

Expression of a novel neuropeptide, NVGTLARDFQLPIPNamide, in the larval and adult brain of *Drosophila melanogaster*

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Abstract

Advances in mass spectrometry and the availability of genomic databases made it possible to determine the peptidome or peptide content of a specific tissue. Peptidomics by nanoflow capillary liquid chromatography tandem mass spectrometry of an extract of 50 larval *Drosophila* brains, yielded 28 neuropeptides. Eight were entirely novel and encoded by five not yet annotated genes; only two genes had a homologue in the *Anopheles gambiae* genome. Seven of the eight peptides did not show relevant sequence homology to any known peptide. Therefore, no evidence towards the physiological role of these 'orphan' peptides was available. We identified one of the eight peptides, IPNamide, in an extract of the *Drosophila* adult brain as well. Next, specific

antisera were raised to reveal the distribution pattern of IPNamide and other peptides from the same precursor, in larval and adult brains by means of whole-mount immunocytochemistry and confocal microscopy. IPNamide immunoreactivity is abundantly present in both stages and a striking similarity was found between the distribution patterns of IPNamide and TPAEDFMRFamide, a member of the FMRFamide peptide family. Based on this distribution pattern, IPNamide might be involved in phototransduction, in processing sensory stimuli, as well as in controlling the activity of the oesophagus.

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The completion of the *Drosophila* genome (Adams *et al.* 2000) made it possible to identify novel fruit fly neuropeptides by *in silico* data mining, based on sequence homology to known peptides of other species. (Vanden Broeck 2001; Hewes and Taghert 2001; De Loof *et al.* 2001) However, potential novel peptides without significant sequence homology to any known peptide cannot be found by this method. Furthermore, data mining itself does not immediately yield any information about where and when predicted peptide genes are expressed in *Drosophila*, if at all.

The presence of a specific peptide in a particular tissue can be elegantly examined by mass spectrometry. Thanks to the recent developments in mass spectrometry, the study of peptidomics or peptide profiling became possible. This is well illustrated by the studies of (Clynen *et al.* 2001) and (Clynen *et al.* 2003) who conducted peptide-profiling analyses of several neuroendocrine organs in locusts. However,

in addition to several known peptides that could be mapped in a straightforward manner, many ion peaks in these locust neuroendocrine preparations remained unidentified because *de novo* sequencing of these peaks by tandem MS did not reveal the complete sequence, due to the lack of a genomic data base.

Having the genome available makes it easy to deduce peptide identities by comparing mass fragments against all possible peptides encoded by the genome by computer

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Abbreviations used: BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; FITC, fluorescein isothiocyanate; LC, liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline.

programs like Mascot. There are two insect genomes available at this moment (*Drosophila melanogaster* and *Anopheles gambiae*) and others are coming on stream in the coming months (*Aedes aegypti*, *Apis mellifera* and *Bombyx mori*). Thus, we expect that mass spectrometric experiments will provide us with a database of most insect neuropeptides of species of interest, as has been achieved for *Drosophila* (Baggerman *et al.* 2002). The sequenced genome of *Drosophila melanogaster* played a significant role in this peptidomic study, in which a total of 28 neuropeptides were isolated and identified using a CNS extract, derived from 50 *Drosophila* larvae only. No more than seven of these peptides had previously been isolated from *Drosophila*; others were predicted by bioinformatic analyses. Eight peptides encoded by five different genes were entirely novel, and named MTYamide (YIGSLARAGGLMTYamide), IPNamide (NVGTLARDFQLPIPamide), NAP peptide (SVAALAAQGLLNAP), Drm-MT2 (SVPFKPRLamide), NEF peptide (TKAQGFNEF), SHA peptide (VVSVP-GAISHA), VVIamide (SVHGLGPVVIamide) and YSY peptide (pQYYYGASPYAYSSGGYYDSPYSY), respectively. BLAST (Basic Local Sequence Alignment Tool) searches with these peptides or their precursors as input queries do not reveal any homologous peptides or precursors in vertebrate databases, although some do have homologues in the *Anopheles gambiae* genome. Only Drm-MT2 from the Hugin gene (Meng *et al.* 2002) has an FXPRLamide C-terminus, typical for myotropin/pyrokinin peptides.

In this study, we demonstrate by means of nanoflow capillary liquid chromatography (LC) tandem mass spectrometry (MS) that one of these recently identified larval peptides, IPNamide, is not only present in the *Drosophila* larval brain, but in the adult brain as well. Polyclonal antisera to IPNamide, MTYamide and NAP peptide, all three derived from the same precursor, were developed and used to study the neuronal distribution of their immunoreactivity in both the larval and adult CNS.

Materials and methods

Insects

Drosophila melanogaster were raised at 20°C in 250 mL milk bottles on a diet made of 17 g sucrose, 0.45 g yeast, 0.9 g agar, 70 mL water, 0.5 mL 8% nipagin and 0.36 mL propionic acid. *Anopheles gambiae* was supplied by L. Koopman from the University of Wageningen, the Netherlands. Adult flies, wandering stage larvae and mosquito pupae were collected; their CNS was dissected and rinsed in physiological saline.

