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FOCUS: MASS SPECTROMETRY-BASED STRATEGIES FOR NEUROPROTEOMICS AND PEPTIDOMICS: RESEARCH ARTICLE

Peptidomics of Neuropeptidergic Tissues of the Tsetse Fly *Glossina morsitans morsitans*

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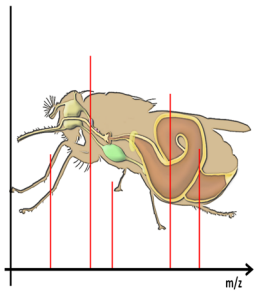

Abstract. Neuropeptides and peptide hormones are essential signaling molecules that regulate nearly all physiological processes. The recent release of the tsetse fly genome allowed the construction of a detailed in silico neuropeptide database (International Glossina Genome Consortium, Science **344**, 380–386 (2014)), as well as an in-depth mass spectrometric analysis of the most important neuropeptidergic tissues of this medically and economically important insect species. Mass spectrometric confirmation of predicted peptides is a vital step in the functional characterization of neuropeptides, as in vivo peptides can be modified, cleaved, or even mispredicted. Using a nanoscale reversed phase liquid chromatography coupled to a Q Exactive Orbitrap mass spectrometer, we detected 51 putative bioactive neuropeptides encoded by 19 precursors: adipokinetic hormone (AKH) I and II, allatostatin A and B, capability/pyrokinin (capa/PK), corazonin, calcitonin-like diuretic hormone (CT/DH), FMRFamide, hugin, leucokinin, myosuppressin, natalisin, neuropeptide-like precursor (NPLP) 1, orcokinin, pigment dispersing factor (PDF), RYamide, SIFamide, short neuropeptide F (sNPF) and tachykinin. In addition, propeptides, truncated and spacer peptides derived from seven additional precursors were found, and include the precursors of allatostatin C, crustacean cardioactive peptide, corticotropin releasing factor-like diuretic hormone (CRF/DH), ecdysis triggering hormone (ETH), ion transport peptide (ITP), neuropeptide F, and proctolin, respectively. The majority of the identified neuropeptides are present in the central nervous system, with only a limited number of peptides in the corpora cardiaca–corpora allata and midgut. Owing to the large number of identified peptides, this study can be used as a reference for comparative studies in other insects.

Keywords: Tsetse fly, Neuropeptide, *Glossina*, Peptidomics, Insect, Mass spectrometry

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Introduction

Tsetse flies (*Glossina* spp.) (Figure 1a) are insects with a peculiar life cycle. They reproduce by adenotrophic viviparity, meaning that the female fly nourishes the developing larvae within its abdomen with a milk secretion and subsequently gives live birth to a full-grown larva, one around every 10 days. More importantly, they are obligate blood feeding insects and are vectors of trypanosomes, protozoan parasites that are the cause of a lethal disease in humans when untreated. An estimated 70 million people are at risk of contracting human African trypanosomiasis (HAT), or sleeping sickness, in 36 countries of Sub-Saharan Africa [2]. In addition, the parasites also cause Nagana or animal African trypanosomiasis (AAT), a disease that is responsible for 3 million deaths amongst livestock in Africa and is considered a major constraint in alleviating poverty in the affected regions [3, 4]. Agricultural losses due to AAT are estimated to be in the range of US$ 4.75 billion annually [5]. Drugs for treatment of sleeping sickness are limited and can be fatal to the patient in itself [6–8]. For AAT, only two trypanocidal drugs are currently available to which resistance has already been observed in many endemic regions. In addition, new drugs have not been developed since

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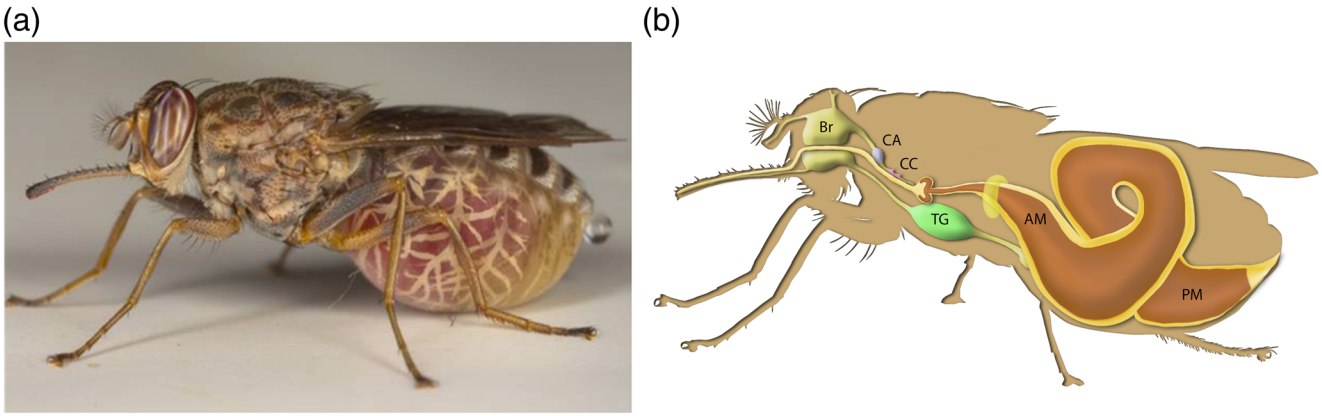


Figure 1. **(a)** The tsetse fly *Glossina morsitans morsitans*. **(b)** Schematic overview of the internal anatomy of the tsetse fly. Brain (Br), thoracic ganglion (TG), corpora cardiaca (CC), corpora allata (CA), anterior midgut (AM), posterior midgut (PM). Photo and scheme by Dr. Geoffrey M. Attardo, Yale Public School of Health

1990. Even though new therapies are being explored such as anti-trypanosome nanobodies [9], targeting the tsetse fly vector remains a cornerstone in the control and elimination of HAT and AAT [10, 11]. Owing to their small population size, low reinvasion rate [12], and low reproductive rate, tsetse flies cannot recover quickly from a collapse of their population [11]. However, in order to combat the flies proficiently, novel compounds are needed that specifically and efficiently kill them. Currently, most used insecticides are detrimental to the environment or toxic to non-target organisms such as honey bees and birds [13]. In addition, resistance to these noxious agents is witnessed in thousands of arthropod species [14]. Nevertheless, to date, insecticide resistance has not yet been observed in tsetse flies. As neuropeptides and their receptors regulate almost all vital biological processes such as development, behavior, metabolism, and reproduction, they are ideal targets for the generation of new pesticides [15]. A first major step in this direction was taken recently with the publication of the genome sequence of *Glossina morsitans morsitans* [1], which predicted 39 neuropeptide precursor-encoding genes and 43 genes that encode a neuropeptide receptor. As a follow-up of this in silico work, we report here the detection of neuropeptides from the primary neuropeptidergic tissues of the adult tsetse fly using nanoscale reversed phase liquid chromatography coupled to Q Exactive mass spectrometry. As a database, we used the tsetse fly transcriptome and the recently released genome sequence readily available from VectorBase. In total, we detected 788 different peptides belonging to 26 neuropeptide precursors. These peptides include propeptides, spacer peptides, truncated and post-translationally modified peptides, and mature peptides. As genomic and transcriptomic data alone is insufficient to reveal the neuropeptides that are ultimately produced, the present study provides important information. As the processing of a neuropeptide precursor can differ during developmental stages or between tissues, and because post-translational modifications are hard to predict when solely based on genomic sequence information [16, 17], we compared the presence of peptides across different neuropeptidergic tissues.

Methods

Animal Rearing Conditions

Tsetse flies (*G. m. morsitans*) were obtained from the insectarium at the Institute of Tropical Medicine Antwerp. This tsetse fly colony was originally derived from pupae collected from Zimbabwe and Tanzania [18]. The flies were kept at 26°C and 65% relative humidity and fed four times a week on sterile defibrinated bovine blood through an in vitro membrane system.

