Mass spectrometric analysis of putative capa-gene products in *Musca domestica* and *Neobellieria bullata*

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**Abstract**

Neuropeptides of the capa-gene are typical of the abdominal neurosecretory system of insects. In this study, we investigated these peptides in two widely distributed and large pest flies, namely *Musca domestica* and *Neobellieria bullata*. Using a combination of MALDI-TOF and ESI-QTOF mass spectrometry, periviscerokinins and a pyrokinin were analyzed from single perisympathetic organ preparations. The species-specific peptide sequences differ remarkably between the related dipteran species. These differences could make it possible to develop peptide-analogs with group- or species-specific efficacy.

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**I. Introduction**

With the completion of the *Drosophila melanogaster* genome project, the importance of the fruitfly in neurogenetics increased dramatically. A number of neuropeptide/neuropeptide-receptor encoding genes have been described\cite{13} and the possibility to study fruitflies with specific peptide deficiencies will contribute to a better understanding of peptide functions in the future. The small size of *Drosophila*, however, often hampers behaviorally oriented research, and studying related but larger species can help to overcome these difficulties. However, specific differences may occur between related species and this problem has to be clarified before starting such experiments. In this study, we focused on neuropeptides of the capa-gene in the house fly, *Musca domestica*, and the fleshfly, *Neobellieria bullata*. Both species are widely distributed pest insects and knowledge about their specific peptide inventory may contribute to a more specific biological pest control which, if peptide-analogs are involved, focuses not on complete removal of a given species but rather on inhibiting their fitness. The capa-gene from *D. melanogaster* encodes three neuropeptides\cite{13} which are expressed in cells of the abdominal ganglia and are accumulated in abdominal perisympathetic organs\cite{11}. These neurohemal release sites are known to store capa-gene related products in other insects as well\cite{3,9,10}, and studies on different insect species indicate a role for these peptides in water balance\cite{4,14}.

Since the localization of capa-gene products (two periviscerokinins/CAP\textsubscript{2b} and a pyrokinin) is known from *D. melanogaster*, we used single perisympathetic organs of adult *M. domestica* and *N. bullata* as sources for putative periviscerokinins/pyrokinins. Such a mass spectrometric strategy was recently successfully used for the detection of *Drosophila* peptides\cite{11}. In this study, however, a comparison of partially obtained sequences with annotated genes was impossible. Thus, because the capa-gene products of *M. domestica* and *N. bullata* were not identical with those of *D. melanogaster*, de novo sequencing was necessary. We describe here the remarkable differences occurring in all of the capa-gene products of three related cycloraphous flies.
2. Materials and methods

2.1. Insects

House flies (M. domestica) were obtained as pupae from the Knipland-Bushland U.S. Livestock Insects Laboratory in Kerrville, TX and kept in cages at 26 °C. Flesh flies (N. bullata) were obtained in colonies maintained at the Areawide Pest Management Research Unit, Southern Plains Agricultural Research Center in College Station, TX. This flesh fly colony was propagated by placing pupae in a cage containing water, sugar cubes, and a powdered milk:sugar mixture. The flies were fed beef liver provided fresh each day for a total of 4 days.

2.2. Dissection and sample preparation for mass spectrometry

Body cavities of non-anaesthetised adult flies were opened surgically; the central nervous system was made visible by removal of other tissues, and flushed with insect saline. Subsequently, preparations of dorsal ganglionic sheath portions were dissected rapidly using fine scissors and micro-needles, and transferred to a stainless steel sample plate for MALDI-TOF mass spectrometry or into a solution of 5% TFA for electrospray mass spectrometry (MALDI-TOF mass spectrometry). The ganglionic sheath was transferred with the help of a micro-needle and rinsed in a drop of water before moved to the final position on the sample plate. A limited amount of matrix solution (o-cyan-4-hydroxycinnamic acid dissolved in methanol/water) was pumped onto the dried preparations over a period of approximately 10 s using a Nanoliter injector (World Precision Instruments, Berlin, Germany). Each preparation was allowed to dry and then covered with pure water for some seconds, which was removed by cellulose paper. Five preparations of each species were prepared for mass spectrometric analysis. Electrospray mass spectrometry: Aqueous extracts of five ganglionic sheath preparations were shortly sonicated and loaded on to a home-made micro-column (purification capillary for electrospray mass spectrometric analysis). Electrospray mass spectrometry: Aqueous extracts of five ganglionic sheath preparations were shortly sonicated and loaded on to a home-made micro-column (purification capillary for electrospray mass spectrometry).

