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Functional Proteomics of Neuropeptidome Dynamics during the Feeding Process of *Rhodnius prolixus*

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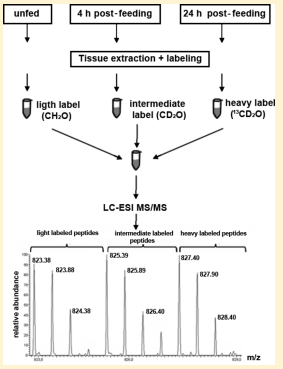
ABSTRACT: In hematophagous insects, blood intake triggers a prompt response mediated by neuropeptides, which regulates a variety of physiological processes. Here we report a quantitative proteomic analysis of the postfeeding response in the central nervous system of *Rhodnius prolixus*, a vector of Chagas disease. The concentration of neuropeptides NVP-like, ITG-like, kinin-precursor peptide, and neuropeptide-like precursor 1 (NPLP1) significantly changes in response to blood intake. We also performed a neuropeptidomic analysis of other feeding-related organs, namely salivary glands and gut. We identified NPLP1 in salivary glands and myosuppressin in midgut. This is the first report suggesting a role for NPLP1, involving the peptides processed from this precursor in the hormonal control of the production and/or release of saliva. Our results contribute to the understanding of the postprandial neuroendocrine response in hematophagous and provide important information for physiological and pharmacological studies aimed to the design of next-generation insecticides such as peptidomimetics.



KEYWORDS: quantitative proteomics, peptidomics, *Rhodnius prolixus*, Chagas disease, neuropeptides, feeding

INTRODUCTION

Rhodnius prolixus is one of the vectors of *Trypanosoma cruzi*, the protozoan that causes Chagas disease. The disease is an important but still neglected tropical disease that affects 10 million people in Central and South America (http://www.who.int/mediacentre/factsheets/fs340/en/). Due to the lack of vaccines and effective treatments for the chronic stage of the disease, the constraint of vector populations is crucial for disease control; this has been achieved by the use of insecticides. However, the use of conventional insecticides in the endemic areas has resulted in the rise of resistant populations,[1] and in consequence, new strategies need to be devised. The study of the neuropeptidome is a promising approach to discover new targets and design novel insecticides of improved selectivity and environmentally safe.[2] The characterization of the complete set of neuropeptides in different tissues and its dynamics in response to changes in the environment or in the physiology of the insect will provide fundamental information required to develop peptidomimetics, pseudopeptides or small molecules capable of disrupting physiological processes, to replace and/or complement conventional neurotoxic insecticides.

In hematophagous insects, feeding is a key process that triggers a series of physiological stimuli, which, in the case of *R. prolixus*, are closely related to the transmission of the disease. Feeding stimulates a prompt and profuse diuresis to maintain the water and ion equilibrium after the intake of large volume of liquid, a process that slows down 3−4 h after the blood intake.[3] Feeding also triggers a long-term neuroendocrine response, which results in growth, molting, and reproduction. Therefore, we can distinguish two steps in postfeeding control: the immediate one—diuresis—and the delayed ultimate—growth and reproduction. Although they are both regulated by the neuroendocrine system, only a few pieces of the signaling complex are known, but the overall network of regulatory signals involved is not accurately known yet. The genome sequence of *R. prolixus* and the technical advances in peptidomics have allowed a comprehensive characterization of neuropeptide precursor genes in this vector.[4]

The salivary glands of hematophagous arthropods are also a fundamental player in the initial feeding steps and contain many active molecules that counteract the host's hemostatic response, that is, coagulation, platelet aggregation and vasoconstriction.[5] Proteomic and transcriptomic analyses have been performed in *R. prolixus*, *Triatoma infestans* and *T. dimidiata*,[6] but the regulatory peptides in the salivary glands have not been identified yet. On the other hand, it is known that regulatory peptides coordinate the digestive physiology. These peptides are produced mostly by endocrine cells dispersed in the gut epithelium and by neurons innervating the gut of insects. As a means to further understand the neuroendocrine regulation of events associated with blood intake in hematophagous insects, in this work we studied the neuropeptidome of *R. prolixus* salivary glands and gut.

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Table 1. Precursor from which Peptides Were Identified in *Rhodnius prolixus* Salivary Glands and Midgut[a]

SALIVARY GANDS: NEUROPEPTIDE-LIKE PRECURSOR 1

*MIATSLAPLI LTLLLLSKAS G*ENNDNTKSS SKHALVTDTG EEHNLEKR**HV SSLLGNRASG PYQT**GKR**SSP**

SKSSLDELAE RLEEAAQEDK RYLGALARSG DLRVVARDRQ EKR**EDLDSLI DELASTEEMR** RMQFDALRDD

LFEEEPDKR**G VVSLARAGYL KPTTHDFLED DEESSFYPAE DED**KR**GGIAS LARNGYYQ**KR TVDAELEQLM

SEVYGIGEKR **SVASLARSYN LPNAVKGGYE NDDE**KRNIPS LLRDRTSPLG EGKRHIGSFV ANHGIPFVNN

KEGGKRSVGS LARNRDPFYA VKFGKRDAPD EEGEEMSKR**Y VATLLRQ**GRL PIGIDSPDHG EMSSMKEDND

DHDVDKDEMS QVMQDEASKL SIRKKKSVPT VEGPTRIKR**E AAADEFGGVR DDDSLAQFAD DAADSPIN**KR

YFGVRGGGKM PGGRLPKVGG RSRNRHENSG RRRH

MIDGUT: MYOSUPPRESSIN

*MILAWMCVTL LAGVLA*APGP DCSPSALQVQ SSRVRNMCAL YQISSALQAY LDEQNNYQTA LRDTNIPYNI

PEKR**QDIDHV FMRF**GRRR

[a] Consensus cleavage sites are underlined, identified peptides are in bold face, predicted signal peptides are in italic face.

