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Neuropeptides in Heteroptera: Identification of allatotropin-related peptide and tachykinin-related peptides using MALDI-TOF mass spectrometry

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ABSTRACT

Recently, the peptidomic analysis of neuropeptides from the retrocerebral complex and abdominal perisympathetic organs of polyphagous stinkbugs (Pentatomidae) revealed the group-specific sequences of pyrokinins, CAPA peptides (CAPA-periviscerokinins/PVKs and CAPA-pyrokinin), myosuppressin, corazonin, adipokinetic hormone, and short neuropeptide F. In this study, we used mass spectrometric profiling of nervous tissue from the species-rich taxon Hemiptera to identify products of two previously unobserved neuropeptide genes from these species, namely allatotropin-related peptide (ATRP) and tachykinin-related peptides (TKRPs). Since neither TKRPs nor allatotropin are accumulated in neurohemal organs, immunocytochemical data were analyzed to find potential accumulation sites within the central nervous system. By mass spectrometry, TKRPs were found to be accumulated in the antennal lobes, and ATRP was identified in the most posterior region of the abdominal ventral nerve cord and fourth abdominal nerves. In addition to neuropeptides from stink bugs, TKRPs and ATRP were also identified from the distantly related bugs *Oncopeltus fasciatus* (Lygaeidae) and *Pyrrhocoris apterus* (Pyrrhocoridae). In total, six TKRPs and one ATRP from each species could be elucidated by tandem mass spectrometry. The ATRP of all species is sequence-identical with *Locusta migratoria* accessory gland myotropin-1 (Lom-AG-MT-1), a member of the highly conserved insect allatotropin family.

1. Introduction

The largest group of hemimetabolous insects, Hemiptera, contains vectors of diseases as well as numerous economically important pest species; among them aphids, cicadas and true bugs. The peptidome of these insects is poorly understood. Neuropeptides occupy a key position in the modulation of physiological events and knowledge of these neuropeptides might lead to specific control methods that do not rely on chemical insecticides. The development of synthetic peptide mimetics that affect the reproductive success of pest species depends, however, on the knowledge about specific ligands that are typical of the respective insect groups. A first comprehensive and comparative peptidomic analysis of peptide hormones of four related hemipteran pests, namely the southern green stinkbug *Nezara viridula* (*L.*), the green stinkbug *Acrosternum hilare* (*Say*), the red-backed stinkbug *Banasa dimiata Say* and the brown stinkbug *Euschistus servus* (*Say*) (all belonging to the Pentatomidae), has been published recently [16]. In that study, the peptidome of *corpora cardiaca* (CC) and abdominal perisympathetic organs (aPSO) were analyzed. This analysis resulted in the identification of products of six different neuropeptide precursors (pyrokinins, myosuppressin, corazonin, adipokinetic hormone, sNPF and PVK/CAPA-peptides). Only pyrokinins and CAPA-peptides showed sequence variations between these species. Adipokinetic hormones of distantly related hemipteran groups, however, differ remarkably [2]; data from other peptide families are still largely missing. In this study, we extended our analysis of neuropeptides from pentatomid insects to ATRP and tachykinin-related peptides (TKRPs). Allatotropin was first isolated from head extracts of the adult sphinx moth *Manduca sexta* (*L.*) [4]. Meanwhile allatotropins (AT) are known from different holometabolous insects but only a single ATRP could be identified from hemimetabolous insects (*L. migratoria* [Lom-AG-myotropin 1]) [3]. TKRPs were first identified from locusts [19,20];

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tkrp-gene of hemimetabolous insects are known from the cockroaches *Leucophaea maderae* (*F.*) [15], *Periplaneta americana* (*L.*) [15] and the locust *Locusta migratoria* (*L.*) [1]. In contrast to ATs, multiple closely related TKRPs were found on the precursors of cockroaches, locusts and homologous precursors of holometabolous insects such as flies, mosquitos or honey bees (see Nässel chapter 26 in book chapter Kastin [5]). Neither ATRP nor TKRPs are accumulated in neurohemal organs of Heteroptera and were therefore missed in our earlier peptidomic studies. To reveal the potential accumulation sites of these neuropeptides in the CNS, we screened published immunostainings with antisera against TKRPs and performed anti-ATRP-immunostainings in different species of Heteroptera (*O. fasciatus, N. viridula, E. servus, P. apterus, Pentatoma rufipes*). The distribution of TKRP-immunoreactive material in the CNS of different insects such as cockroaches [9], locusts [3,12], flies [7] and mosquitos [8] is in part very similar. This is particularly true for the antennal lobes, which are the first odor processing centres of the insect brain [18], and ususally showed distinct immunoreactivity. Kwok et al. did not mention TKRP immunoreactivity in the antennal lobes of *R. prolixus* [6]. By mass fingerprinting, Utz et al. confirmed the occurrence of native TKRPs in antennal lobes of moths [21]. Thus, we used these easily identifiable brain structures to search for TKRPs of Heteroptera. Indeed, direct tissue profiling using MALDI-TOF/TOF mass spectrometry allowed de-novo sequencing of six different TKRPs in all species that were studied. The mass spectrometric analysis of putative ATRP-expressing cells, which were detected in the abdominal ventral nerve cord (VNC) by immunocytochemistry, could be performed after dissection of lateral link nerves (LN) which contained neurites of the Manse-AT immunoreactive somata. The sequence of ATRP was found to be identical in all pentatomid species; this peptide could later also be identified from distantly related Hemiptera such as *Oncopeltus fasciatus* (Lygaeidae) and *Pyrrhocoris apterus* (Pyrrhocoridae). The TKRPs of the latter species, however, differed from those of the pentatomid Hemiptera.