Sample Preparation

Tissues were collected from female flies, 3 days after their last blood meal, in ice-cold phosphate buffered saline (PBS) (composition: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM; pH 7.4). They were rinsed three times with fresh PBS, and finally collected in ice-cold acidified methanol (methanol/water/acetic acid – 90/9/1 – v/v/v) to extract peptides, inactivate protease and peptidase activity, and for denaturation and precipitation of large proteins. Following tissues were dissected: the entire brain (Br), including the optical lobes, antennal lobes, and sub-esophageal ganglion, the thoracic ganglion (TG), the corpora cardiaca–corpora allata (CC-CA), the posterior midgut (PM) and anterior midgut (AM) (Figure 1b) [19]. Samples contained between 15 and 20 pooled tissues. The number of samples for each tissue depended on the size and state of the tissues. The tissues were homogenized by sonicating three times for 10 s (Sanyo MSE Soniprep 150, London, UK) on ice. Following centrifugation (10 min at 10,000 rcf, 4°C) the supernatants were collected. The remaining pellets were resuspended in extraction solution, sonicated, and centrifuged again. First and second supernatants were pooled and filtered through 0.22 µm Millipore spindown filters (Millipore, Bedford, MA, USA). The samples were lyophilized and the posterior and anterior midgut samples were resuspended in a 2% acetonitrile and 0.1% trifluoroacetic acid solvent for delipidation by n-hexane extraction to remove intestinal debris. The aqueous phase was collected and lyophilized. The pellets

of all samples were resuspended in 25 µL 2% acetonitrile and 0.1% trifluoroacetic acid, desalted according to the manufacturer's instructions (ZipTip C18 Millipore, Billerica, MA, USA) and lyophilized again.

Nanoscale Reversed Phase Liquid Chromatography Coupled to Q Exactive Mass Spectrometry

The lyophilized samples were resuspended in 18 µL of a 2% acetonitrile 0.1% formic acid solution and analyzed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA), online coupled to an Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) instrument (Thermo Scientific). The UHPLC system has a 2 µm particle size, 100 Å pore size Easy Spray Pepmap RSLC C18 column with dimensions 50 µm×15 cm (Thermo Scientific) and a 3 µm particle size, 100 Å pore size, nanoviper, Acclaim Pepmap 100 C18 precolumn with dimensions 75 µm×2 cm (Thermo Scientific). A sample volume of 8 µL was injected. Buffer A consisted of 0.1% formic acid in water and buffer B of 80% acetonitrile and 0.08% formic acid. After 10 min equilibration, buffer B increased from 4% to 10% in 5 min, 10% to 25% in 50 min, 25% to 45% in 18 min, and a steep increase to 95% in 1 min. The flow rate was 300 nL/min. The mass spectrometer operated in data-dependent mode and all MS1 spectra were acquired in the positive ionization mode with an *m/*z scan range of 400 to 1600. Up to 10 most intense ions in MS1 were selected for fragmentation in MS/MS mode. A resolving power of 70,000 full width at half maximum (FWHM), an automatic gain control (AGC) target of 3,000,000 ions, and a maximum ion injection time (IT) of 256 ms were set for the generation of precursor spectra. To obtain fragmentation spectra, a resolving power of 17,500 FWHM, an AGC target of 1,000,000 ions, and a maximum IT of 64 ms were used as settings. In order to prevent repeated fragmentation of the most abundant ions, a dynamic exclusion of 10 s was applied. Ions with one or more than six charges were excluded. Ions of interest were selected with a 3.0 *m/z* isolation window.

Data Analysis

Analysis of the MS/MS data was performed with the PEAKS 7 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada). Spectra were first selected by quality, and fragmentation spectra with the same mass (less than 5 ppm difference) and retention time were merged. The PEAKS DB, PTM, and Spider function [20] were used to assign the MS/MS spectra to peptide sequences by matching the experimental spectra to theoretical spectra generated from the automatically annotated *G. m. morsitans* protein database available on VectorBase [21], supplemented with self-predicted annotations. The following search parameters were used: a precursor mass tolerance of 8 ppm using monoisotopic mass and a fragment mass tolerance of 15 mmu. The -10logP score was set for every sample to allow a maximal false-discovery rate of 5% for peptide spectrum matches. No digestion enzyme was selected. The following post-translational modifications (PTMs) were selected as variable modifications: oxidation (+15.99), acetylation (+42.01), amidation (–0.98), glycine-loss + amidation (–58.01), phosphorylation (+79.97), pyro-glu from E (–18.01), pyro-glu from Q (-17.03), sulfation (+79.96), sodium adduct (+21.98) with a maximum of three allowed variable PTMs per peptide.

Results

We prepared four brain (Br), three thoracic ganglion (TG), and two corpora cardiaca–corpora allata (CC-CA), anterior midgut (AM), and posterior midgut (PM) samples for analysis. The fragmented spectra yielded in total 2352 nonredundantly identified peptide sequences. Here, 788 unique sequences could be identified as peptides derived from 26 neuropeptide precursors (see Supplementary Data). Based on similarity with known neuropeptides of other insect species, 51 of these peptides were predicted to be bioactive neuropeptides (Table 1). The majority of the identified peptides in all samples belonged to common non-neuropeptide cytosolic proteins such as actin, tubulin, ribosomal proteins, histones, or heat shock proteins (Supplementary Data). Most of the neuropeptides were found in the tissues of the central nervous system (CNS). We found 45 mature neuropeptides in the brain and 46 in the thoracic ganglion. As far as we could detect, the tissues of CC-CA (7), AM (3), and PM (3) contained far less mature neuropeptides.

Typical peptide fragmentation spectra with their accompanying ion tables and error maps generated by PEAKS 7 are shown for CAP3/PK1, myosuppressin, orcokinin A, and TK2 (Figures 2, 3, 4 and 5). The spectrum alignments show how the fragment ions generated from the peptides correspond with the mass spectra. The accompanying ion tables display the calculated mass of possible fragment ions. If a fragment ion is found in the spectrum, its mass value is displayed in blue (b-ions) or red (y-ions). The error map shows the mass errors of the matched fragment ions. The fragmentation spectra of the other putative bioactive peptides can be found in the Supplementary Data (File S2).

Discussion

Tissue Distribution of Neuropeptide Sequences

By far the most neuropeptide sequences were found in the brain (467) and in the TG (501) (Supplementary Data). Apart from AKH I, which was only found in the CC-CA, all predicted bioactive neuropeptides were detected in the Br and/or TG tissues, supporting the notion that these are the main sites of neuropeptide synthesis. Other peptides found in the CC-CA belong to the AKH II, sNPF, myosuppressin, hugin, FMRFamide, corazonin, CRF/DH, or capa precursor proteins. In addition, the CC-CA was the only tissue in which a peptide from the ion transport peptide (ITP) precursor was detected. In the AM, we detected peptides from the sNPF, myosuppressin, hugin, capa, and AKH precursors. Samples from the PM contained peptides from AKH II, sNPF, PDF,

Table 1. Summary of Detected Neuropeptides in the Primary Neuropeptidergic Tissues