This detailed description of the neuropeptidome led us to investigate gene expression and peptide release in response to blood intake. Here we also report the use of dimethyl labeling, nano liquid chromatography (LC) and mass spectrometry (MS), to study the dynamics of the peptidome of *R. prolixus* nervous system.

Quantitative peptidomics has been used for the study of the neuroendocrine regulation of feeding in mice and crabs.[7] In insects, pioneering quantitative peptidomics has been only used to study foraging behavior in honeybees.[8] Dimethyl labeling has been recently introduced for quantitative proteomics and peptidomics.[9] The method utilizes isotopomers of formaldehyde and sodium cyanoborohydride to incorporate dimethyl labels at the α- and ε- amino groups of peptides. This technique increases the complexity of the analysis by the use of triplex quantification, instead of the traditional two-point comparisons provided by other methods. Compared to other multiplex labeling techniques such as SILAC or iTRAQ, dimethyl labeling is fast, affordable, sensitive, accurate, and applicable to any sample.[9] In the study presented here, triplex labeling determined changes in neuropeptide concentration in the central nervous system (CNS) of *R. prolixus*, in a time course experiment that spanned the immediate and delayed response to blood intake, linking each to a subset of peptides related to the feeding process. Altogether, our results contribute to the characterization of the neuroendocrine response and of the events triggered by blood intake, and provide the first quantitative proteomic analysis of a vector of human disease.

RESULTS AND DISCUSSION

Neuropeptidomic Analysis of *R. prolixus* Salivary Glands and Gut

To investigate the occurrence of neuropeptides that could regulate the feeding process, we extended the proteomic analysis that we performed in the brain[4] to other tissues related to blood intake, namely salivary glands and gut. Our neuropeptidomic analysis of salivary glands detected the presence of seven mature neuropeptides that derive from the NPLP1 precursor (Table 1). Interestingly, we also detected the peptide EDLDSLIDELASTEEMR (Figure 1), a peptide-processing intermediate that still contains the C-terminal basic residue R, which has not yet been removed by carboxypeptidases. The presence of processing intermediates could indicate that NPLP1 is being expressed and processed in salivary gland cells, and not exclusively in the soma of the neurons of the hypocerebral ganglion (which reach the salivary glands through the esophageal nerve) or the subesophageal ganglion (which reach the salivary glands through the salivary nerve).[10] However, is it plausible to consider that the endopeptidase that cleaves the site RRM (at the C-terminus of the fragment EDLDSLIDELASTEEM in the NPLP1 precursor) acts very slowly, owing to the presence of an aliphatic amino acid residue after the R doublet.[11] The site RR is usually cleaved with less efficiency than KR; that is why we cannot rule out that this particular peptide could reach the salivary glands from the CNS partially uncleaved. In addition, it is interesting to note that in the neuropeptidomic analysis of the mosquito *Aedes aegypti*, authors also found a C-terminal extended form of a peptide encoded in NPLP1 precursor.[12] As this form is always more abundant than the predicted peptide, authors suggest that the extended form could actually be the mature biologically active neuropeptide. Although in our work we found the nonextended form of the peptide in CNS (see Table 2), we cannot rule out the possibility that the extended form acts in the salivary gland as a mature biologically active peptide. In any case, our results suggest a role of NPLP1 in the hormonal control of salivary secretion, although further experiments are required to determine the site of transcription and maturation of the peptides.

Regarding the digestive system, we performed the neuropeptidomic analysis of crop, midgut and hindgut separately and determined the presence of myosuppressin in midgut (Table 1). None of the other digestive structures analyzed revealed the presence of neuropeptides. Our results agree with *in situ* hybridization experiments that

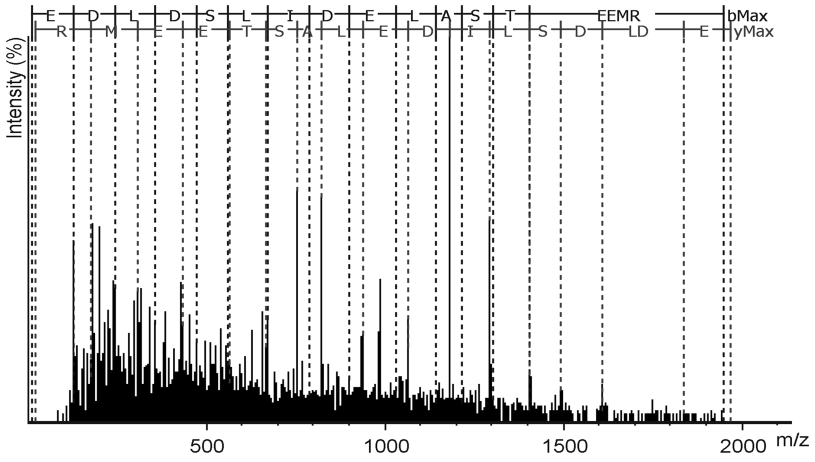

Figure 1. MS/MS spectrum verifying the presence of the peptide EDLDSLIDELASTEEMR in salivary glands. Prominent fragment ions are labeled.

