterolateral packets (Fig. 1A). On dorsal surface of segmental ganglia M2–M21, two pairs of neurons are CNP-IR: one pair in anterolateral packets and another one in posterolateral packets. In a few preparations, we observed two additional pair of cells also (Fig. 1B). The first segmental ganglion M1 has a different pattern of CNPs immunoreactivity—only one asymmetric neuron on the dorsal surface (Fig. 1E). Processes of all these cells, except the last one, were always unstained in our preparations. Surprisingly, many immunopositive fibers were observed in the neuropil (Fig. 1B). No differences in pattern were found in ganglia of "sexual segments" M5 and M6.

The cerebral ganglia contained about 20–30 miniature immunopositive cells on the ventral surface (Fig. 1C). Higher magnitude of reaction was found in 3 cell pairs: 2 pairs located in the medial region, packets 7 and 8, approximately; and the third pair in packet 5, near the D1 nerve. In the dorsal neuropil of cerebral ganglia, a widespread net of CNP-IR fibers was found. The source of origin of at least some of those fibers in the cerebral ganglia was