Precursor	NP	Sequence	Mass [M]	Br	TG	CC	AM	PM
AKH I	**AKH I**	**pQLTFSPGWa**	916.4443			xx		
		+26 identified peptides		xx	x	xx	x	
AKH II	**AKH II**	**pQLTFSPDWa**	974.4498			x		
		+35 identified peptides		xx		xx	x	xx
AstA	**AstA1**	**VERYAFGLa**	952.5130	x	x			
	AstA2	**GYTYTNGNGM**	1076.4233	x	x			
	AstA3	**LPVYNFGLa**	920.5120	xx	xx			x
	AstA4	**APYTFDLD**	940.4178	xxx	x			
	AstA5	**EPSEDVSHRASYPMRYGFGLa**	2296.0693	xxx	xx			x
		+18 identified peptides		xxx	x			x
AstB/MIP	**AstB2**	**AWKALNVAWa**	1056.5869	xxx	xx			
	AstB3	**RNAPTWNKFRGAWa**	1601.8131	xxx	xx			
	AstB4	**EPNWSNLKGMWa**	1359.6394	x	x			
	AstB5	**SKNWQKLQGAWa**	1343.7098	x	x			
		+19 identified peptides		xx	xx			
Ast C		2 identified peptides			x			
Capa/PK	**CAP1/PVK1**	**GGSSSSKLLAFPRIa**	1417.8042	xxx	xxx			
	CAP2/PVK2	**ASLRAFPRIa**	1028.6243		xxx			
	CAP3/PK1	**AGPSATTGVWFGPRLa**	1514.7993	xxx	xxx	xx	x	
		+30 identified peptides		xxx	xx	x		
CCAP		4 identified peptides		xxx	xx			x
Corazonin	**CRZ**	**pQTFQYSRGWTNa**	1368.6211	x		x		
CT/DH	**DH31**	**TVDFGLTRGYPGSAEARHRM GIASANSPEGPa**	3199.5581	xx	xx			
		+17 identified peptides			x			
CRF/DH		15 identified peptides		x	xx	x		
ETH		1 identified peptide	1782.0670	x				
FMRFa	**FMRFa1**	**KSLQENFMRFa**	1297.6602	xx	xx			
	FMRFa2	**GDNFMRFa**	884.3963		xx			
	FMRFa3	**VTDDFMRFa**	1028.475		xx			
	FMRFa4	**SNGGSVDFMRLa**	1180.5659	xxx	xxx			
	FMRFa5	**SGEDFMRFa**	986.4280	xxx	xx			
	FMRFa6	**NPGNQGFMRFa**	1165.5450	xx	xxx			
	FMRFa7	**SLGNQDFMRFa**	1212.5709	x	xx			
	FMRFa8	**NSDFMRFa**	914.4069	xx	xxx			
	FMRFa9	**SSSSPDFMRFa**	1158.5128	xx	xxx	x		
	FMRFa10	**SDNFMRFa**	914.4069	xx	xx			
	FMRFa11	**ASKENFMRFa**	1127.5546	x	xx			
		+140 identified peptides	1143.5494	xxx	xx			
Hugin	**PK2**	**SVPFKPRLa**	941.5811	xxx	xxx	xx	x	
		+15 identified peptides		xx	xx	x		
ITP		1 identified peptide	830.4399			x		
Leucokinin	**LK**	**NLVIMGKKQRFHSWGa**	1798.978		xx			
		1 identified peptide	2695.3757		xx			
Myosuppressin	**MS**	**SDVDHVFLFRa**	1232.6301	xxxx	xxx	xx	x	
		+10 identified peptides		xxx	x	x		
Natalisin	**NTL3**	**LSKTDINLLLKSMEPFTPTRa**	2302.2717	x	x			
		+1 identified peptide	2318.2668	xx	xx			
NPF		9 identified peptides		x	x			
NPLP1	**NPLP1-1**	**SVAALAAQGLLNHNH**	1514.7954	xxxx	xxx			
	NPLP1-2	**SLATLAKNGQLPTTDPNIIPDDEEERTED**	3181.526	xxx	xx			
	NPLP1-3	**YVGALARSGGLAGYa**	1352.7201	xxxx	xxx			
	NPLP1-4	**NIGTLARDYQLPQNa**	1600.8322	xxxx	xxx			
	NPLP1-5	**NLATMARLGMLGNRYPT**	1877.9604	xxx	xxx			
	NPLP1-6	**NLAALARYNSQRYYDMAE**	2148.0059	xxx	xx			
	NPLP1-7	**SLAALKASPVHGGGFQQ**	1665.8950	xxx	xxx			
	NPLP1-9	**HIGSVYRSGFLPSYRALRGFTGGGGGG GGGGDLKGRFSRSa**	3999.0476	xx	x			
		+340 identified peptides		xxxx	xxx			
Orcokinin	**OrcA**	**NFDEIDKTSASFSTLNQLL**	2142.0481	x	x			
PDF	**PDF**	**NSELINSLLSLPKNMNDAa**	1971.0095	xxx				x
		+8 identified peptides		xxx				x
Proctolin		1 identified peptide	1100.4563		x			
RYamide	**RYa-1**	**PSFYVGSRYa**	1073.5293	xxx	xx			
	RYa-2	**FSVVPRNDRFFLSSRYa**	1988.038	xxx	xx			
	RYa-2$_{7\text{-}17}$	**NDRFFLSSRYa**	1302.6470	xxx	xx			
		+6 identified peptides		xx	x			
SIFamide	**SIFamide**	**AYRKPPFNGSIFa**	1394.7458	xxxx	xx			
		+5 identified peptides		x	xx			

Table 1. (continued)

Precursor	NP	Sequence	Mass [M]	Br	TG	CC	AM	PM
sNPF	**sNPF1**	**AQRSPSLRLRFa**	1240.7153	x	x			
	sNPF2	**SPSLRLRFa**	973.5821	xxxx	xxx	x		
		+32 identified peptides		xxxx	xxx	xx	x	x
Tachykinin	**TK1**	**APSGFIGMRa**	933.4854	xx	xx			
	TK2	**APTAFYGVRa**	979.5239	xx	x			
	TK3	**APNGFTGVRa**	916.4879	xx	x			
	TK4	**AIKPFTGIDNNSFSVIRa**	1877.016	x				
		+19 identified peptides		xxx	xx			

Only sequences of the putative mature neuropeptides (NP) are shown. An N-terminal pyroglutamation and a C-terminal amidation are, respectively, indicated with "pQ" and "a". Additional identified peptides consist of spacer peptides, propeptides, and truncated peptides belonging to the neuropeptide precursors. Sequences for these peptides are shown in the supplementary data (Supplementary Table S1). Masses are given in Dalton (Da) and represent monoisotopic uncharged mass (M). Primary neuropeptidergic tissue: brain (Br), thoracic ganglion (TG), corpora cardiaca-corpora allata (CC), anterior midgut (AM), posterior midgut (PM). "x" represents the number of samples in which each peptide was detected. Note that the names of the NPs correspond to the order in which they appear in the precursor and do not necessarily correspond with their counterpart in other insects. AKH: adipokinetic hormone; Ast: allatostatin; MIP: myoinhibiting peptide; capa/PK: capability/pyrokinin; CCAP: crustacean cardioactive peptide; CT/DH: calcitonin-like diuretic hormone; CRF/DH: corticotropin releasing factor-like diuretic hormone; NPLP1: neuropeptide-like precursor 1; NPF: neuropeptide F; PDF: pigment dispersing factor; sNPF: short neuropeptide F

CCAP, and allatostatin type A precursors. In total, the CC-CA, AM, and PM contained far less neuropeptide-related peptides than the CNS (88, 12, and 8, respectively). This may in part be due to their relative size and smaller number of neuropeptidergic cells. In addition, the midgut samples were filtered twice because of their high protein content. All peptides referred to in the discussion can be found in the Supplementary Table S1. Sequences of the neuropeptide precursors are shown in the Supplementary Data (File S3).