have shown the presence of myosuppressin transcripts in the midgut endocrine cells of *Diploptera punctata*,[13] *Pseudaletia unipuncta*[14] and *Manduca sexta*.[15] Myosuppressin has also been isolated and sequenced from whole gut tissue in *Locusta migratoria*.[16] Our results in *R. prolixus* presented here support the hypothesis that myosuppressin may have a role in digestion, acting either as a paracrine mediator or as a hormone released into the circulation.

Quantitative Proteomics of the Nervous System Neuropeptidome in Response to Feeding

By means of nano-LC−ESI-Orbi-Trap MS analysis of *R. prolixus* CNS, we identified a total of 75 neuropeptides from 18 precursors (Table 2). In some cases, multiple truncated peptides were detected from a single precursor. Different fragments from a particular peptide were found in different chromatographic fractions of independent experiments. Therefore, it cannot be the result of fragmentation during the ionization process. Although we cannot rule out the possibility that this was a consequence of the extraction process, this seems to be a common observation in other peptidomic studies performed with different extraction methods.[4,17] Therefore, based on this evidence, we consider that the truncated forms naturally occur in the nervous system. In addition to bioactive peptides and their fragments, in many cases we detected nonconserved peptides from a precursor ("spacer peptides"; see Table 2). These spacer peptides occur in the precursor sequence flanked by endopeptidase recognition sites, but they lack the characteristic domain of the core bioactive peptides.[18]

Among the identified peptides, the expression of Allatostatin C, NVP-like, ITG-like, CAPA1, CAPA2 and Diuretic hormone (DH) 44 was not detected by MS in the previous comprehensive analysis of the *R. prolixus* brain.[4] This indicates that these precursors could be present at higher concentration in the nerve ganglia than in the brain. In agreement with our hypothesis, Paluzzi et al. have observed that the CAPA 1 gene is predominantly expressed in abdominal ganglia.[19] CAPA1 and CAPA2 expression has been indeed detected in the *R. prolixus* abdominal nerves and subesophageal ganglion by MS.[20] Nevertheless, differences among peptides that could be detected either in our studies or in studies from other authors could also be the consequence of the different LC−MS/MS protocol applied.

Using dimethyl labeling, we examined the quantitative change of individual neuropeptide expression in CNS of *R. prolixus* over three different moments in the response to feeding: unfed, 4 h after blood intake, and 24 h after blood intake. This experimental design spans the immediate and delayed physiological processes related to feeding. Figure 2 shows the experimental strategy used for peptide quantification. Changes in concentration of 19 mature neuropeptides, fragments of mature neuropeptides and/or spacer peptides from 9 precursors were analyzed. Variations in the abundance of prohormone fragments would reflect the dynamics of the bioactive peptides. However, it could be argued that an increase in the concentration of a truncated fragment reflects an increase in its extracellular degradation, indicating that it is not the result of the synthesis but the consequence of the release of the peptide. Nevertheless, previous quantitative peptidomic studies in mice,[7b,21] crabs[7a] and bees[8] show that in those cases where multiple neuropeptides and truncated fragments derived from the same precursor were detected, most of them change in a similar way.

Peptides that Show Significant Changes in the CNS after Blood Intake

NVP-like and ITG-like peptides (also called Apis prohormones 2 and 3). NVP-like and ITG-like peptides are putative neuropeptides, first identified in the peptidomic study of honeybee brain,[17a] and, later on, in *Tribolium castaneum* and *R. prolixus*.[4b,22] The genes that encode the propeptides show the hallmarks of a neuropeptide precursor (a signal peptide and several putative mature neuropeptides flanked by dibasic proteolytic sites), but their functions, and even their status as neuropeptides, have remained elusive until now. In our quantitative analysis, we found reduced levels of a peptide derived from the NVP-like precursor (EHVLNPEEFLAL), 4 h ($p < 0.01$) and 24 h ($p < 0.001$) after blood intake (Figure 3). Peptide levels were higher at 4 h than at 24 h after feeding ($p < 0.05$; Figure 3). These results indicate that the peptide is released from CNS in response to feeding. Taking in consideration that the levels are reduced 4 and 24 h after blood intake, we hypothesize that NVP-like is involved in the regulation of rapid events such as diuresis and antidiuresis, and in delayed events related to mating and reproduction. Further experiments should be performed in order to dissect the different functions of NVP-like peptides in insect physiology.