Prior to mass spectrometric analysis, batches of 50 adult fly CNSs were extracted with 20 µL of methanolic/water/formic acid (90 : 9.9 : 0.1). After homogenization, the extract was centrifuged at 3000 × g for 10 min. The supernatant was filtered through Millipore spindown filters, dried and stored at -20°C until further use.

CNSs dissected for immunohistochemistry were immediately fixed in 2% paraformaldehyde in 10 mM PBS (phosphate-buffered saline, pH 7.2).

Capillary LC-tandem MS and identification

Capillary LC-tandem MS was performed using an Ultimate HPLC pump, a column-switching device (Switchos) and a Famos auto sampler (LC Packings, the Netherlands) coupled to a Q-ToF hybrid quadrupole/time of flight mass spectrometer (Micromass, UK).

Ten microlitres of the sample (containing an equivalent of 50 adult *Drosophila* brains) was loaded on a precolumn (µ-guard column MGU-30 C18, LC-Packings, the Netherlands) with an isocratic flow of 2% acetonitrile in milliQ-water with 0.1% formic acid (FA) at a flow rate of 10 µL/min. After 2 min the precolumn was switched online with the analytical capillary column (Pepmap C18, 3 µm, 75 µm × 150 mm nano column, LC Packings, the Netherlands). We imposed a linear gradient from 95% solvent A, 5% solvent B to 50% A, 50% B in 65 min [solvent A: milliQ-water, FA; 99.9, 0.1 (v/v); solvent B: acetonitrile, FA; 99.9, 0.1 (v/v)]. The flow rate was 150 nL/min.

The Ultimate capillary LC was connected in series to the electrospray interface of the Q-ToF mass spectrometer. Needle voltage was set at 1300 V and cone voltage at 35 V. Nitrogen was used as nebulizing gas. Ions with two or three protons (typical for small peptides) were automatically selected for fragmentation during the nano-LC tandem MS separation. Argon was used as a collision gas; collision energy was set at 25–40 eV depending on the selected mass.

Fragmentation spectra were subjected to the Mascot search program (<http://www.matrixscience.com>). This program analyses fragmentation spectra of peptides and uses the computed partial sequences to search a genome for possible matches, after which it compares the theoretical fragmentation spectrum of matching sequences to the experimentally obtained spectrum. (Perkins *et al.* 1999)

Antisera production

Polyclonal antisera against IPNamide, MTYamide and NAP peptide were raised by immunizing six New Zealand White rabbits with synthetic peptide (Resgen) coupled to thyroglobulin in two different ways, using EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] and glutaraldehyde as cross-linkers. Each time, a total of 1 mg of conjugate was injected subcutaneously at multiple sites. Initial immunization was performed with Freund's complete adjuvant, subsequent boosts every 2–3 weeks with Freund's incomplete adjuvant. Antisera titres were analyzed by solid phase dot blot assay.

Dot blot assay

One microlitre of a dilution series of IPNamide, MTYamide, NAP peptide, TPAEDFMRFamide, AYAAPAALSVPNPYAGLGA and GTVLIQTDNTQYIRamide ranging from 1 µg to 1 pg/µL was spotted onto a nitrocellulose membrane (Hybond-C, Amersham). Membranes were heated for 30 min at 120°C, blocked with 1% skimmed milk in 50 mM Tris-buffered saline and incubated overnight at 4°C with the primary antiserum, diluted 1 : 500. After rinsing, blots were incubated in anti-rabbit horseradish peroxidase-conjugated antibody (Dako, Glostrup, Denmark) for 45 min, rinsed and developed using the substrate 3,3'-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA).

Immunohistochemistry

After dissection, CNSs were fixed in 2% paraformaldehyde in 10 mM PBS (pH 7.2) for 18–24 h and subsequently washed with cold PBS for 4–5 h. Tissues were incubated overnight at 4°C in 4% Triton X-100, 2% normal porcine serum and 2% bovine serum albumin (BSA). Primary antiserum was applied in a dilution of 1 : 500 or 1 : 1000 in PBS for 24 h at 4°C on a flatbed shaker. Tissues were washed with cold PBS for 4–5 h, and subsequently incubated with the secondary antibody. FITC (fluorescein isothiocyanate)-conjugated swine anti-rabbit Ig (Dako), diluted 1 : 20 in PBS, were applied for 24 h at 4°C. To prevent degradation of FITC, tubes containing the tissue samples were wrapped in foil. Finally, the tissues were briefly washed in cold PBS before being mounted in 90% glycerol containing 0.1% *p*-phenylenediamine.

Tissue samples were viewed with a Zeiss LSM 410 Kr-Ar inverted laser-scan confocal microscope, using a 20 × or 40 × objective and excitation wavelength 488 nm. With the extended depth of focus function, we obtained stacks of serial optical sections, which were electronically superimposed along the *Z*-axis. Data were processed using Adobe Photoshop software, version 6.0.