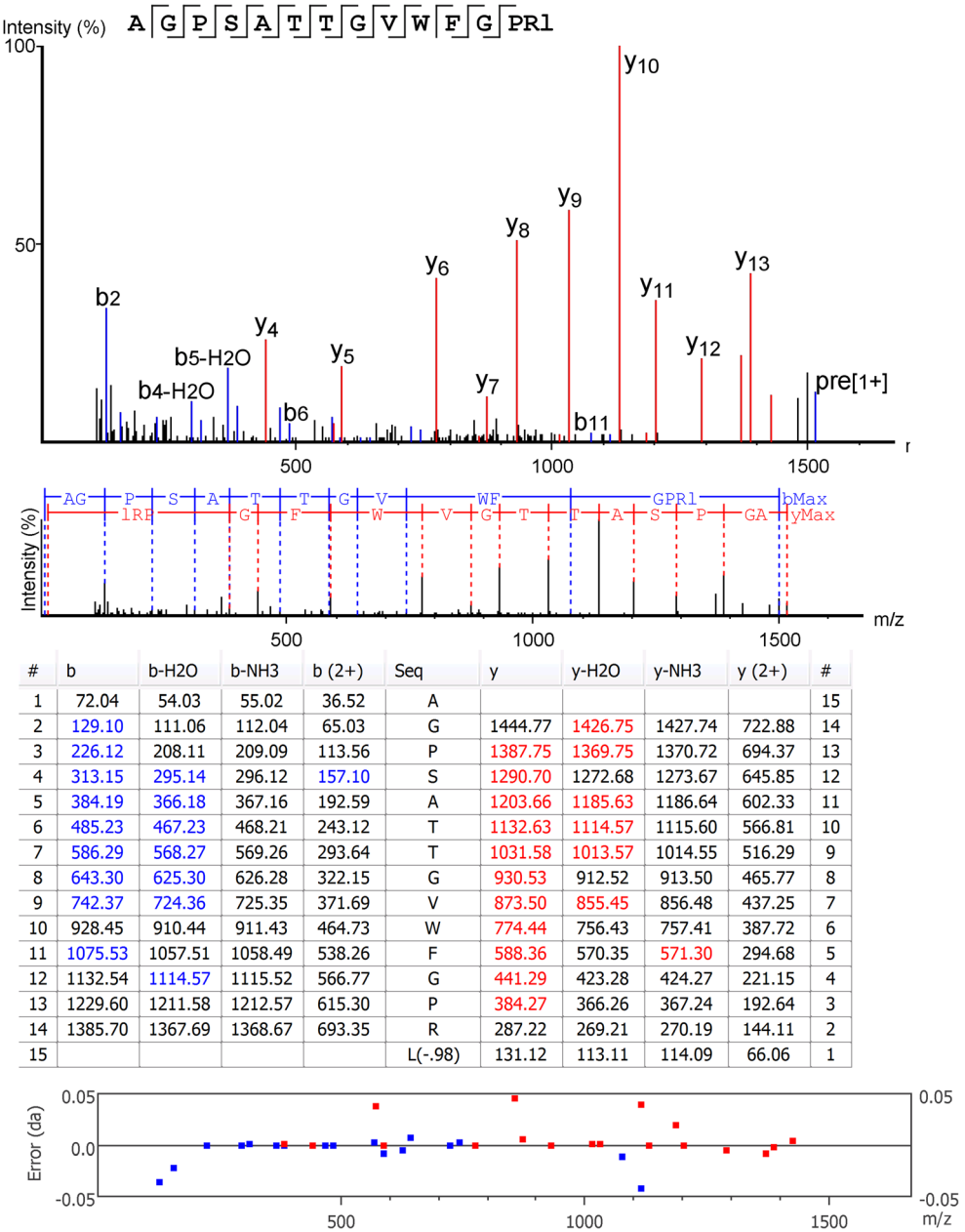
Distribution of Detected Neuropeptides

Adipokinetic Hormones Adipokinetic hormones are mainly involved in the mobilization of fuels (lipids, carbohydrates, or proline) from the fat body during energy-requiring processes. The CC neurohemal glands most likely function as the site for AKH synthesis and storage, similar to what has been described for all other insects studied so far [22]. This is also in agreement with an immunohistochemical study that demonstrates the presence of AKH peptides in the tsetse CC [23]. Two *akh* genes are predicted in the tsetse fly [1, 24]. One of these genes encodes a peptide, designated AKH II (pQLTFSPDWa), which is identical to AKH in other dipterans, including *Drosophila* [25, 26]. As it is also identical to the hypertrehalosemic hormone (HrTH) of the blowfly *Protophormia terraenovae* [27], it is sometimes called *Gmm*-HrTH. However, recently this peptide was shown to increase lipid mobilization in tsetse flies [23]. The second gene encodes AKH I, or *Gmm*-AKH (pQLTFSPGWa), a sequence that differs from any known insect AKH [27].

Mature AKH I and AKH II were found only in the CC-CA samples. Incompletely processed AKH I peptides containing the C-terminal -GK or -GKR were also detected in the CNS and AM, whereas peptides from AKH II were found in the brain and both the AM and PM (Supplementary Table S1). In accordance with this finding, it has been shown that AKH peptides are present in the brain and in neuronal projections innervating the midgut in mosquitoes [24, 28]. In several peptidomics studies on *D. melanogaster*, the signal of mature AKH was very weak [29, 30], whereas intermediate AKH peptides were clearly detected [31]. The same was found in the Australian sheep blowfly *Lucilia cuprina*, and the anautogenous flesh fly *Sarcophaga crassipalpis* [25]. This may be due to the more efficient fragmentation of peptides with additional C-terminal basic amino acids, indicating that propeptides, even though they occur in lower concentrations, have higher quality MS/MS spectra and are more easily identified [32].

Allatostatins Three types of allatostatins exist in invertebrates. Although four allatostatin genes (AstA, AstB, AstC, and the AstC paralog AstCC) are predicted in the genome of the tsetse fly [1], only peptides encoded by the first three genes were detected in our experimental setup. AstA and C peptides are inhibitors of juvenile hormone biosynthesis in cockroaches, crickets, and termites. In addition, they exhibit myoinhibitory properties in most insects [33, 34]. Five AstA neuropeptides are predicted (AstA1-5) corresponding to Drostatin-1, -5, -2, -3, and -4, respectively. All five predicted mature AstA neuropeptides were identified in the brain and TG. The PM contained two of the predicted neuropeptides. The presence of AstA peptides in the CNS and midgut has previously been reported in other dipterans including *D. melanogaster* [33, 35–37], the cabbage root fly *Delia radicum* larvae, the midge *Chironomus riparius*, and mosquitoes *Aedes aegypti* and *Anopheles albimanus* [36, 38–41].

It is important to note that the ortholog of Drostatin-3 (SRPYSFGL) in tsetse flies (AstA4: APYTFDLD) lost the characteristic Y/FXFGL/Ia C-terminus of most AstA peptides. In addition, an amidated spacer peptide (SGYNRLDDELLAKQELDKAFNTATMLa) located between AstA4 and AstA5 in the prepropeptide (File S3) was also found in the brain, TG, and PM. Since both these peptides lack the characteristic AstA C-terminus, it remains to be seen whether they can activate a G protein-coupled receptor.



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.04	54.03	55.02	36.52	A					15
2	129.10	111.06	112.04	65.03	G	1444.77	1426.75	1427.74	722.88	14
3	226.12	208.11	209.09	113.56	P	1387.75	1369.75	1370.72	694.37	13
4	313.15	295.14	296.12	157.10	S	1290.70	1272.68	1273.67	645.85	12
5	384.19	366.18	367.16	192.59	A	1203.66	1185.63	1186.64	602.33	11
6	485.23	467.23	468.21	243.12	T	1132.63	1114.57	1115.60	566.81	10
7	586.29	568.27	569.26	293.64	T	1031.58	1013.57	1014.55	516.29	9
8	643.30	625.30	626.28	322.15	G	930.53	912.52	913.50	465.77	8
9	742.37	724.36	725.35	371.69	V	873.50	855.45	856.48	437.25	7
10	928.45	910.44	911.43	464.73	W	774.44	756.43	757.41	387.72	6
11	1075.53	1057.51	1058.49	538.26	F	588.36	570.35	571.30	294.68	5
12	1132.54	1114.57	1115.52	566.77	G	441.29	423.28	424.27	221.15	4
13	1229.60	1211.58	1212.57	615.30	P	384.27	366.26	367.24	192.64	3
14	1385.70	1367.69	1368.67	693.35	R	287.22	269.21	270.19	144.11	2
15					L(-.98)	131.12	113.11	114.09	66.06	1