A significant decrease in the levels of the peptide ITGK-TAAFNHL (derived from the ITG-like precursor) was observed only 24 h after feeding ($p < 0.05$; Figure 4), indicating a delayed release from CNS, and a role of this precursor in the regulation of delayed physiological events. Our data strongly support a role for

Table 2. Peptides identified by nano-LC and ESI-Orbi-Trap MS/MS in *Rhodnius prolixus* Central Nervous System

name of the precursor	peptides identified	retention time (min)	molecular monoisotopic mass
Allatostatin A	AHNEGRLYSFGL-NH_2	22.96−23.23	1361.68
	SNPNGHRFSFGL-NH_2	19.74−20.09	1330.65
	TQLDPANNLHN[a]	15.24−15.82	1235.58
Allatostatin B	SWKDLQSSGW-NH_2	25.04−25.82	1191.56
	GWKDMQTVGW-NH_2	24.55−24.86	1205.56
	AWSDLQSSGW-NH_2	28.06−28.53	1134.50
	AWNSLHGGW-NH_2	23.82−24.18	1025.48
	TADWGSFRGSW-NH_2	25.49−25.93	1267.57
	EPAWQNLKGLW-NH_2	31.13−31.70	1339.70
Allatostatin C	Pyro-QPTPDKEKLLNELS	25.48−25.84	1593.85
	Pyro-QPTPDKEKLL	21.45−21.79	1150.65
	LVEDDGSIDRAVIDYLY	33.16−33.49	1954.95
	LVEDDGSIDRA	18.19−18.44	1188.56
	RAQAGAAEIQQ-NH_2	10.04−10.29	1140.59
	AQAGAAEIQQ-NH_2	20.03−20.56	984.49
Allatotropin	GFKNVQLSTARGF-NH_2	21.44−21.77	1422.77
CAPA1	ARNFPATWGMLVGDDKN[a]	32.53−32.91	1890.90
	SPISSVGLFPFLRA-NH_2	34.21−34.53	1488.84
CAPA2	ARNFPITWGMLVGDDKY[a]	32.53−32.01	1981.97
Diuretic hormone 44	MQRPQGPSLSVANPIEVLRSRLLLE[a]	35.31−35.69	2802.55
	MQRPQGPSLSVANPIEVL[a]	29.46−29.87	1935.02
FMRF-amide related peptides	IKDNFIRF-NH_2	22.81−23.04	1050.59
Insect kinin	FLDHEDALI[a]	25.52−26.03	1072.18
ITG-like precursor	ITGKTAAFNHL	19.49−19.74	1171.63
Long neuropeptide F	FASPDDLRTYLDQLGQY	34.55−34.76	2002.16
	YAVAGRPRF-NH_2	16.17−16.47	1035.21
	AGGINPRLHLAVDGVN[a]	21.03−21.5	1602.81
	YRYPLADASDLYDLL[a]	36.54−37.12	1787.98
Myosuppressin	LDEQNNYQTALRDTNIPYNIPE[a]	32.50−32.80	2620.24
	Pyro-QDIDHVFMRF-NH_2	31.16−31.63	1288.62
	Pyro-QDIDHVF(M−OH)RF-NH_2	29.03−29.67	1304.59
Neuropeptide-like precursor 1	HVSSLLGNRASGPYQT-NH_2	22.93−23.08	1684.86
	SSPSKSSLDELAERLEEAAQED	32.03−32.53	2390.10
	EDLDSLIDELASTEEM	41.80−42.10	1808.78
	FDALRDDLFEEEPD	28.61−28.99	1709.74
	GVVSLARAGY	18.96−19.40	991.54
	GGIASLARNGYYQ	20.11−20.66	1368.67
	TVDAELEQLMSEVYGIGE	38.81−39.10	1981.91
	SVASLARSYNLPNAVKGGYENDDE	24.59−25.06	2568.20
	AVKGGYENDDE	10.04−10.32	1195.49
	SVGSLARNRDFPYAVKF-NH_2	24.01−24.30	1925.02
	YVATLLRQ-NH_2	20.39−20.80	961.57
NVP-like precursor	AQEFIMFGNQQNRAPSFSNIRND	26.77−27.18	2683.25
	EHVLNPEEFLALM	38.95−39.24	1540.75
	EHVLNPEEFLAL(M−OH)	31.35−31.77	1556.75
	EHVLNPEEFLAL	33.25−33.82	1409.71
Orcokinin	NHDTLDSLSGITFGSQ[a]	26.91−27.36	1690.77
	NFDEIDRSGFNSFI	31.40−32.00	1659.75
	NFDEIDRSGFNS	21.16−21.60	1399.60
	NFDEIDRSGFDGFV	30.32−30.86	1616.71
	NFDEIDRSGFDG	22.10−22.60	1370.57
	NFDEIDRVGFGSFI	34.85−35.51	1614.76
	NFDEIDRVGFGS	25.26−25.71	1354.61
	NFDEIDRVG	19.80−20.29	1063.49