Bio-assays

In order to measure the effect of IPNamide, MTYamide and NAP peptide on muscle contractions of the gut, isolated foreguts and hindguts of both *Leucophaea maderae* and *Neobellieria bullata*, were mounted in a chamber filled with physiological saline, as described by (Holman *et al.* 1991). IPNamide, MTYamide and NAP peptide were also tested in the *in vitro* crop assay as described by (Duttlinger *et al.* 2003). The crop of both *Drosophila melanogaster* and *Neobellieria bullata* were used.

The concentrations of the peptides, applied in both assays, varied between 10^{-5} and 10^{-7} M.

Results

Capillary LC-tandem MS

The analysis of adult brains by LC-tandem MS revealed a lot of fragmentation spectra, although fewer than those obtained with larval fruit flies (Baggerman *et al.* 2002). This is largely due to the high signal intensity of eye pigments throughout the entire separation. However, one particular fragmentation spectrum was identified as the recently discovered IPNamide (Baggerman *et al.* 2002). Despite the lack of several fragment ions an exactly matching amino acid sequence, NVGTLARDFLQPIPNamide, could be found, with high confidence, through a Mascot search (Fig. 1). This peptide is part of a peptide precursor predicted by a genomic sequence which was first annotated in the flybase as CG3441, now named neuropeptide like precursor 1 (NPLP1) by (Baggerman *et al.* 2002).

Immunohistochemistry

Specificity of the antisera was demonstrated. For example, IPNamide was detected up to a concentration of 100 pg per dot in a solid phase dot blot assay, using a dilution of 1 : 500. IPNamide does not display significant sequence similarities with other *Drosophila* neuropeptides and the IPNamide

a	δ	87.06	186.12	243.15	344.19	457.28	528.31	684.42	799.44	946.51	1074.57	1187.65	1284.71	1397.79	1494.84	1608.89
b	δ	115.05	214.12	271.14	372.19	485.27	556.31	712.41	827.44	974.51	1102.56	1215.65	1312.70	1425.79	1522.84	1636.88
i	δ	87.06	72.08	30.03	74.06	86.10	44.05	129.11	88.04	120.08	101.07	86.10	70.07	86.10	70.07	87.06
		0.01	0.00	---	---	0.01	---	0.01	---	0.01	0.00	0.11	---	0.01	0.01	0.01
		Asn	Val	Gly	Thr	Leu	Ala	Arg	Asp	Phe	Gln	Leu	Pro	Ile	Pro	Asn-amide
y	δ	1653.91	1539.86	1440.80	1383.77	1282.73	1169.64	1098.61	942.50	827.48	690.41	552.35	439.27	342.21	229.13	132.08
z	δ	1636.88	1522.83	1423.77	1366.74	1265.70	1152.61	1081.58	925.47	810.45	663.38	535.32	422.24	325.18	212.10	115.05
		---	---	0.07	---	0.07	0.03	---	---	---	---	---	0.01	0.01	0.00	---
		---	---	---	---	---	---	---	---	---	---	---	-0.00	---	-0.01	-0.01

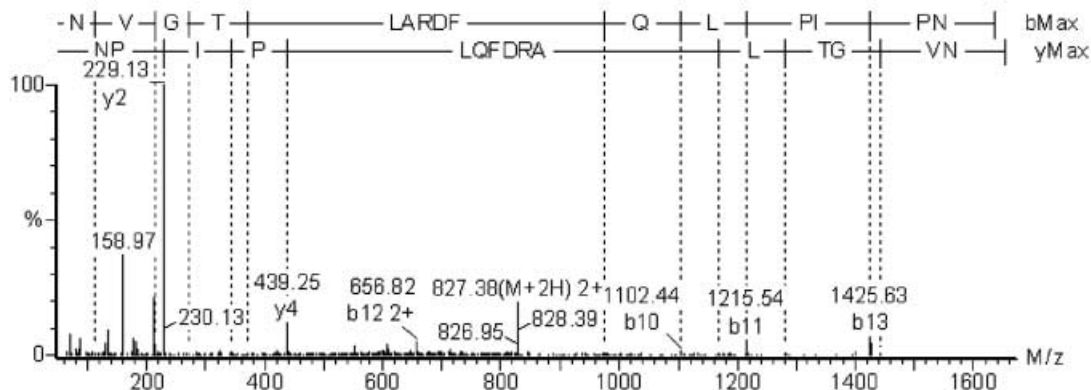


Fig. 1 CID (collision induced dissociation) spectrum of NVGTLARDFLQPIPNamide, originating from the gene annotated as CG3441. a-type, b-type, y-type, z-type and immonium (i) fragment

ions are indicated in the table. The theoretical fragment ion masses found in the spectrum are indicated in bold. Mass differences between expected and observed fragment ion masses are indicated by δ .

antisera did not react with any other of the synthetic peptides we tested, not even in a concentration 10 000 times higher than IPNamide. The antisera derived from both rabbits yielded the same results in the dot-blot assay as well as in the immunohistochemical experiments. The control experiments using preimmune serum did not reveal any dots in the dot-blot experiments, nor were any cells or fibres in the whole-mount preparations revealed. Finally, specificity was confirmed by incubating the antisera with synthetic IPNamide at 0.0001 M for 24 h on a flatbed shaker at 4°C prior to immunohistochemical analysis.