2.3. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS)

Mass spectra were acquired in positive ion mode on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in reflectron mode using a Nanoliter injector (World Precision Instruments, Berlin, Germany). Each preparation was allowed to dry and then covered with pure water for some seconds, which was removed by cellulose paper. Five preparations of each species were prepared for mass spectrometric analysis. Electrospray mass spectrometry: Aqueous extracts of five ganglionic sheath preparations were shortly sonicated and loaded on to a home-made micro-column (purification capillary for electrospray mass spectrometry).

Mass spectra were acquired in positive ion mode on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analyzed in reflectron mode using a delayed extraction time of 175 ns and an accelerating voltage of 20 kV. Laser strength was adjusted to provide the optimal signal-to-noise ratio. All spectra were the result of signal averaging of between 50 and 100 laser shots. An external mass spectrum calibration was performed using the calibration mixture 1 of Sequazyme Peptide Mass Standards Kit (Perseptive Biosystems, Framingham, MA), including known peptide standards in a mass range from 900 to 1600 Da. MALDI-PSD (post-source decay) analyses were performed on the same instrument. To generate a composite PSD spectrum eight spectral segments were acquired and stitched together using the Biospectrometry Workstation software.

2.4. Electrospray quadrupol time-of-flight mass spectrometry (ESI-QTOF)

Nanoelectrospray mass spectra were acquired in the positive-ion mode using an MDS Sciex (Concord, Canada) API Qstar Pulsar [5] fitted with a Protana (Odense, Denmark) nanoelectrospray source and using an Ionwerks (Houston, TX) time-to-digital converter [2]. Typically 1000–1400 V was applied as an ionspray voltage. Additional instrument settings are as follows: GS1 1–4, curtain gas 20, DP 50, FP 220, and DP2 35. Samples were purified using a homemade spin column. Approximately 2–3 mm of Poros R2 (PerSeptive Biosystems) was loaded into a 2 cm capillary column with a needle tip. Liquids are passed through the column by securing the capillary column to a purification needle holder (Proxeon Biosystems A/S, Odense, Denmark) and centrifugation. After the column was equilibrated in 5% formic acid, the samples were loaded and rinsed with 5% formic acid followed by an additional rinse step of 10% methanol, 5% formic acid. Peptides were eluted from the column with a solution of 25% methanol, 5% formic acid and collected into a metal coated nanoelectrospray capillary. The sample was then loaded onto the source and analyzed. After determining the m/z of the peptides in MS mode, a collision energy (typically 20–60 V) and collision gas (setting of 5, arbitrary units) was applied. The m/z of interest was isolated and fragmented with the instrument in “enhance all” mode. Data was typically acquired for 20 s. Additional peptides were eluted from the column in a solution of 50% methanol, 5% formic acid and mass analyzed. A final elution step of 80% methanol, 5% formic acid was performed and mass analyzed.

3. Results

Products of the capa-gene were earlier found to accumulate in perisym pathetic organs of Drosophila [11]. During the metamorphosis of cycloraphous diptera, these neurohemal organs become incorporated into the dorsal ganglionic sheath. Hence, preparations of ganglionic sheath portions should contain peptides typical of perisym pathetic organs. To check the dorsal ganglionic sheath of the fused ventral nerve cord of adult M. domestica and N. bullata for the occurrence of peptides, this sheath was subsequently dissected from both species and prepared for MALDI-TOF mass spectrometry. Preparations of the more posterior por-
tion of the ganglionic sheath revealed the occurrence of three abundant substances in both species (Fig. 1), namely at \( m/z \) 1000.7, 1321.7, 1515.8 (Neobellieria) and 973.6, 1294.7, 1515.8 (Musca). Although only a single mass signal was identical with a capa-gene product from *D. melanogaster* (Drm-PVK-1; \( m/z \) 1294.7), these substances were thought to be products of the capa-genes from *M. domestica* and *N. bullata*. This assumption was subsequently confirmed by post-source decay fragmentations using the same single organ preparations (Fig. 2). In both species, the larger peptide (\( m/z \) 1515.8) was found to be a member of the FXPRLamides (pyrokinins) whereas the other peptides turned out to be putative periviscerokinins. The complete sequence, however, could not be deduced from any of these fragmentations.