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Table 2. Continued

name of the precursor	peptides identified	retention time (min)	molecular monoisotopic mass
Short neuropeptide F	AIQDYDSMRDLYELL[a]	36.58−37.02	1843.86
	LQREALPDSWAHKVV[a]	21.52−21.94	1747.93
Tachykinin	APSTMGFQGVR-NH_2	23.08−23.50	1148.57
	APST(M−OH)GFQGVR-NH_2	16.61−17.08	1164.57
	DDLIGEPDDTFLEEF[a]	37.86−38.15	1753.75
	APAAMGFQGMR-NH_2	18.14−18.70	1134.54
	APAA(M−OH)GFQG(M−OH)R-NH_2	13.18−13.73	1166.54
	APASGFFGMR-NH_2	25.46−25.93	1038.50
	GPSSSAFFGMR-NH_2	23.73−24.00	1141.53
	GPSSSAFFG(M−OH)R-NH_2	20.25−20.73	1157.53
	GPSGFMGVR-NH_2	16.30−16.77	905.45
	GPSGF(M−OH)GVR-NH_2	15.44−15.81	921.45
	SPDDLNHLLQL[a]	37.14−37.52	1263.64

[a] Spacer peptide, lacking the characteristic domain of core bioactive peptides from the precursor.

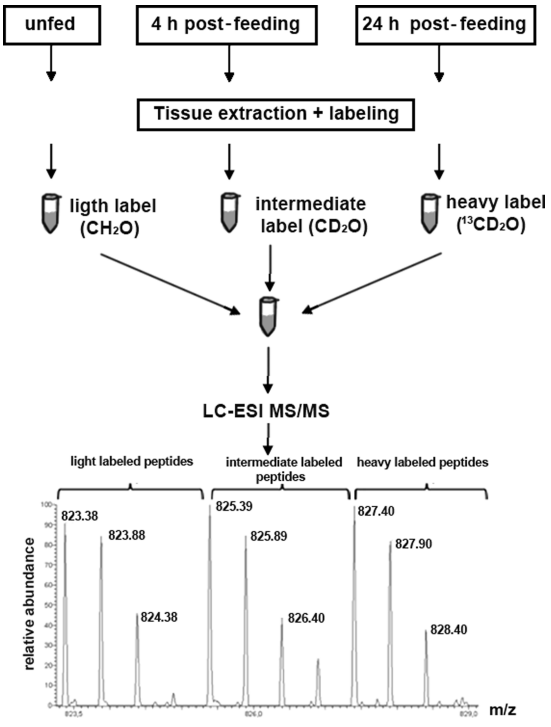

Figure 2. Experimental design used for the analysis of changes in peptide concentrations during *R. prolixus* response to feeding. CNS from insects were dissected either unfed, 4 or 24 h after blood intake. Peptide extracts from the different experimental groups were labeled with different dimethyl isotopes, pooled, and finally analyzed by LC−ESI−MS/MS. MS spectra of the orcokinin labeled peptides with the sequence NFDEIDRSGFDGFV are presented as an example. Quantification was performed by comparing the peak intensity of the light, intermediate and heavy forms of the peptide, by using the sum of three abundant isotopic peaks from each form.

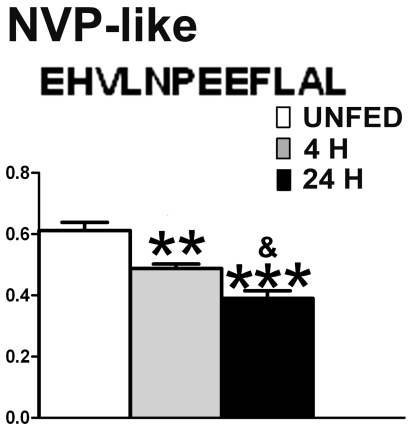

Figure 3. Changes in EHVLNPEEFLAL peptide (encoded in NVP-like precursor) levels in *R. prolixus* CNS after blood intake. Peptide abundance in the different groups are represented (means ± SEM; $N = 4-5$). ** = $p < 0.01$ vs unfed group, *** = $p < 0.001$ vs unfed group, and = $p < 0.05$ vs 4H group. 4H: peptide levels 4 h after blood intake. 24H: peptide levels 24 h after blood intake.

NVP-like and ITG-like precursors in the neuroendocrine response to feeding, opening the path to further studies regarding the detailed function of these molecules in insect physiology.

Kinin. Kinin neuropeptides have been considered diuretic peptides by their ability to stimulate fluid secretion from the Malpighian tubules of several insect species.[23] In *R. prolixus,* however, these assays failed to demonstrate the diuretic activity of kinins in Malpighian tubules.[24] In *R. prolixus,* leucokinin produces a reduction of the trans-epithelial voltage in the anterior midgut, although the physiological significance of this event remains unclear.[24c] Our data show an increase of the level of the peptide FLDHEDALI, derived from the kinin precursor, 4 h after blood intake ($p < 0.01$; Figure 5). Although this is a peptide fragment and not a bioactive peptide, we assume that it reflects the dynamics of the Kinin precursor after feeding (see above).

Neuropeptide-like precursor 1. NPLP1 was first identified in *Drosophila melanogaster* by peptidomic analysis[25] and, later on, in other holometabolous insects such as *Apis mellifera,*[17a] *Bombix mori,*[26] *Tribolium castaneum*[22] and *Neobellieria bullata.*[27] Orthologs of NPLP1 have recently been identified in the hemimetabolous *Acyrthosiphon pisum*[28] and *R. prolixus.*[4b] The physiological role of NLPL1 is unknown so far.

