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Identification and distribution of products from novel *tryptopyrokinin* genes in the locust, *Locusta migratoria*

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ABSTRACT

A recent analysis of the genome of *Locusta migratoria* indicated the presence of four novel insect neuropeptide genes encoding for multiple tryptopyrokinin peptides (tryptoPKs); hitherto only known from *pyrokinin* or *capa* genes. In our study, mature products of *tryptoPK* genes 1 and 2 were identified by mass spectrometry; precursor sequences assigned to the *tryptoPK* genes 3 and 4 are likely partial sequences of a single precursor. The expression of *tryptoPK* genes 1 and 2 is restricted to two cells in the subesophageal ganglion, exhibiting not only a unique neuropeptidome but also a very distinctive axonal projection. Comparative neuroendocrinology revealed that homologous cells in other insects also produce tryptoPKs but use other genes to generate this pattern. Since *capa* and *pyrokinin* genes are discussed as ancestors of the *tryptoPK* genes, we completed the hitherto only partially known precursor sequences of these genes by means of transcriptome analyses. The distribution of mature products of CAPA and pyrokinin precursors in the CNS is compared with that of tryptoPKs. In addition, a novel pyrokinin-like precursor is described.

1. Introduction

Most of the neuropeptide genes in insects and crustaceans have largely similar ancestors in arthropods [e.g. 1, 2]. There is sufficient evidence to suggest that orthologous neuropeptide genes are even known from insects and mammals which supports a long evolutionary history and conservation of neuropeptide genes [3]. On the other hand, many lineage-specific developments of neuropeptide genes are known in metazoa and these developments can usually be traced back to gene duplications. Representatives for the development of lineage-specific neuropeptide genes in insects are *pyrokinin* (*pk*) and *capa* genes. Current knowledge about these genes and their mature products suggests an ancient condition in arthropods of a single gene encoding periviscerokinins (PVKs) and pyrokinins (PKs) [4]. At the origin of hexapods, the ancestor of *pk* and *capa* genes rather suddenly encodes a third peptide species, differentiated from conventional PKs by the presence of Trp preceding the C-terminal core motif of PKs; namely FXPRLamide [5]. This is important since it is known from *Drosophila melanogaster*, that PVKs, PKs and tryptoPK each activate specific receptors [6–8]. It can only be speculated that the presence of three different receptor ligands in a single precursor posed a challenge for a ligand-specific release. It is also not known yet, how long in the evolution of hexapods the trypto-PK receptor evolved as functionally separated unit. Clearly however, the appearance of tryptoPKs was followed in Hexapoda by a gene duplication which led to *pk* (*pban* of Lepidoptera, *hugin* of *D. melanogaster*) and *capa* genes [see 4]. Following gene duplication, both genes evolved differentially. In today's insects, the *pk* gene is expressed in neurosecretory cells of the subesophageal ganglion (SEG) and the *capa* gene is mainly expressed in neurosecretory cells of abdominal ganglia. Although there are several exemptions known from insects, most *pk* genes encode for PKs and a single tryptoPK and the *capa* genes encode for PVKs and also a single tryptoPK. A separate release of CAPA-tryptoPK is known from many insects and likely the result of differential processing of the CAPA precursor [9–11]. On the other hand, no data exist regarding a differential processing of PK precursors in insects which likely results in a consequent co-release of PKs and tryptoPKs from the *pk* expressing cells. A recent analysis of the genome of the migratory locust *Locusta migratoria* indicated an

Abbreviations: NCA-2, nervus corporis allati-2; NCC-3, nervus corporis cardiaci-3; PSO, perisympathetic organ; PK, pyrokinin; PVK, periviscerokinin; RC, retrocerebral complex; tryptoPK, tryptopyrokinin; VPMN, ventral posterior median neuron.

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alternative option for a separate release of tryptoPKs that was not known from insects so far. In fact, not less than 4 genes coding for multiple tryptoPKs were described [12]. However, not a single tryptoPK which can be deduced from these genes is known from the well-studied locusts [13].

Here, we describe the identification of mature products from two *tryptoPK* genes in *L. migratoria* as well as their cellular origin and axonal projection. In addition, only partially known precursor sequences of *pk* and *capa* genes have been completed and their distribution in the CNS analyzed. Finally and for the first time in insects, a second PK precursor and the neuropeptides processed from this precursor have been identified.

2. Materials and methods

2.1. Transcriptome sequencing, library construction and BLAST search

Total RNA was extracted from the CNS of single adult locusts (*L. migratoria*) kept at 4 °C during the preparation. CNS samples were preserved in RNAlater solution (QIAGEN GmbH, Hilden, Germany) and sequenced using the Illumina Truseq RNA Sample Preparation Kit (Illumina, San Diego, USA) at Beijing Genomics Institute (China). Isolation of mRNA, generation of cDNA and PCR amplification of suitable fragments were performed as described in Ref. [4]. Raw data were initially filtered by removing adapters, reads with more than 5% of unknown basses and reads with low quality sequences (reads having more than 20% bases with quality value lower or equal to 10). Transcripts were then *de novo* assembled using Trinity [14] with default options and used for tBLASTn on a local computer.