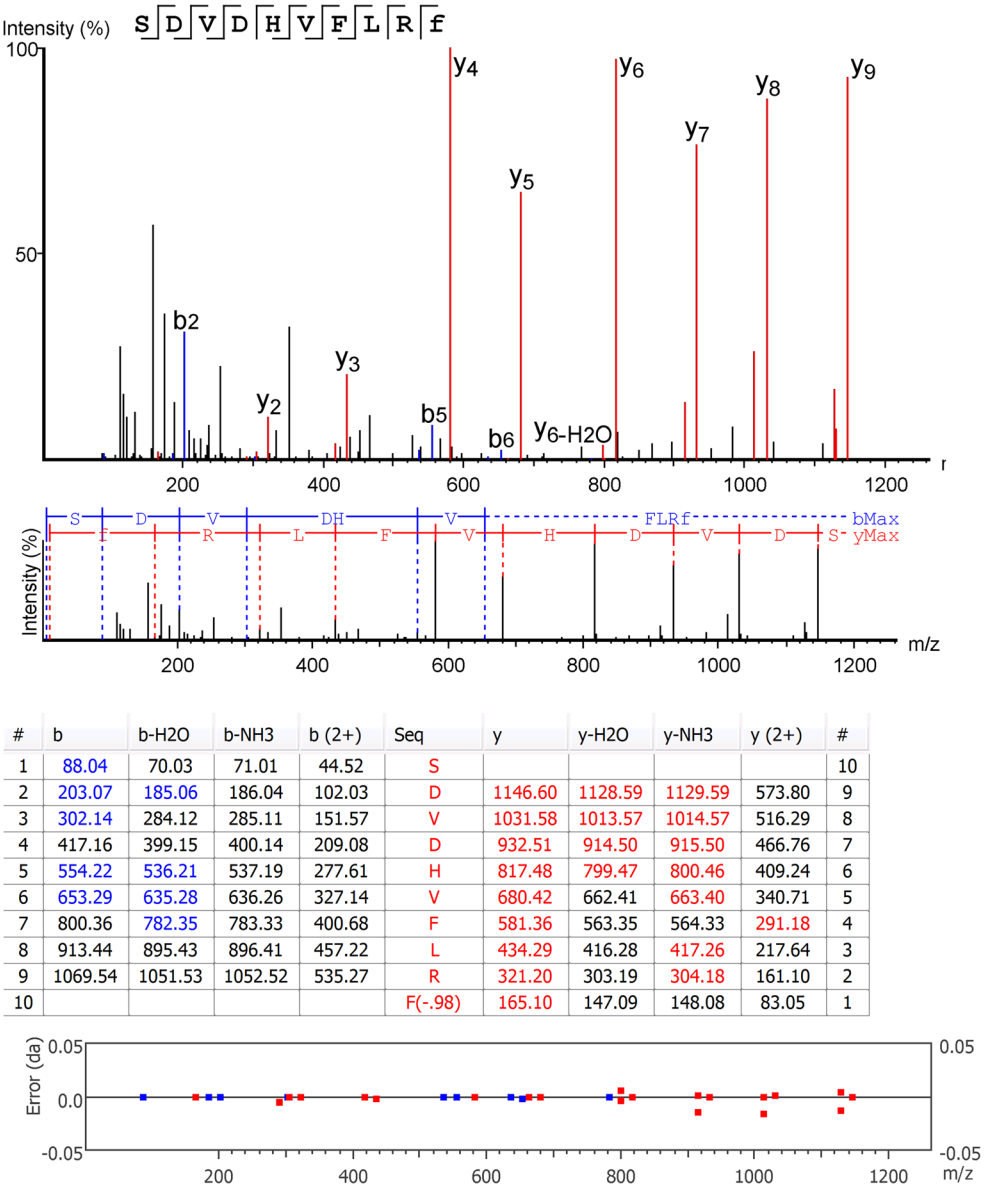
Figure 2. Fragmentation spectrum, ion table, and error map of CAP3/PK1. Data was obtained from a brain extract of *G. m. morsitans* using PEAKS 7 software. N-terminal ions (b-ions) are shown in blue, C-terminal ions (y-ions) in red. The (–.98) indicates a C-terminal amidation. AAs with PTMs are written in lowercase

In crickets, AstBs are known for their allatostatic effect, whereas in the silkworm they inhibit ecdysteroid synthesis by the prothoracic gland. However, in most insects studied so far, they act by reducing muscle contractions (reviewed by [34]). AstBs have a characteristic WX_6Wa motif at their C-terminal end. Four of the five predicted AstB peptides, also known as myoinhibiting peptides (MIPs), were detected in the CNS of the tsetse fly. In contrast to *Drosophila* and *Aedes* [36, 39], but similar to *D. radicum* [41], we did not detect MIPs in the midgut. AstB2 was wrongly predicted in the genome. Although we could not detect the (uncorrected or) corrected sequence in our analyses, we did detect the accurate mass of the corrected sequence (including those of truncated versions lacking the amidation and/or N-terminal Arg) and a six amino acid long tag (PTWNKF).

Two peptides originating from the AstC precursor were detected in the TG. Localization experiments by immunohistochemistry indicated the presence of AstC in neuronal cells in the brain with projections to the thoracic and abdominal ganglia in mosquitoes [38].

Capability Peptides – Hugin

Capa-derived neuropeptides include the periviscerokinins (PVKs), characterized by a carboxy-terminal FPRI/Va motif,



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	88.04	70.03	71.01	44.52	S					10
2	203.07	185.06	186.04	102.03	D	1146.60	1128.59	1129.59	573.80	9
3	302.14	284.12	285.11	151.57	V	1031.58	1013.57	1014.57	516.29	8
4	417.16	399.15	400.14	209.08	D	932.51	914.50	915.50	466.76	7
5	554.22	536.21	537.19	277.61	H	817.48	799.47	800.46	409.24	6
6	653.29	635.28	636.26	327.14	V	680.42	662.41	663.40	340.71	5
7	800.36	782.35	783.33	400.68	F	581.36	563.35	564.33	291.18	4
8	913.44	895.43	896.41	457.22	L	434.29	416.28	417.26	217.64	3
9	1069.54	1051.53	1052.52	535.27	R	321.20	303.19	304.18	161.10	2
10					F(-.98)	165.10	147.09	148.08	83.05	1

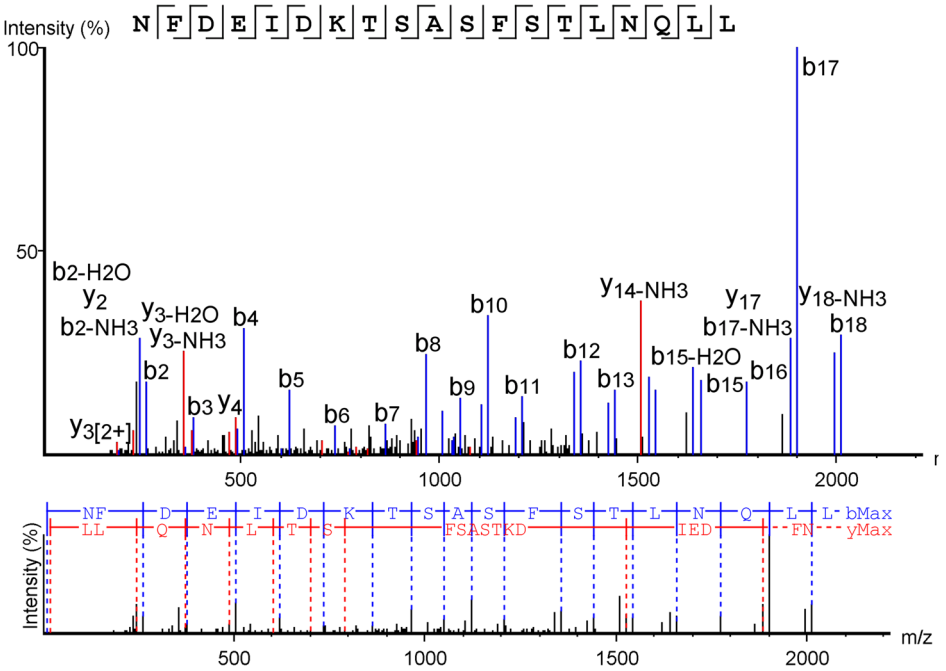
Figure 3. Fragmentation spectrum, ion table, and error map of myosuppressin. Data was obtained from a brain extract of *G. m. morsitans* using PEAKS 7 software. N-terminal ions (b-ions) are shown in blue, C-terminal ions (y-ions) in red. The (–.98) indicates a C-terminal amidation. AAs with PTMs are written in lowercase

and usually one pyrokinin (PK), with a conserved WFXPRL/Va motif. PVKs are mainly involved in myomodulatory and osmoregulatory processes (reviewed in [42, 43]). Knowledge on the function of the capa-derived pyrokinin peptide is scarce. We detected all three predicted neuropeptides of the capa prepropeptide precursor in the TG. Whereas the capa-derived pyrokinin peptide was identified widespread in the nervous system (brain, TG, CC-CA) and AM, periviscerokinins displayed a more restricted distribution pattern (brain and TG). Previous peptidomic studies in dipteran insects did reveal the presence of capa peptides in the CNS, CC [36, 39, 41] and the midgut [39].