Two NPLP1 peptides are differentially regulated after blood intake (Figure 6). TVDAELEQLMSEVYGIGE is upregulated at 4 h ($p < 0.01$) and downregulated at 24 h ($p < 0.05$) after feeding, whereas SVASLARSYNLPNAVKGGYENDDE is downregulated at 24 h after feeding ($p < 0.01$). Although these two peptides are processed from the same precursor, the variation

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in their concentration after feeding is not identical. Similar observations have been reported for some families of neuropeptides in previous studies.[7,8] In the case of Rhopr-NPLP1, this could be explained by differences in the rate at which convertases generate the two peptides, which are adjacent in the precursor.

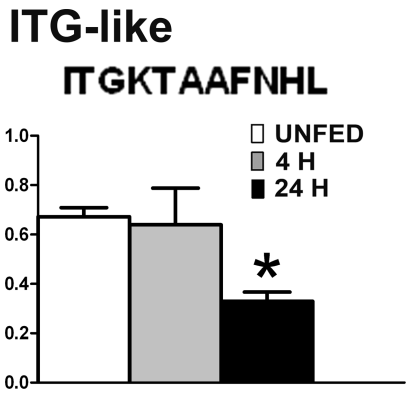

Figure 4. Changes in ITGKTAAFNHL peptide (encoded in ITG-like precursor) levels in *R. prolixus* CNS after blood intake. Peptide abundance in the different groups are represented (means ± SEM; $N = 4-5$). * = $p < 0.05$ vs unfed and 4H groups. 4H: peptide levels 4 h after blood intake. 24H: peptide levels 24 h after blood intake.

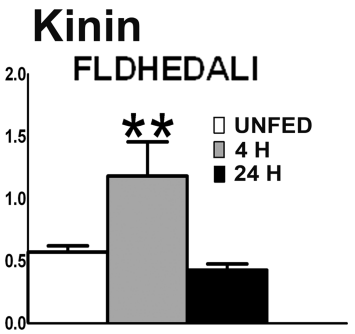

Figure 5. Changes in FLDHEDALI peptide (encoded in kinin precursor) levels in *R. prolixus* CNS after blood intake. Peptide abundance in the different groups are represented (means ± SEM; $N = 4-5$). **= $p < 0.01$ vs unfed and 24H groups. 4H: peptide levels 4 h after blood intake. 24H: peptide levels 24 h after blood intake.

Although both peptides are flanked by KR sites, TVDAELEQLMSEVYGIGE is likely generated faster than SVASLARSYNLPNAVKGGYENDDE, owing to the presence in the precursor of an R residue at its −6 position (separated by 5 N-terminal amino acids from the KR site). The sequence R-X-X-X-X-X-K-R favors the activity of endopeptidases.[11] Thus, the different amino acid residues around the KR cleavage sites influence the efficiency of processing and could account for the differences observed in the dynamics of NPLP1-derived peptides. Either way, we conclude that NPLP1 peptides are involved in the feeding response, providing the first clues to elucidate their function.

Peptides that Do Not Significantly Change after Blood Intake

We could detect and quantify peptides derived from Allatostatin C, Long Neuropeptide F, Short Neuropeptide F, Orcokinin and Tachykinin precursors, none of which show significant differences in their concentration in the CNS after feeding (Figure 7A−D).

CONCLUDING REMARKS

In this work we present a comprehensive, pioneering quantitative peptidome analysis related to the feeding process in a human disease vector. We showed the expression of NPLP1 in salivary glands, which suggests the involvement of NPLP1 in the production and/or secretion of saliva. We also detected myosuppressin in midgut, indicating its probable role as a paracrine and/or endocrine hormone regulating digestion. We determined the implication of neuropeptides in the immediate and delayed neuroendocrine activation after blood intake in *R. prolixus*. Up to this work, the neuropeptides NVP-like, ITG-like, and NPLP1 were molecules with unknown function in insects. We show evidence that they are involved in processes triggered by the blood intake. Our study provides clues for the characterization of the postprandial neuroendocrine activation in a hematophagous insect. It contains relevant information for future studies aimed at the design of next-generation insecticides such as peptidomimetics, which are expected to be species-specific and environmentally friendly.