The terminology used to identify the cells stained by antisera is consistent with previous publications on *Drosophila* (White *et al.* 1986; Schneider and Taghert 1988). Because we observed all immunoreactivity bilaterally symmetric to the midline of the nervous system, referring to one cell indicates the presence of a pair of cells positioned bilaterally symmetric to one another. If corresponding cells do not appear perfectly symmetrically positioned, this is due to the manipulation or distortion of the tissue.

IPNamide immunoreactivity was observed in both larval CNS and adult brain. In the CNS of the third larval instar, almost all staining was found in the ventral ganglion (Fig. 2). The fused ventral ganglion consists of the three thoracic and eight abdominal ganglia. Thirteen cells were observed, from which 10 form a row along the length of the ventral ganglion.

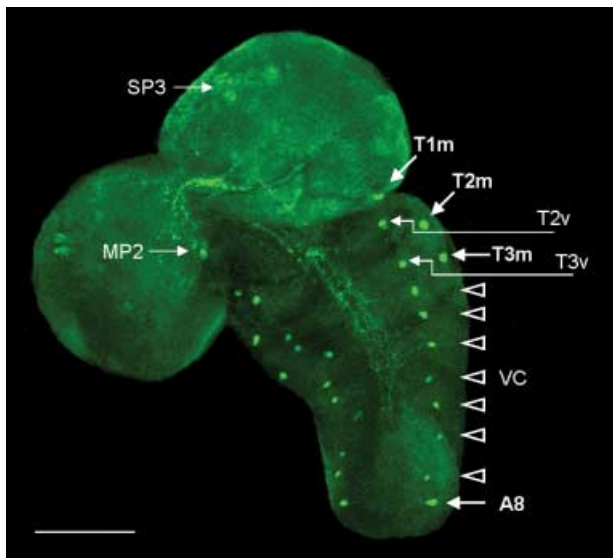


Fig. 2 IPNamide immunoreactivity in a whole-mount tissue preparation of a *Drosophila* third-instar larval CNS. The image was obtained by superimposing nine optical sections each 4 µm. Immunoreactivity is strongly expressed in the abdominal A8 and VC (open arrows) cells and in the thoracic T1–3 m and T2–3v cells of the ventral ganglion. Immunoreactive fibres derived from these cells and encircling the oesophageal opening are faintly visible. Slightly stained in the brain lobe are the presumed SP3 and MP2 cells. Bar = 100 µm.

Eight (VCs, A8) were found in the abdominal ganglia, two in the second and third thoracic ganglion (T2–3v). The other three cells (T1–3m) are more intensely stained and positioned further away from the midline; one cell per thoracic ganglion. All these cells show immunoreactive processes, which project towards the midline and turn to the anterior part of the brain where they encircle the oesophageal opening (Fig. 4). These immunoreactive fibres also laterally branched, projecting into the brain. As can clearly be seen on Fig. 3(b), the processes cross the midline to contralaterally extend to the oesophageal opening. From here the cells form a ladder-like structure along the length of the nerve cord. In particular, the processes seem to cross the midline near the three thoracic ganglia to outline two boxes. However, in many cases an additional and intensely stained 14th cell was observed in the first thoracic ganglion. This cell did not follow the row of 10 cells as one would expect, but was positioned more laterally at about the same distance to the midline as the three other thoracic cells (Fig. 4). This cell also projected processes towards the midline.

As far as the dorsoventral position of the cells is concerned, the three or four laterally thoracic cells are situated in the middle of the dorso-ventral axis. The last abdominal cell (A8) and the ladder-like processes are at the same depth, while the remaining cells (VCs and T2–3v) are found more ventrally. Furthermore, several faintly staining cells were detected (Figs 2 and 4). Three of these cells were found in the superior protocerebrum, two in the proximity of the suboesophageal ganglion and about four dorsal cells in the middle of the abdominal ganglion. Based on their position these cells probably correspond to, respectively, SP3, MP2 and DC cells. No processes seemed to extend from these cells.

In the adult brain the arborization of the projections from the thoracic and abdominal cells was firmly increased with immunoreactive fibres extending throughout the brain and into the optic lobes (Fig. 5a,b). The fibres outline a symmetrical pattern with the oesophageal opening at the midline. At the ventral basis of the brain, immunoreactive fibres form a cross-like structure (not shown), extending in four directions: anterior, posterior and towards the lobes. In addition one intensely immunoreactive cell appeared in the upper part of the lateral protocerebrum. A few weakly stained cell-like structures are also present in the lateral protocerebrum. As no processes seemed to extend from these cells, all processes most likely originate from cells in the ventral ganglion.