2.2. Tissue preparation and cell dissection for MALDI-TOF mass spectrometry

Corpora cardiaca, *corpora allata*, perisympathetic organs and nerves were dissected as described for cockroach samples by Predel [15] and directly transferred into a drop of water on the sample plate for MALDI-TOF mass spectrometry. Immediately after the transfer, the water was removed and the tissue samples were dried prior to deposition of matrix. Preparations of neuroendocrine cells or cell cluster from isolated SEGs were performed as described in Predel et al. [16].

2.3. MALDI TOF mass spectrometry

10 mg/ml 2,5−dihydroxybenzoic acid (Sigma-Aldrich, Steinheim, Germany) dissolved in 20% acetonitrile/1% formic acid or alternatively 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 60% ethanol, 36% acetonitrile, 4% water (Sigma-Aldrich, Steinheim, Germany) were used as matrix salts and loaded onto the dried samples using a glass capillary (10–20 nl for single cell preparations) or 0.1–10 μl pipettes (about 0.3 μl for direct tissue profiling). Mass fingerprint spectra were acquired in positive ion mode with an UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). MSMS fragment spectra were acquired in gas-off mode using an ABI 4800 proteomics analyzer (Applied Biosystems, Framingham, USA). Due to the limited nature of all samples, acquisitions were generally taken in manual mode.

2.4. Immunocytochemistry

An antiserum raised against the CAPA peptide *Periplaneta* PVK-2 [17], which recognizes PKs, tryptoPKs and PVKs, was used for whole-mount immunostainings. Tissue samples were fixed with Histofix (Sigma-Aldrich) at 4 °C for 12 h, then washed with phosphate-buffered saline (ph 7.2) containing 1% TritonX-100 (1% Tx) for 24 h and incubated for 5 days at 4 °C in a polyclonal anti-PVK-2 serum at a concentration of 1:4000 diluted in PBS-1% Tx containing 2,5 mg/ml bovine serum and 10% normal goat serum. Subsequently, the samples were washed in PBS 1% Tx for 24 h at 4 °C followed by incubation with Cy3-coupled goat anti-rabbit antiserum at a concentration of 1:3000 (Jackson ImmunoResearch) for 5 days at 4 °C. After washing in PBS-1% Tx for 24 h at 4 °C, the samples were embedded in Mowiol (Merck KGaA, Darmstadt, Germany). A confocal laser scanning microscope (Zeiss LSM 510 Meta system; Jena, Germany), equipped with a C-Apochromat 10×/0.45W objective, a Plan-Apochromat 20x/0.75 objective and a Helium-Neon1 laser was used to scan serial optical sections at optical section thicknesses from 0.3 to 0.8 μm. Images were exported and processed to adjust brightness and contrast using Adobe Photoshop 7.0 software (San Jose, CA).

3. Results

As a first step, transcriptome analysis of the CNS was performed to complete sequences of PK and CAPA precursors, which were only partially known from genome data [12], and to confirm the sequences of the four tryptoPK precursors. The resulting sequences of tryptoPK precursors 1 and 2 (Supplementary Material 1) show a surprisingly high number of amino acid substitutions when compared with the respective precursors predicted from the locust genome (e. g. 25 in the tryptoPK 2 precursor). Although different populations of *L. migratoria* were used in both studies, such variability is without precedent even for neuropeptide precursors of closely related insect species [18]. Transcriptome data indicated that the published tryptoPK precursors 3 and 4 [12] contain in fact partial sequences of a single precursor; designated as tryptoPK 3 precursor here. It can also be concluded from the transcriptome data, that most of the already known PKs from *L. migratoria* [see 13] are processed from the PK precursor (Supplementary Material 1), except for pQSVPTFTPRL-NH_2 [Lom-PK-2; 19]. A blast search in the transcriptome assembly of the CNS, using the sequence of this peptide, revealed a second PK precursor containing two PKs only. Thus, *L. migratoria* not only has *tryptoPK* genes, which were hitherto unknown from insects, but also an unusual second *pk* gene which shows low similarity with the other *pk* gene, except for the C-terminus of the PKs. To avoid confusion in the assignment of mature peptides, we name the novel gene *pk-like* gene (*pkl*). Sequences of putative neuropeptides which can be predicted from PK, PKL, CAPA and tryptoPK precursors are listed in Table 1 insofar as mature products from these precursors were found by mass spectrometry. With the complete precursor sequences in hand, these peptides were renamed according to their position in the different precursors. The resulting nomenclature is used throughout this manuscript. Theoretical masses ($[M+H]^+$) of peptides predicted from the different precursors provided the basis to search for tryptoPKs and related PKs, PKLs, and PVKs in the CNS and neurohemal organs by means of mass spectrometric direct tissue profiling.

It was already known from cockroaches that, except for a few *capa*-interneurons in the brain/SEG, all *capa* and *pk* expressing cells in the CNS store their products in neurohemal organs [16]. Therefore, we searched for neuropeptides deduced from the three tryptoPK precursors first in major neurohemal organs of *L. migratoria*. As expected [19,20], mass spectra of abdominal perisympathetic organs (PSOs) verified the presence of CAPA peptides including hitherto unknown PVKs and CAPA-tryptoPKs (Table 1) but products of the *tryptoPK* genes were not detected. Mass spectra of thoracic PSOs did not contain any ion signals typical of PKs, PKLs, PVKs or