MATERIAL AND METHODS

Food Intake Experiment

A colony of *R. prolixus* was maintained in our laboratory in a 12 h light/dark schedule at 28 °C. Adult male and female insects

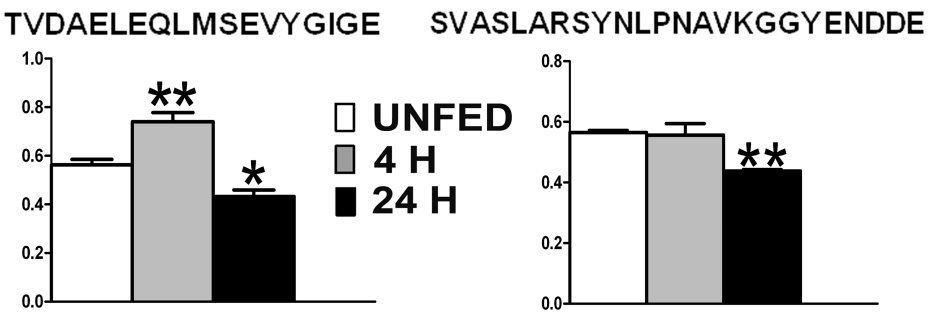

Figure 6. Changes in TVDAELEQLMSEVYGIGE and SVASLARSYNLPNAVKGGYENDDE peptides (encoded in neuropeptide-like precursor 1 gene) levels in *R. prolixus* CNS after blood intake. Peptide abundance in the different groups are represented (means ± SEM; $N = 4-5$). *= $p < 0.05$ vs unfed group, **= $p < 0.01$ vs the other groups, and = $p < 0.001$ vs 4H group. 4H: peptide levels 4 h after blood intake. 24H: peptide levels 24 h after blood intake.

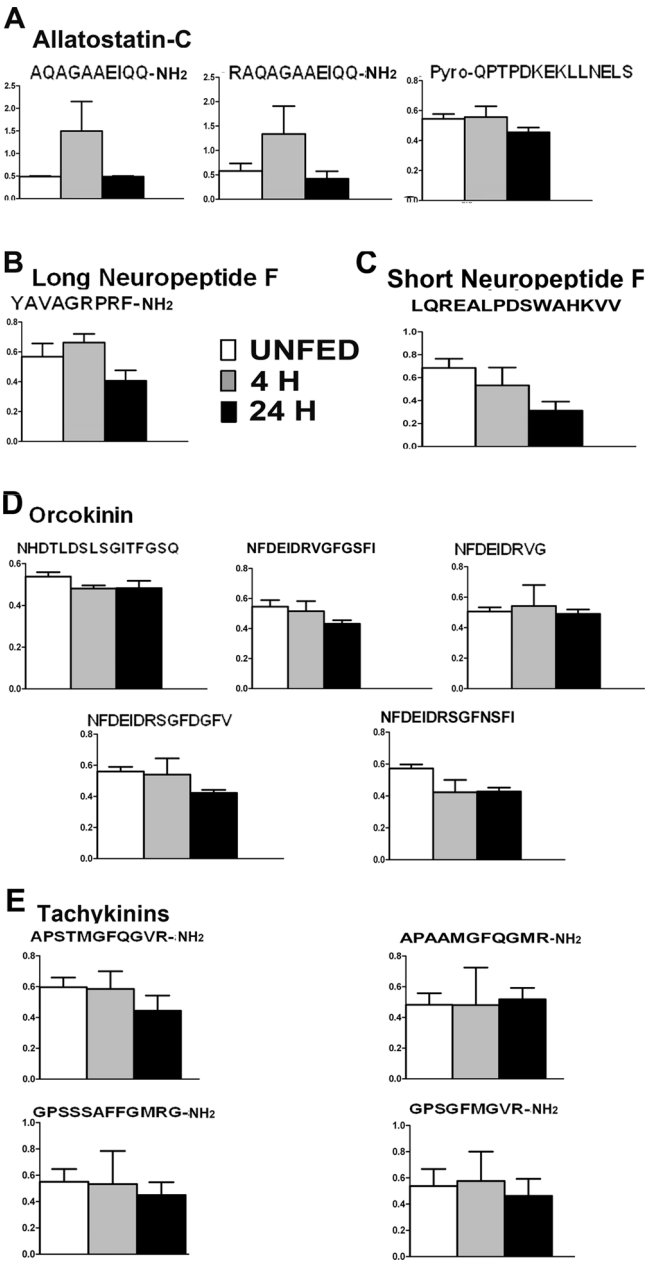

Figure 7. Changes in peptide levels in *R. prolixus* CNS after blood intake. Peptide abundance in the different groups are represented (means ± SEM; $N = 4-5$). 4H: peptide levels 4 h after blood intake. 24H: peptide levels 24 h after blood intake. (A) Peptides encoded in Allatostatin C precursor. (B) Peptide encoded in long neuropeptide F precursor. (C) Peptide encoded in the short neuropeptide F precursor. (D) Peptides encoded in the orcokinin precursor. (E) Peptides encoded in the tachykinin precursor.

were unfed 2−3 weeks postemergence, from a group of fifth instar individuals previously fed on chickens. In a feeding experiment, the insects were analyzed unfed or (a) 4 h (4H) or (b) 24 h (24H) after the beginning of feeding on chickens (N = 10 for each experimental group; 5 experimental replicas for groups unfed and 24H, and 4 replicas for group 4H were analyzed). The whole CNS from each insect was manually dissected, immediately placed in ice-chilled *R. prolixus* saline (NaCl, 129 mM; KCl, 8.6 mM; CaCl2 2.0 mM; MgCl2 8.5 mM; NaHCO3 10.2 mM; NaH2PO4 4.3 mM; Hepes (Sigma, St. Louis, MO) 8.6 mM, PH 7) and stored at −80 °C until peptide extraction.