Antisera raised against MTYamide (not shown) or NAP peptide (Fig. 3a) resulted in identical immunoreactive distribution patterns as obtained with IPNamide antisera. Moreover, IPNamide, MTYamide and NAP peptide antisera, revealed a similar distribution pattern in both larval and adult CNSs of the grey flesh fly, *Neobellieria bullata* (data not shown). However, applied on the CNS of *Anopheles* pupae,

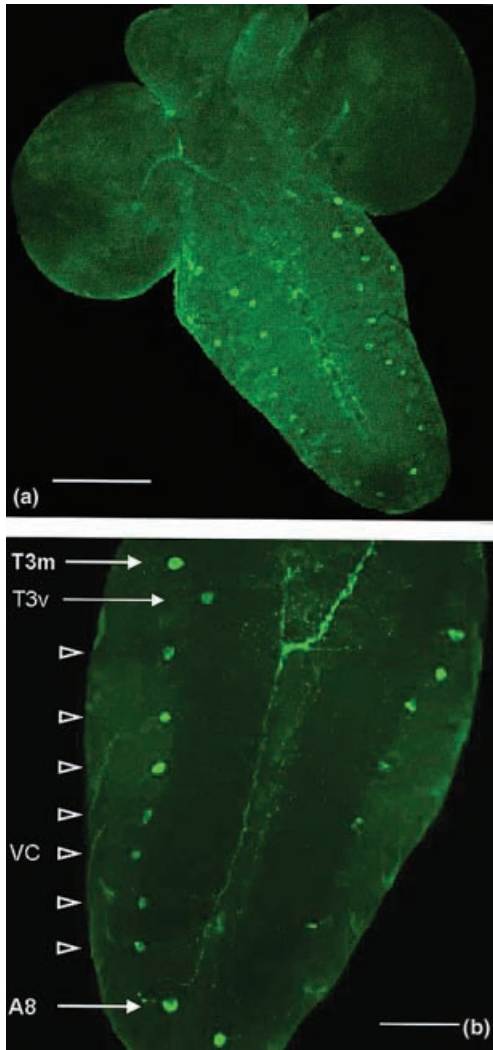


Fig. 3 (a) NAP peptide immunoreactivity in the larval CNS. This image was obtained by superimposing five optical sections each 3 μm . Bar = 100 μm . (b) IPNamide immunoreactivity in the larval CNS. This image was obtained by superimposing six optical sections each 3 μm . Enlargement of the ventral ganglion. Immunoreactive material is strongly expressed in the abdominal A8 and VC (open arrows) cells and the thoracic T3m and T3v cells. An immunoreactive fibre projecting from an A8 cell follows the midline until the third thoracic ganglion, where it crosses the midline and contralaterally proceeds. Bar = 50 μm .

immunoreactivity was found only with IPNamide antisera (Fig. 6). No immunoreactivity was found in the brain or in the suboesophageal ganglion. The pro- and mesothoracic (ms) ganglion, on the other hand, contained two immunoreactive cells; the metathoracic ganglion, which is fused with the small first abdominal ganglion, contained three cells. Immunoreactive fibres were only faintly visible, along the length of the nerve cord, on the midline of the metathoracic ganglion (Fig. 6). Abdominal ganglia from *Anopheles* were not dissected.

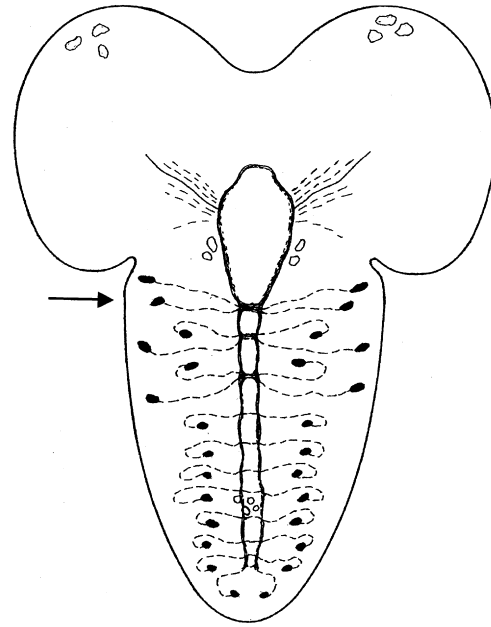


Fig. 4 IPNamide immunoreactivity in the larval CNS. Schematic drawing based on whole-mount preparations. Both strong and weak immunoreactive cells are indicated. The strong immunoreactive cells project towards the midline and contralaterally proceed to encircle the oesophageal opening. While each abdominal ganglion contains one cell, the second and third thoracic ganglion contains two. The first thoracic ganglion contains either one or two cells (the additional cell is indicated by an arrow).

Discussion

After it had first been discovered in an extract of larval brains of *Drosophila* by nanoflow capillary LC-tandem mass spectrometry (Baggerman *et al.* 2002), we identified the peptide IPNamide in an extract of the *Drosophila* adult brain, using the same experimental set-up. Next, by means of whole-mount immunocytochemistry we revealed the distribution pattern of IPNamide and confirmed the reliability of the peptidomics approach. In other words, the present immunocytochemical experiments demonstrate that the presence of IPNamide in the CNS peptidome is not due to contamination of the CNS extract with haemolymph, fat tissue or to degradation products of larger proteins and establishes IPNamide as a genuine neuropeptide.