Hugin-derived pyrokinin neuropeptides have roles in various biological processes, including pupariation [44], diapause, pheromone biosynthesis, regulation of gustation and feeding behavior [45, 46], muscle contraction [47, 48], and cuticular melanization. The *hugin* gene also harbors a second (predicted) neuropeptide, hug-γ, for which a function in ecdysis was suggested [47]. Although we predicted two hugin-derived neuropeptides in the tsetse fly genome (corresponding to hug-γ and PK2), we could only detect PK2 in the brain, TG, CC, and AM. This is similar to the situation in *Drosophila*, where hug-γ was never detected by means of mass spectrometry [29, 49]. *Hugin* was found to be expressed in 20 cells in the sub-esophageal ganglion, with projections to the pharynx, CC, and ventral nerve cord [45, 50].

Crustacean Cardioactive Peptides – Corazonin – Ecdysis Triggering Hormone

CCAP is a neuropeptide that was initially discovered in the shore crab *Carcinus maenas* for its ability to raise the heart rate



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	115.05	97.04	98.02	58.03	N					19
2	262.12	244.09	245.08	131.56	F	2029.01	2011.00	2011.97	1015.01	18
3	377.15	359.13	360.12	189.09	D	1881.93	1863.93	1864.92	941.43	17
4	506.19	488.18	489.16	253.59	E	1766.92	1748.91	1749.89	883.96	16
5	619.27	601.28	602.26	310.14	I	1637.87	1619.86	1620.85	819.39	15
6	734.29	716.29	717.27	367.65	D	1524.80	1506.78	1507.77	762.90	14
7	862.40	844.38	845.37	431.70	K	1409.76	1391.75	1392.74	705.38	13
8	963.44	945.44	946.43	482.22	T	1281.67	1263.66	1264.64	641.33	12
9	1050.48	1032.44	1033.46	525.74	S	1180.62	1162.61	1163.59	590.81	11
10	1121.51	1103.50	1104.49	561.26	A	1093.59	1075.58	1076.53	547.29	10
11	1208.54	1190.55	1191.52	604.77	S	1022.55	1004.54	1005.52	511.78	9
12	1355.61	1337.60	1338.58	678.31	F	935.52	917.51	918.49	468.26	8
13	1442.64	1424.65	1425.64	721.82	S	788.45	770.43	771.42	394.73	7
14	1543.70	1525.68	1526.70	772.36	T	701.41	683.41	684.39	351.21	6
15	1656.79	1638.76	1639.75	828.89	L	600.38	582.36	583.34	300.69	5
16	1770.83	1752.81	1753.79	885.91	N	487.29	469.28	470.26	244.14	4
17	1898.88	1880.87	1881.85	949.94	Q	373.24	355.23	356.19	187.14	3
18	2011.97	1993.95	1994.94	1006.48	L	245.19	227.14	228.16	123.09	2
19					L	132.10	114.09	115.07	66.55	1

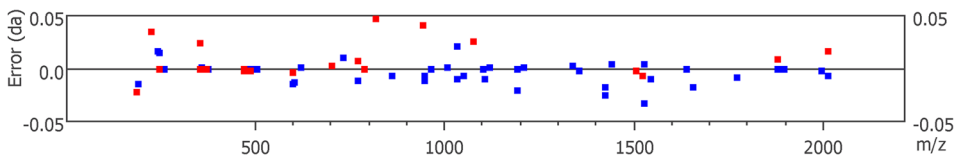
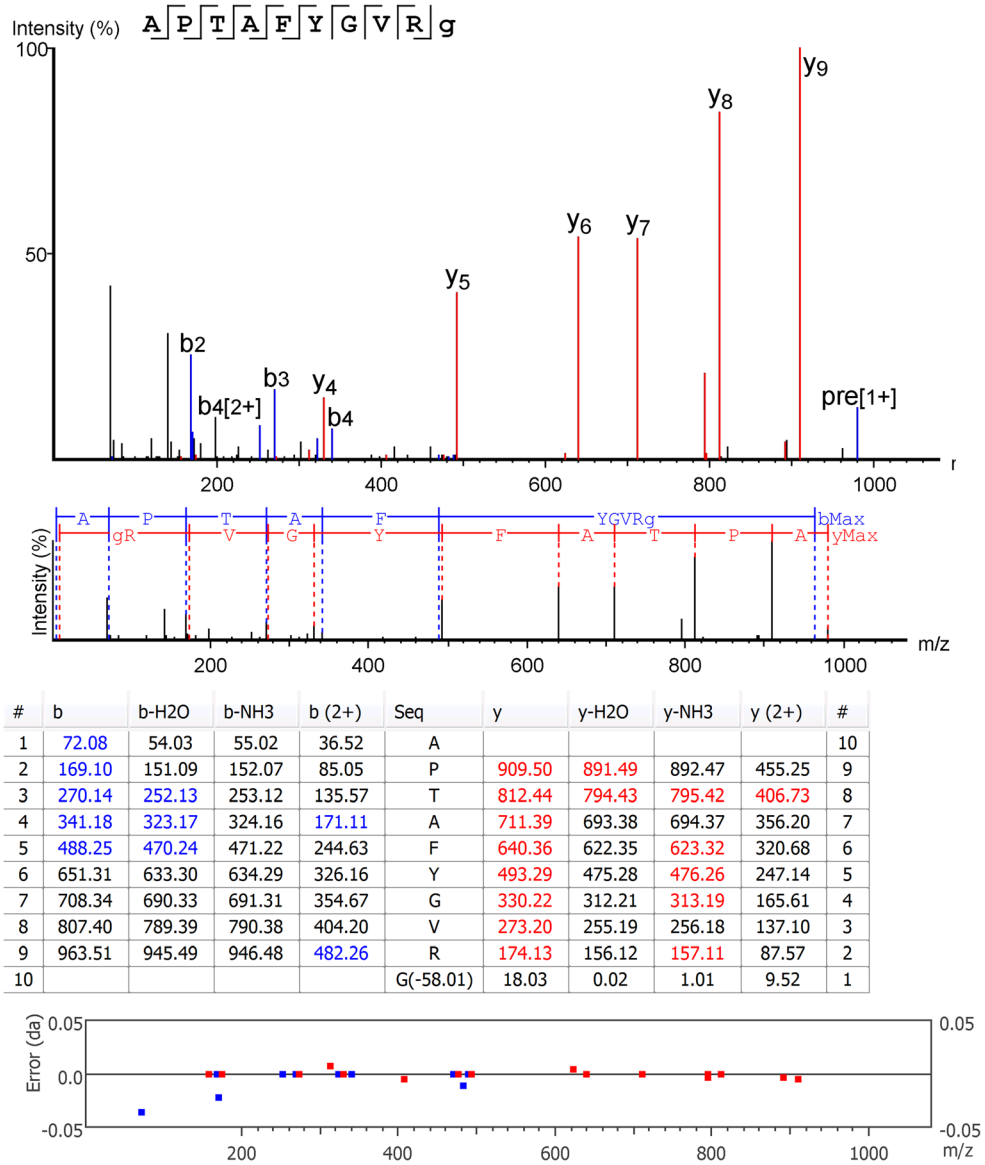


Figure 4. Fragmentation spectrum, ion table and error map of orcokinin A. Data was obtained from a brain extract of *G. m. morsitans* using PEAKS 7 software. N-terminal ions (b-ions) are shown in blue, C-terminal ions (y-ions) in red

[51]. CCAP is highly conserved within the arthropod phylum, being identical in most species [52]. It is a pleiotropic neuropeptide, mediating numerous processes. Besides promoting cardiac muscle contractions, it is involved in initiating ecdysis, stimulating digestive secretions, and stimulating visceral muscles [53–56]. Our study failed to detect the mature PFCNAFTGCa, but did show the presence of CCAP-precursor-derived spacer peptides in the CNS and PM. Peculiarly, in adult *Aedes* mosquitoes, the presence of CCAP has recently been shown by immunological methods in the brain and CC, but not in the PM [52].