Peptide Extraction and Isotope Labeling

For tissue extraction, 10 CNS were placed in 100 μL methanol/water/acetic acid (90, 9, 1, v/v/v), sonicated for 5 min and centrifuged for 10 min at 7500× *g*. The supernatant was collected and the pellet was re-extracted twice. The collected supernatant was placed under vacuum to remove organic solvents. For the labeling reaction, samples were rediluted in 100 μL triethyl ammonium bicarbonate buffer 100 mM (Sigma, St. Louis, MO). Four microliters of the corresponding dimethyl isotope was added to each sample at 4% v/v as follows: the sample from S group was labeled with CH_2O (light isotope), the sample from 4H group was labeled with CD_2O (middle weight isotope) and the sample from 24H group was labeled with $^{13}CD_2O$ (heavy isotope). Four microliters of $NaBH_3CN$ 0.6 M was then added to S and 4H samples, and 4 μL of $NaBD_3CN$ 0.6 M was added to 24H sample. Samples were incubated for 1.5 h at 20 °C to allow the labeling reaction to proceed. After the labeling was completed, the samples were transferred to ice. Then 16 μL of NH_3-H_2O and 8 μL of formic acid (FA) were added to each sample to stop the reaction. Samples from the three experimental groups were mixed together in a 1:1:1 ratio. Solvent was removed under vacuum, and samples were rediluted in 20 μL 0.1% trifluoroacetic acid (TFA) and desalted using a C18 extraction disk (Varian, Darmstadt, Germany) activated with 80% acetonitrile (ACN)/0.1% TFA. C18 extraction material was washed with 0.1% TFA, and peptides were eluted with 70% ACN/0.1% TFA. Organic solvent was removed under vacuum and the sample rediluted in 12 μL 5% ACN/1% FA for MS analysis.

Liquid Chromatography (LC)−MS

Samples were analyzed using an online nano-LC−Electrospray (ESI)−Orbi-Trap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA). Each experimental replica was split into two LC−MS runs, resulting in two replicated spectra for each experimental replica. The samples were loaded onto the precolumn at a flow rate of 10 μL/min of buffer A (0.1% v/v TFA) for 5 min. After valve switching, the peptides were eluted by a linear gradient of 10−60% v/v solvent B (80% v/v ACN, 0.1% v/v TFA in water) for 38 min. Finally, the peptides were eluted with 100% of solvent B for 7 min.

Peptide Identification and Quantification

MS/MS spectra were processed and the peak lists were used for database search against a database containing all *R. prolixus* sequences submitted to Genbank using MASCOT 2.1.0 (Matrix Science, Boston, MA) and PEAKS STUDIO 5.2 (Bioinformatic Solutions, Waterloo, ON, Canada) software. Further settings included: enzyme: none; variable modification; Pyroglutamic of Glutamine, C-terminal amidation; oxidation of Met; Lys and N-terminal dimethyl modifications (CH_2O, CD_2O or $^{13}CD_2O$); parent ion mass error tolerance: 10 ppm; fragment ion mass error tolerance: 0.5 Da. For peptide identification, the peptides which mass matched with predicted neuropeptides, and that were pointed with high score using both software, were considered and manually analyzed. All results reported here are from careful manual analysis of each spectrum, identifying accurate series of b-and y- ions, and a-, z-ions and immonium ions confirming sequences. Additional criteria to confirm peptide identity were (a) peptides identified as the same molecule in different

LC−MS/MS runs should present the same retention time and (b) the presence of particular post translational modifications and cleavage in sites that are predicted from the primary structure of the precursor.

MSQuant software and manual analysis were used for peptide quantification, with the aid of XCalibur software (Thermo Fisher Scientific, Waltham, MA). Peaks generated from known neuropeptides were selected. Quantification was performed by comparing the peak intensity of the light, intermediate and heavy forms of the peptide, by using the sum of three abundant isotopic peaks from each form. For the statistical analysis, the intensity of the light, intermediate or heavy labeled peaks, was normalized dividing by the sum of the intensities of light and heavy peaks. In this way, differences in ionization efficiency between different acquisitions were eliminated. The ratios from spectra derived from two replicates were averaged. One-way ANOVA was performed to evaluate differences in the concentration of neuropeptides among the experimental groups. When necessary, data were log-transformed to achieve homogeneity of variances.

Peptidomics of *R. prolixus* Salivary Glands and Gut

Salivary glands, crop, midgut and hindgut were dissected as described for CNS from insects belonging to the groups U and 24H, and pooled. Peptide extraction and desalting was performed as described for CNS.[4] Samples were then analyzed using an online nano-LC−ESI-Quadrupole-TOF Mass Spectrometer (Thermo Fisher Scientific). Each sample contained tissue from 4 insects. Four replicates were processed for each. The LC protocol employed was the same as the one used for CNS. Peptide identification was performed as described for neuropeptides in CNS. Further settings in MASCOT and PEAKS STUDIO were: enzyme, none; variable modification; Pyroglutamic of Glutamine (Q), N-terminal amidation; C-terminal acetylation, oxidation of Met (M); parent ion mass error tolerance, 100 ppm; fragment ion mass error tolerance, 0.25 Da.

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