The combination of nanoflow capillary LC-tandem mass spectrometry with genome data mining again proved to be a powerful technique for peptide identification. This statement is especially illustrated by the identification of IPNamide solely based on a rather imperfect CID-spectrum as shown in Fig. 1. In comparison with traditional purification techniques (Nichols 1992), the advantages of the peptidomics approach are obvious. First, only a small number of flies are needed for the extraction. As a result, specific tissues can be dissected carefully for extraction instead of making whole body

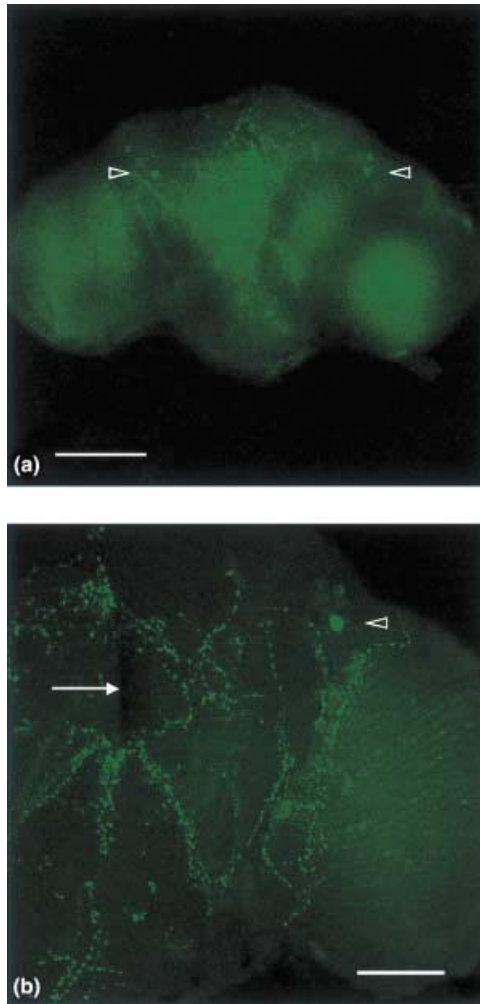


Fig. 5 IPNamide immunoreactivity in a whole-mount tissue preparation of a *Drosophila* adult brain. (a) This image was obtained by superimposing 10 optical sections each 3.5 μm . Immunoreactive processes extend throughout the brain and optic lobe. In addition, a lateral protocerebrum cell is intensely stained (open arrow). Bar = 100 μm . (b) This image was obtained by superimposing 10 optical sections, each 1.1 μm . Enlargement of the upper right part of the brain. Extensive arborization of fibres throughout the brain and optic lobes. Filled arrow points at the oesophageal opening. Open arrow indicates an intensely stained lateral protocerebrum cell, without projections. Bar = 50 μm .

extracts. Second, multiple purification steps are no longer necessary. Third, time-consuming screening of fractions is avoided. Finally, identification of a peptide happens immediately and automatically after the LC separation and more than one peptide can be identified at a time. Thus, peptidomics or peptide profiling of a specific tissue during development has become possible.

IPNamide is encoded by the *Drosophila* neuropeptide like precursor 1 (NPLP1) gene, situated at chromosome arm 2R. NPLP1 encodes a signal peptide, according to Nielsen *et al.*

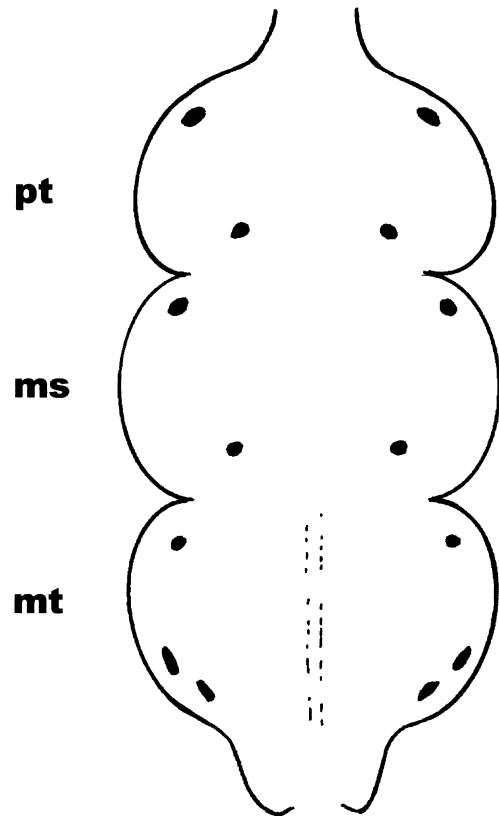


Fig. 6 IPNamide immunoreactivity in the thoracic ganglia of *Anopheles gambiae*. Schematic drawing based on whole-mount preparations. The pro- (pt) and mesothoracic (ms) ganglion contain two immunoreactive cells. The metathoracic (mt) ganglion, which is fused with the small first abdominal ganglion, contains three cells. Immunoreactive fibres were only faintly visible in the metathoracic ganglion.