2. Material and methods

2.1. Insects

Adult *N. viridula*, *E. servus*, *A. hilare* and *B. dimiata* (Pentatomidae) were captured with 40 W blacklight traps (with live insect canisters) located adjacent to fields cultivated in corn, cotton, sorghum and soybeans in Burleson County, Texas. Adult *O. fasciatus* were initially collected from flowering butterfly weed, *Asclepius tuberosa,* in a home garden at College Station, TX and reared in the laboratory. Adult *P. rufipes* (Pentatomidae) and *P. apterus* were caught during field trips around of Jena, Germany by S. Neupert and kept alive until dissection.

2.2. Imunohistochemistry

Dissected VNCs of *P. apterus* were fixed overnight at 4 °C with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.2 (whole mounts). Subsequently, preparations were washed in PBS+4% Triton X-100 (24 h) and PBS+1% Triton X-100 for 24 h, respectively. The preparations were then incubated for 4 days at 4 °C in anti-Manse-allatotropin serum (1:4000) (no. 13.3.91, kindly provided by Dr J. Veenstra, University of Bordeaux, Talence, France; [22]) diluted with PBS containing 0.25% bovine serum albumin, 1% Triton X-100 and normal goat serum. The antiserum recognizes Manse-allatotropin and Locmi-AG-MT-1 [22], both ending at the C-terminus with TARGFamide. Following overnight washing in 0.1 mol/L Tris–HCl, 3% NaCl, 1% Triton X-100 (pH 7.6), the secondary Cy3-conjugated antibodies were diluted in PBS-bovine serum albumin (2.5 mg/mL) at a concentration of 1:3000 for 4 days. Finally, the preparations were washed again overnight in 0.1 mol/L Tris–HCl-3%, NaCl-1%, Triton X-100, pH 7.6, and transferred in glycerine. For visualization, tissues were mounted in glycerine/moviol. Fluorescence staining was examined with a confocal laser scanning microscope (ZEISS LSM 510 Meta system; Jena, Germany), equipped with a HeliumNeon1 laser (wavelength 543 nm). Serial optical sections were assembled into combined image. The image was exported and processed with Adobe Photoshop 7.0 software.

2.3. Dissection of nervous tissues and sample preparation for mass spectrometry

For antennal lobe (AL) preparations, the pronotum of the adult insect was removed; the neck region of the head was opened with scissors and fixed with insect needles. Subsequently, the brain was dissected out of the head capsule, and transferred in a separate drop of saline. All attached tissues were removed (e.g. muscles, fat body, trachea) and the ganglionic sheath disrupted with fine forceps. The ALs were easily identified in the species under study and could be directly transferred using an uncoated glass capillary (Hilgenberg GmbH, Malsfeld, Germany) fitted to a tube with a mouthpiece onto a stainless steel sample plate for mass spectrometry. For the identification of allatotropin, the fused ventral nerve cord of adult stinkbugs was transferred onto a separate chamber filled with insect saline. For mass spectrometric analyses, we dissected the fourth posterior abdominal nerve or small pieces of the posterior part of the VNC part and prepared these tissues directly for matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The respective nerves as well as the AL-preparations were rinsed in a drop of water before being moved to the final position on the sample plate. The samples were air-dried. A limited amount of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water (1:1)) was pumped on the dried preparations. Each preparation was air-dried again and covered with pure water for a few seconds, which was removed by cellulose paper.

2.4. MALDI-TOF/TOF mass spectrometry

Mass spectrometric analysis was performed on the ABI 4800 proteomics analyzer (Applied Biosystems, Framingham, MA). All

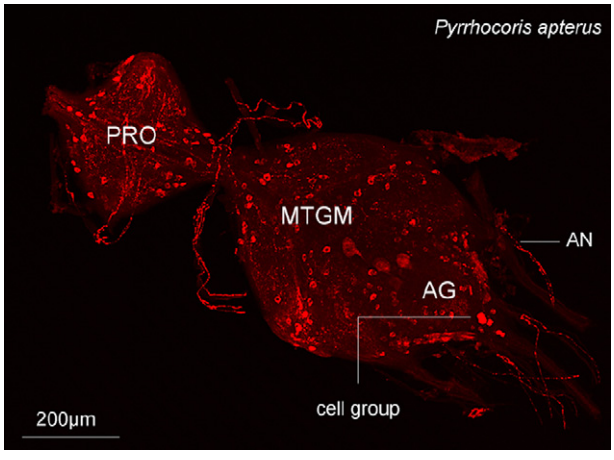

Fig. 1. Allatotropin-*like* immunofluorescence staining in a whole mount preparation of the ventral nerve cord (VNC) of *P. apterus*. A distinct allatotropin-like immunoreactive cell group is marked in the posterior abdominal VNC. Only preparations of this part of the VNC yielded distinct AT-signals in MALDI-TOF mass spectra. In addition, AT could be identified in mass spectra from the 4th abdominal nerve. PRO, prothoracic ganglion; MTGM, mesothoracic ganglionic mass; AG, abdominal ganglion; AN, abdominal nerve. Scale bar: 200 µm.