In a second step, we used an extract of five dorsal ganglionic sheath preparations from both species for electrospray mass spectrometry. After removing salts and other contaminants on a purification capillary column that was filled with Poros R2 material, peptides were washed off with 10/25/50% ACN (5% HCOOH). The putative capa-gene products were detected in the 25 and 50% ACN fractions and subsequently fragmented by tandem mass spectrometry. The fragment series resulting from the shorter of the two putative periviscerokinins (\( m/z \) 973.4 and 1000.4) revealed the following sequences: ASL/IFNAPRV\( _a \) (Fig. 3; *M. domestica*) and AGT/ILFVPRFL\( _a \) (N. bullata). Mass spectra cannot distinguish between the conservative Leu and Ile alternates. The same procedure was performed with the second putative periviscerokinin of both species (\( m/z \) 1294.7 and 1321.4). The resulting fragments (not shown) were interpreted as originating from the parent sequences AGGTSGL/IYAFPRV\( _a \) (M. domestica) and NG-GTSGL/IYAFPRV\( _a \) (N. bullata); the sequence of the two

![Fig. 1. MALDI-TOF mass spectra from preparations of the posterior (abdominal) dorsal sheath of the ventral nerve cord of N. bullata and M. domestica. The dorsal sheath represents a neurohemal release site homologous to perisympathetic organs which become incorporated into the dorsal ganglionic sheath during the metamorphosis of cycloraphous flies. Abundant mass signals represent putative products of the capa-genes—the mass of the substance at \( m/z \) 1234.71 (asterisk) is identical with Dem-PVK-1. \([M + Na]^+ \) and \([M + K]^+ \) adduct ions are not labelled.](image1)

![Fig. 2. MALDI-TOF post-source decay spectrum of a putative periviscerokinin from N. bullata (\( m/z \) 1321.7). Different fragments are labelled. Although the complete sequence could not be deduced, fragments strongly suggest that the peptide is a member of the periviscerokinins with a C-terminal PRVamide (arrows) typical of that peptide family.](image2)
Fig. 3. CID spectra (ESI-QTOF) of the peptide at m/z 973.54 ([M + 2H]²⁺: 487.27; asterisk). Mainly y-type fragment ions are labelled. The fragments were analyzed manually and the resulting sequence is included in the figure. The y12-fragment was not clearly observable because of mass-related b3-fragments but internal fragments (APR; not labeled) filled this gap.

Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>PVK-1</th>
<th>PVK-2</th>
<th>Pyrokinin</th>
</tr>
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<tbody>
<tr>
<td>D. melanogaster</td>
<td>GSGIDLEAFPRV-NH²</td>
<td>ASLGFVPRV-NH²</td>
<td>TGFGASSDLWFGPRL-NH²</td>
</tr>
<tr>
<td>N. bullata</td>
<td>MGTDLEAFPRV-NH²</td>
<td>ASLGFVPRV-NH²</td>
<td>TGFGASSDLWFGPRL-NH²</td>
</tr>
<tr>
<td>M. domestica</td>
<td>MGTDLEAFPRV-NH²</td>
<td>ASLGFVPRV-NH²</td>
<td>TGFGASSDLWFGPRL-NH²</td>
</tr>
<tr>
<td>A. gambiae</td>
<td>MGTDLEAFPRV-NH²</td>
<td>ASLGFVPRV-NH²</td>
<td>TGFGASSDLWFGPRL-NH²</td>
</tr>
</tbody>
</table>

PVKs/CAP2b are designated according to the position in the respective capa-genes, which is known for D. melanogaster and A. gambiae. Underlined amino acids: substitution of Leu to Ile cannot be ruled out due to mass ambiguity for this amino acid pair.