(1997), and 11 other putative neuropeptides, flanked by dibasic cleavage sites (Fig. 7). At least three of them, IPNamide, MTYamide and NAP peptide (Fig. 7, in bold type), named according to their final three C-terminal amino acids, are expressed in the larval CNS (Baggerman *et al.* 2002) and this study. The eight other peptides vary in length from two to 73 amino acids. The longest peptide is situated between the signal peptide and the first dibasic cleavage site and may not be bioactive. However, the seven following sequences flanked by dibasic cleavage sites, are potential neuropeptides, varying in size between 14 and 31 amino acids. These putative peptides share some sequence similarity at their N-termini. They do not, however, display any important sequence similarities with other *Drosophila* peptides.

Subjecting NPLP1 to a BLAST search in all currently available databases reveals only one significant hit, i.e. in the *Anopheles gambiae* genome. The predicted transcript agCP10689 encodes a similar neuropeptide precursor structure and is therefore named agamNPP1 (Riehle *et al.* 2002).

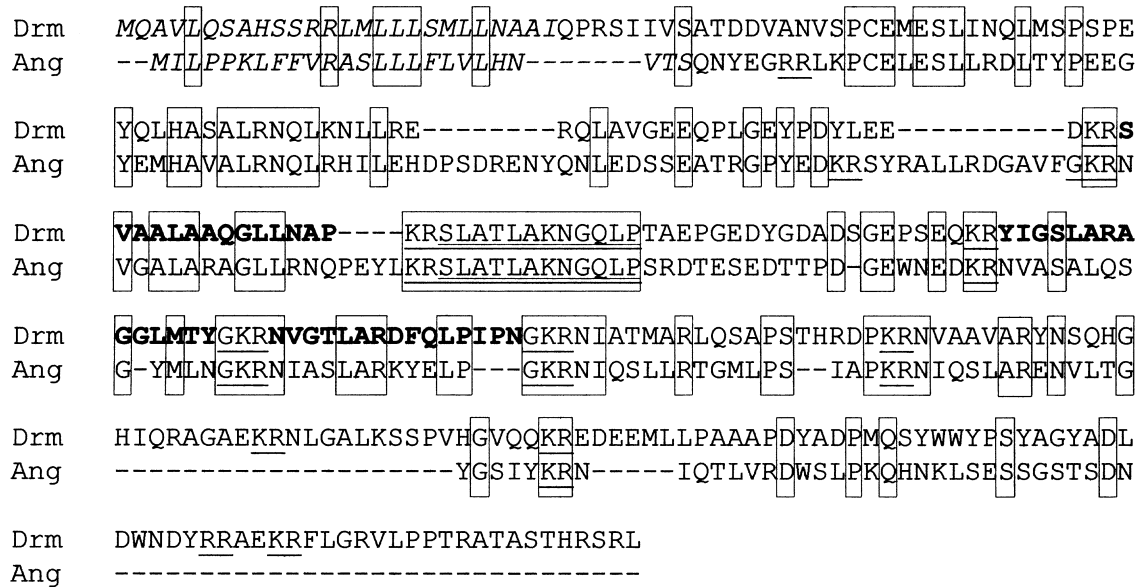


Fig. 7 Drm is *Drosophila melanogaster* neuropeptide like precursor 1, accession AAF47313 (CG3441); Ang is *Anopheles gambiae* neuropeptide precursor 1, accession EAA07164 (agCP10689). Amino acid sequences retrieved by BLAST search, CLUSTALW multiple alignment

(<http://www.ebi.ac.uk/clustalw>). Signal peptides are shown in italics. Dibasic cleavage sites: underlined. Peptides identified with nano LC-tandem MS in bold. Most conserved sequence is double-underlined.

Similar to *Drosophila* NPLP1, *Anopheles* NPP1 encodes a signal peptide and 11 putative neuropeptides, flanked by dibasic cleavage sites (Fig. 7). The first two peptides after the signal peptide are, respectively, four and 61 amino acids in length. The seven following sequences share mutual similarities at their N-termini and are between 12 and 30 amino acids long. Three of them are C-terminally amidated: AVFamide, MLNamide and ELPamide. ELPamide contains an internal RK dibasic cleavage site, but according to (Veenstra 2000) RK sites are not processed in insect peptide precursors. The most highly conserved peptide within the NPLP precursors of *Drosophila* and *Anopheles* is the second longest peptide, counting, respectively, 31 and 30 amino acid residues, of which the first 12 N-terminal amino acids are identical (Fig. 7, double-underlined).

Based on this comparison, the neuropeptide-like precursor 1 seems to be well conserved within the order of the *Diptera*. Because no homologues are found in vertebrates, this precursor may perhaps harbour possibilities for developing anti-dipteran control methods. The conservation of the precursor within the *Diptera*, is also confirmed by our immunohistochemical data. Antisera to IPN amide, MTYamide and NAP peptide showed similar distribution patterns in *Neobellieria*. With IPNamide antisera, a few thoracic cells are also stained in *Anopheles* (Fig. 6). The fact that only antisera to IPNamide detect immunoreactivity, is most likely due to the greater sequence similarity between IPNamide and the putative *Anopheles* peptides, in particular ELPamide and EYL peptide (Fig. 7).