Corazonin (Crz) was found in the CC-CA and the brain. This corresponds to findings in *D. melanogaster* where Crz is expressed in neurons in the brain that send projections to the CC and CA [57]. The expression pattern is very similar in other dipterans, such as *Musca domestica* and *P. terraenovae* [58, 59]. Crz was first identified as a cardioaccelerator in *P. americana* [60], but progressive research proved its



#	b	b-H2O	b-NH3	b (2+)	Seq	y	y-H2O	y-NH3	y (2+)	#
1	72.08	54.03	55.02	36.52	A					10
2	169.10	151.09	152.07	85.05	P	909.50	891.49	892.47	455.25	9
3	270.14	252.13	253.12	135.57	T	812.44	794.43	795.42	406.73	8
4	341.18	323.17	324.16	171.11	A	711.39	693.38	694.37	356.20	7
5	488.25	470.24	471.22	244.63	F	640.36	622.35	623.32	320.68	6
6	651.31	633.30	634.29	326.16	Y	493.29	475.28	476.26	247.14	5
7	708.34	690.33	691.31	354.67	G	330.22	312.21	313.19	165.61	4
8	807.40	789.39	790.38	404.20	V	273.20	255.19	256.18	137.10	3
9	963.51	945.49	946.48	482.26	R	174.13	156.12	157.11	87.57	2
10					G(-58.01)	18.03	0.02	1.01	9.52	1

Figure 5. Fragmentation spectrum, ion table and error map of tachykinin TK2. Data was obtained from a brain extract of *G. m. morsitans* using PEAKS 7 software. N-terminal ions (b-ions) are shown in blue, C-terminal ions (y-ions) in red. G(–58.01) indicates the loss of glycine, plus a C-terminal amidation. AAs with PTMs are written in lowercase

pleiotropic nature, which also includes a role in ecdysis (reviewed in [61]). In conjunction with corazonin and CCAP, two other neuropeptides, the eclosion hormone (EH) and the ecdysis triggering hormone (ETH), are crucial in the onset of pre-ecdysis behavior [62]. ETH is released from the Inka cells of the trachea upon stimulation by EH and/or Crz [63], and acts on the CNS. While we could not detect EH in tsetse fly tissues, we could detect the presence of a single truncated ETH peptide ($ETH_{3\text{-}17}$) in the brain (Supplementary Data).

Diuretic Hormones – Ion Transport Peptide

Diuretic hormones (DHs) and ITP regulate the salt and water balance in insects. Although other DHs are present in insects, the two most studied are calcitonin-like and corticotropin releasing factor-like diuretic hormone (CT/DH and CRF/DH), which both stimulate the secretion of water in the Malpighian tubules (reviewed in [64, 65]). Besides diuresis, they appear to have roles in feeding behavior [66, 67]. We detected DH peptides in the TG and the brain. We found the mature neuropeptide of CT/DH, but for CRF/DH only detected spacer peptides. Taking into account the nutritional status of the flies upon dissection, it is not surprising that little DH was found. As these flies have to quickly lose water after a blood meal, the expression of these hormones is expected to be high shortly after feeding. In contrast, 3 days after a meal (as with our flies), the flies preferably need to retain water, and expression of CT/DH and CRF/DH is expected to be low. Immunologic studies in *R. prolixus* showed the presence of CT/DH in neurons in the brain and TG, which project to the hindgut and salivary glands, and cells in the midgut (reviewed in [65]). Detection of CRF/

DH showed a similar pattern. However, no reactivity was found in the salivary glands while staining was present in the CC [68].

In addition, we identified one peptide belonging to the ITP precursor in the CC-CA (see Supplementary Data). ITP is involved in ion and fluid transport over the ileum, and recently has been shown to have a role in the regulation of circadian rhythm [69]. While ITP was initially isolated from CC extracts of locusts [70], it has so far not been reported to occur in CCs of dipterans. Transcripts of ITP in insects are generally found in the brain (4–5 pair of cells), the terminal abdominal ganglion and sometimes in the midgut [71].

FMRFamides – Leucokinin – Proctolin

Remarkably, little is known about the functions of FMRFamides. Some FMRFamides stimulate heart and gut contractions, but other roles have been observed (reviewed by [72]). All 11 predicted neuropeptides from the same FMRFamide protein precursor are detected in the TG. In the brain, almost no pro- or spacer peptides were found, whereas nine of the 11 FMRFa neuropeptides were present. Only one FMRFa neuropeptide (SSSSPDFMRFa) was detected in the CC-CA.

Leucokinins were initially discovered as myostimulatory peptides [73]. Later, members of this family were found to have a role in gustatory and olfactory perception [74] and feeding behavior [75], and to act as ion transport regulators [76]. *Drosophila* leucokinin has been shown to be present in the brain and ventral ganglia [75]. We only detected two peptides in the tsetse fly, both in the TG, one being the predicted mature leucokinin (NLVIMGKKQRFHSWGa).

As a myotropic neuropeptide, proctolin has been implicated in feeding behavior, digestion, heart rate regulation, egg laying, and sexual behavior [77–79]. As it is found in neurosecretory cells as well as in the hemolymph in certain insects, it is believed that it can both act as a neuromodulator/co-transmitter and a circulating hormone (reviewed in [80]). As proctolin-expressing neurons innervate a large number of tissues including CC, gut, and heart, we expected to find it in most of our samples. However, we could only detect a spacer peptide in the TG.

Myosuppressin

Myosuppressin is a potent inhibitor of muscle contractions and also inhibits food uptake in several insects (reviewed in [81]). Its presence has been shown in the brain, neurohemal organs, stomatogastric nervous system, and by immunologic means also in midgut endocrine cells [81]. In accordance, we identified the predicted mature neuropeptide in all tissues except the PM.

Natalisin – Orcokinin

Orcokinin and natalisin are two recently discovered arthropod-specific neuropeptides, which were not predicted in the genome analysis study of the tsetse fly [1]. The natalisin protein precursor, thus named as it increases fecundity and sexual activity in several insects [82], contains five putative neuropeptides in *Drosophila*. We identified a natalisin gene in the genome of tsetse flies (GMOY006483) encoding three putative neuropeptides, but could only detect one, NTL3, in the brain and TG.

Insect orcokinin neuropeptides occur in two distinct forms depending on alternative splicing. Orcokinin-A is involved in circadian behavior [83] and ecdysteroidogenesis [84], whereas for orcokinin-B, which has only recently been identified, no functions have been described hitherto [85]. In *G. m. morsitans* we could only detect orcokinin A (originating from gene GMOY009230) in both the brain and TG.

Neuropeptide F

Like its mammalian counterpart neuropeptide Y, NPF is primarily known for its role in feeding behavior [86], but is also involved in several other behavioral processes including mating behavior-associated activity [87–89], sleep [90], aggression [91], and ethanol sensitivity [92]. Dipteran NPF expression is observed in larval and adult brain tissue and endocrine cells in the midgut [93, 94]. We could not detect mature NPF in the brain or midgut. Only the C-terminal peptide was detected in the CNS, indicating that NPF processing likely occurs in these tissues.

Neuropeptide-Like Precursor 1

Neuropeptide-like precursor 1 (NPLP1) is conserved in various insects and contains multiple mono- and dibasic cleavage sites for the processing of several peptides, either bioactive neuropeptides or spacer peptides [29, 30, 95, 96]. Currently, a functional role for only one NPLP-1 derived peptide has been elucidated. NPLP1-VQQ is an activating ligand of a guanylate cyclase receptor, involved in environmental stress responses [97]. *Drosophila* NPLP1 peptides only occur in the CNS as determined by mass spectrometry and immunostainings [98]. Here, we detected seven of the nine predicted mature neuropeptides in the brain and TG. NPLP1-7, the putative analog of NPLP1-VQQ, was detected in the brain only, whereas a mature NPLP1-8 was not detected.