PVK-2 sequences do not show strong similarities at all, PVK-1 sequences have more identities between M. domestica and N. bullata. The single pyrokinin from the capa-gene is even identical between M. domestica and N. bullata; and this is the only identity between the four dipteran species listed in Table 1. Assuming that the amino acid at position 7 from the C-terminus is indeed Leu (which cannot be separated from Ile by mass spectrometric

4. Discussion

Large insects are often better suited for physiological research and biochemical analysis, but lack of genomic information can clearly limit the experimental progress in many cases. In this case, we studied the products of the capa-gene in two widely distributed and large pest flies, namely M. domestica and N. bullata. Although the capa-gene itself was only known from D. melanogaster [11] and A. gambiae [12], earlier studies have unambiguously shown that related products (peptides) appear in all insects investigated so far. These insects include mainly hemimetabolous species (cockroaches and locusts; [3,9,10]) but a single peptide (CAP2b) is also known from the tobacco hawkmoth, Manduca sexta [6]. All these peptides share not only sequence similarities but also a unique distribution in the central nervous system. Capa-gene products are the only abundant peptides produced in the median neurosecretory system of the abdominal ganglia and accumulated in major neurohemal release sites, namely abdominal PSOs. Knowledge of the function(s) of the capa-gene products, however, is still less than complete; although good evidence exists regarding their role in water balance in a number of flies as well as in the regulation of muscle activity in a number of insects (summary in [14]).

The current study shows that the species-specific peptide sequences differ remarkably between the related dipteran species. Whereas the PVK-2 sequences do not show strong similarities at all, PVK-1 sequences have more identities between M. domestica and N. bullata. The single pyrokinin from the capa-gene is even identical between M. domestica and N. bullata; and this is the only identity between the four dipteran species listed in Table 1. Assuming that the amino acid at position 7 from the C-terminus is indeed Leu (which cannot be separated from Ile by mass spectrometric
methods only) in all cases, the periviscerokinins contain two conserved regions in their sequence. As in most insect neuropeptide families, the C-terminus is relatively well conserved; consisting in this case of a PRVamide, usually preceded by Phe. The most distinct exceptions among the known periviscerokinins is the C-terminus of Pea-PVK-1 (MRNamide, [8]). In addition, the PVK-2 of N. bullata has an Leu/Ile as C-terminal amino acid, which resembles the C-terminus of pyrokinnins. A close relationship between periviscerokinins and pyrokinnins is also supported by the recent identification of a peptide from abdominal PSOs of Locusta migratoria which has a C-terminus of PRVamide but otherwise exhibits all attributes typical of the single pyrokinin from the capa-gene [3]. Periviscerokinins, however, do not have Phe at position 5 from the C-terminus which is required for the biological activity of pyrokinnins [7].

In turn, the pyrokinnins from the capa-gene do not have Leu at the position 7 from the C-terminus which is part of the second conserved region of periviscerokinins. Substitution of the leucine at position 2 against isoleucine resulted in a drastic reduction in the efficacy of Mas-CAP2b in the heart bioassay [6]. These data suggest that the products of the capa-gene interact with different receptors. The peptide sequences native to four dipteran species (Table 1) not only show species-specific differences but are also indicative of another phenomenon. Although PVK-1 of M. domestica has a mass identical with that of D. melanogaster, and was identified from the homologous hormone release site, it was shown to have a different N-terminus. Hence, caution is recommended when interpreting mass spectra obtained from species that have not been previously studied.

The specific differences between the dipteran species mentioned in this study could make it possible to develop peptide-analogs with group- or species-specific efficacy. To distinguish between Leu and Ile in the peptide sequences determined here by mass spectrometric techniques, we plan to identify the capa-genes of *M. domestica* and *N. bullata*, using the sequence information we obtained in this study. Subsequently, synthetic peptides, identical with the capa-gene products of *M. domestica*, *N. bullata*, and *D. melanogaster*, will be tested in diuretic assays in all three species to search for putative species-specific efficacy.

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