Discovering a new peptide is one thing; revealing its function is quite another, mainly because no bioassay was involved in the purification. IPNamide can be tested in well established bioassays, but the complete absence of sequence homology to known peptide families makes success rather improbable. Whereas mass spectrometry can provide a clear idea of how peptides are processed exactly, immunocytochemistry can show the precise distribution pattern, revealing clues about the peptides' physiological function. The abundant immunoreactive material in both larval and adult CNS suggests that IPNamide plays a rather important role(s) in both stages of development. In addition, the complexity of the immunoreactive projections implies that there may be several sources of input causing release of IPNamide at different sites. Comparing the immunoreactive pattern of IPNamide with the data on the distribution of other *Drosophila* neuropeptides described in literature, we found a striking resemblance with the distribution pattern of TPAEDFMRFamide (McCormick *et al.* 1999; Nichols *et al.* 1999b). In the larval CNS, 13 cells at similar positions with projections towards the midline and encircling the oesophageal opening were stained with TPAEDFMRFamide antisera. Moreover, an additional fourteenth cell in the first thoracic ganglion was stained, although only in the third instar and puparium stage (Nichols *et al.* 1999b). Faintly stained cells without projections were found in the same regions, i.e. brain lobes and suboesophageal ganglion. Furthermore, the same complex arborization of projections was observed in the adult brain, starting as a cross-like formation in the pupal

stage. Finally, one TPAEDFMRamide immunoreactive cell was present in the adult lateral protocerebrum. Despite limited sequence similarity between TPAEDFMRamide and NVGTLARDFQLPIPamide, our polyclonal antisera did not show cross-reactivity in the dot blot assay nor did the antisera to MTYamide and NAP peptide.

TPAEDFMRamide is contained in the *Drosophila* FMRamide gene, which encodes other members of the FMRamide peptide family. FMRamides are present throughout the animal kingdom and well studied in insects, where they affect many biological activities (Orchard *et al.* 2001; Nassel 2002; Nichols 2002). In *Drosophila*, FMRamides are involved in physiological processes like heart rate, gut motility and synaptic activity (Kaminski *et al.* 2002; Hewes *et al.* 1998; Nichols *et al.* 1999a; Johnson *et al.* 2000). As in other species, the N-terminal extension is critical for activity. SDNFMRamide decreases *in vivo* heart rate whereas the other Drm FMRamides, encoded by the same precursor, DPKQDFMRamide and TPAEDFMRamide, do not (Nichols *et al.* 1999a). TPAEDFMRamide decreases the frequency of spontaneous contractions in the crop, DPKQDFMRamide and SDNFMRamide, do not (Duttlinger *et al.* 2003). The distribution of these three peptides in larval CNS was studied by means of antisera raised against the specific N-terminal fragments of the peptides (McCormick *et al.* 1999; Nichols *et al.* 1999b). The three antisera displayed three non-overlapping cellular expression patterns. According to (McCormick *et al.* 1999; Nichols *et al.* 1999b), these data indicate the presence of cell-specific proteolytic enzymes which differentially process a peptide precursor, resulting in unique expression patterns of neuropeptides. However, this conclusion was questioned in a review (Taghert 1999). Anyhow, as far as it concerns IPNamide, MTYamide and NAP peptide, their identical distribution patterns seem to rule out cell-specific enzymes for the processing of NPLP1. The extent of the colocalization between TPAEDFMRamide and NPLP1 peptides will be further explored with a double-labelling immunocytochemical method. Nevertheless, the colocalization of TPAEDFMRamide and IPNamide suggests that they might be coreleased and involved in the same physiological roles as proposed by (Nichols *et al.* 1999b). First, the presence of immunoreactive processes around the oesophageal opening suggests a role for both peptides related to the function of the oesophagus. However, while TPAEDFMRamide decreases crop contractions (Duttlinger *et al.* 2003), we found this is the case for neither IPNamide nor MTYamide and NAP peptide. These peptides also do not influence the contractions of isolated fore- and hindguts of *Leucophaea* and *Neobellieria*, when applied in concentrations between 10^{-5} and 10^{-7} M. Second, the processes at the midline of the ventral ganglion correspond with a neuronal sheath, which is a neurohemal release site in *Drosophila* (Lundquist and Nassel 1990). Finally, the increasing arborization of immunoreactive

fibres in the brain lobes suggests an extra role for IPNamide in the adult stage. Immunoreactivity in the optic lobes, the lobula and a lateral protocerebral cell, located in the region of the antennal lobe where sensory information is processed, may point to a neurotransmitter function in, respectively, phototransduction, motion and sensory stimuli signalling.

In sum, we have provided biochemical evidence for the presence of IPNamide in the adult *Drosophila* brain. In addition, we have studied the distribution of IPNamide, MTYamide and NAP peptide in CNSs of both larval and adult flies. The distribution pattern provides further evidence that these peptides are genuine neuropeptides, possibly having a neurotransmitter as well as a neurohormonal function.

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