Pigment Dispersing Factor

PDF is the insect homolog of the crustacean pigment-dispersing hormone. It is well known as an important regulator of the insect circadian clock and has been associated with several other processes like activity, reproduction, and geotactic behavior (reviewed in [99]). More recently, PDF has also been associated with ecdysteroid biosynthesis, pheromone production and mating behavior [100, 101]. The PDF neuropeptide occurs in tsetse brain and PM samples. This is in accordance with *Drosophila* where besides expression in certain brain neurons, PDF is also found in neurons innervating the most caudal part of the midgut and hindgut [99].

RYamide

In 2010, RYamides were discovered in the parasitic wasp *Nasonia vitripennis* and their genes have since been found in the genome of most species [102, 103]. Initial studies suggest a role in feeding behavior and/or digestion, a hypothesis strengthened by the expression pattern of its receptor, which mainly occurs in the hindgut [104, 105]. The RYamide gene in *G. m. morsitans* contains at least two RYamide neuropeptides, both of which we were able to detect in the brain and TG.

SIFamide

SIFamide was abundantly found in the tsetse fly CNS. It is an extremely well conserved neuropeptide in arthropods and identical in all dipteran species [25, 41, 106]. It was initially discovered in the flesh fly *Neobellieria bullata* [106], and proved to be a potent stimulator of oviduct contractions in locusts. In *Drosophila*, SIFamides have a role in courtship behavior [107] and sleep [108].

Short Neuropeptide F

The influence of sNPF on feeding behavior and growth is well described [109–111], but numerous other roles have been reported [86, 112]. We detected short NPF-2 (SPSLRLRFa), one of the four predicted sNPF neuropeptides, in the brain, TG, and CC-CA. Short NPF-1 (AQRSPSLRLRFa) was found in brain and TG samples. It has a (nearly) identical sequence in all dipterans [25, 41, 86]. In addition, a few spacer peptides were also detected, one of which (NDPELIRQLPI) was found in every tissue studied, illustrating its wide distribution. Short NPFs have previously been localized in a large number of neurons and endocrine cells in various insects (reviewed in [86]).

Tachykinin

Diverse roles have been described for insect tachykinin neuropeptides (reviewed in [113]). Besides their myotropic activity, they also regulate aggression [114, 115], act as modulators of olfactory perception and locomotion [116, 117], and inhibit insulin signaling in the brain [118]. Tachykinins are expressed in the CNS and endocrine cells of the midgut [114, 119, 120]. Three tachykinin neuropeptides containing the C-terminal -FX_1GX_2R-NH_2 consensus sequence were predicted in *G. m. morsitans,* and we detected all three in the brain and TG. We did not, however, detect any peptides or spacers in the midgut samples. In addition, a fourth atypical tachykinin was identified in brain tissues (AIKPFTGIDNNSFSVIRa), which contains a Val instead of the canonical Gly in the C-terminal core sequence.

Absence of Other Neuropeptides

Besides the neuropeptides described here, additional neuropeptides are predicted in the genome of the tsetse fly [1]. The following peptides were predicted but not detected in our mass spectrometric analysis: bursicon-α and -β, CCHamide-1 and -2, eclosion hormone (EH), glycoprotein hormone-α2 and –β5 (GPA2 and GPB5), insulin-like peptides 1–3 and 7 (ILP1–3,7), prothoracicotropic hormone (PTTH), sulfakinin and trissin. While we could predict a sulfakinin peptide in the genome (CGEELFDDYGHMRFa), we believe this belongs to a pseudogene as a complete gene could not be predicted and no receptor was found in any of the *Glossina* genomes (unpublished data). In addition, AstCC (AYWRCYFNPVSCF; in contig CTG10007078), and a CNMamide (YLTPCHFKICNMa; partially present in GMOY000823), were predicted by genome analysis, but we could not detect these peptides in any of the samples under investigation. The absence of (neuro)peptides in our peptidomics studies may have several reasons. Neuropeptides can be expressed in a large set of neurons or in a single neuron, and they may be abundantly produced or synthesized in trace levels, which could impede their detection. The use of a nanoscale liquid chromatography column already diminishes this problem because of the small elution volumes, which improves the detection limit and thus sensitivity. Second, only tissues of adult female flies were tested in the present peptidomics study. Therefore, neuropeptides that are mainly produced in embryonic or larval stages or in different environmental, developmental, or physiological conditions (e.g., stress, satiety, molting, or sleep) or specifically in males (e.g., sex peptide) are absent in the studied samples. Owing to the presence of high amounts of blood in freshly fed flies, especially in the midgut, we opted to only dissect flies 3 days after their last blood meal, which implies that digestive or satiety related peptides might be absent as well. Third, not all neuropeptides are extracted with equal efficiency by the extraction procedure used in our analysis. Larger neuropeptides/-proteins such as glycoprotein hormone-α and -β, for instance, likely precipitate during acid methanol extraction. It is well known that peptides larger than 3–4 kDa fragment inefficiently. To overcome these problems, the group of L. Li optimized elegant protocols that allow them to cover the mass range of 0.5 to 10 kDa. Peptides with masses lower than 2.5 kDa are analyzed directly by LC-MS/MS (top down), whereas larger peptides are cleaved with trypsin or Lys-C (bottom-up) prior to MS analysis [121, 122]. In future experiments, implementing this technique might yield even more identified peptides. Fourth, peptides do not have the same ionization and fragmentation efficiency. This may explain why for the same precursor protein, we could detect incompletely processed peptides ending in KR, K, or R, a feature that generates predictable fragmentation patterns. Finally, there is no guarantee that the predicted prepropeptide genes are expressed at all in the examined tissues. In addition, the genome of *Glossina* is not yet perfectly annotated, so putative neuropeptides could be missed in our analysis because of mis- or incomplete assemblies. As spacer peptides are more difficult to predict because of their low(er) conservation, they are even more often overlooked.

It should be noted that the PEAKS 7 software is developed for combining de novo sequencing with database searches.

This is beneficial since (neuro)peptides from live animals are derived from prepropeptides that are cleaved by various types of proteases in vivo, instead of a controlled in vitro digestion (e.g., tryptic digest) of a protein sample with predictable cleavage sites, and thus cannot be predicted accurately. Therefore, no protease specification can be indicated, leading to a huge search space and low scores if only a database search is used. Another difficulty associated with neuropeptide MS analyses arises from the frequently occurring PTMs, which need to be taken into account when performing a database search. The Peaks PTM module, in combination with high accuracy and high resolution MS, handles this problem well compared with database search engines, using de novo approaches for PTM identification. Even better rates of peptide identifications and more accurate genome-wide searches including post-translational modifications can be achieved by combining two fragmentation methods, instead of one, to get more structural information [123].

Conclusion

In the past 15 years, mass spectrometry has become the method of choice for detecting and analyzing native (neuro)peptides in complex biological samples. The UHPLC-Q Exactive Orbitrap setup used in these experiments presents an improvement on earlier peptidomics studies using older hyphenated mass spectrometry systems. Where in the past our and other groups had to resort to high dimensional separations of tissue extracts, such as the combination of ion exchange and reversed phase chromatography to fractionate samples before MS analysis, we can now analyze more complex samples in a shorter time period. Thanks to the higher sensitivity and broader dynamic range, we could detect an extensive amount of peptides in several different tissues relatively fast without prior fractionation. This method is complementary to direct tissue profiling using MALDI-TOF, which is also capable of detecting (and simultaneously localizing) neuropeptides, although sequence confirmation is harder to obtain. In the near future, even more sensitive equipment and software enhancements will undoubtedly lead to even smaller tissue samples needed, paving the way for organ-, tissue-, and cell-specific peptidome detections. Higher spatial resolution in peptidomics experiments results in more complete peptidomic analyses since the signal-to-noise ratio is increased and will, therefore, allow us to detect lesser abundant neuropeptides and growth factors. In addition, the higher mass accuracy and resolution of current mass spectrometers allows us to use stricter parameters for database searches and de novo sequencing, resulting in higher scores, longer peptide sequence tags, and ultimately leading to highly reliable results. Owing to these and ongoing improvements to mass spectrometric equipment and tools, peptidomics is currently close to being truly genome-wide and, together with the increased localization information, will deliver more physiologically relevant information.

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