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Original Research Paper

Neuropeptidomics in *Triatoma infestans*. Comparative transcriptomic analysis among triatomines

Lucila Traverso [a], Ivana Sierra [a], Marcos Sterkel [b], Flavio Francini [c], Sheila Ons [a,*]

[a] Laboratory of Insect Neurobiology, Regional Center for Genomic Studies, Faculty of Exact Sciences, National University of La Plata, Bvd 120 Nº. 1459, CP: 1900, La Plata, Argentina
[b] Institute of Medical Biochemistry, Federal University of Rio de Janeiro, Av. Carlos Chagas Filho, 373, bloco D. Prédio do CCS, Ilha do Fundão, Rio de Janeiro 21941-902, Brazil
[c] Center of Experimental and Applied Endocrinology, CONICET-CCT La Plata, National University of La Plata, 60 and 120 Street, CP: 1900, La Plata, Argentina

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ABSTRACT

Chagas' disease, affecting up to 6–7 million people worldwide, is transmitted to humans through the feces of triatomine kissing bugs. From these, *Rhodnius prolixus*, *Triatoma dimidiata*, *Triatoma infestans* and *Triatoma pallidipennis* are important vectors distributed throughout the Latin American subcontinent. Resistance to pyrethroids has been developed by some triatomine populations, especially *T. infestans*, obstructing their control. Given their role in the regulation of physiological processes, neuroendocrine-derived factors have been proposed as a source of molecular targets for new-generation insecticides. However, the involvement of neuropeptides in insecticide metabolism and resistance in insects has been poorly studied. In the present work, the sequences of 20 neuropeptide precursor genes in *T. infestans*, 16 in *T. dimidiata*, and 13 in *T. pallidipennis* detected in transcriptomic databases are reported, and a comparative analysis in triatomines is presented. A total of 59 neuropeptides were validated by liquid chromatography-tandem mass spectrometry in brain and nervous ganglia from *T. infestans*, revealing the existence of differential post-translational modifications, extended and truncated forms. The results suggest a high sequence conservation in some neuropeptide systems in triatomines, whereas remarkable differences occur in several others within the core domains. Comparisons of the basal expression levels for several neuropeptide precursor genes between pyrethroid sensitive and resistant population of *T. infestans* are also presented here, in order to introduce a proof of concept to test the involvement of neuropeptides in insecticide resistance. From the precursors tested, NVP and ITG peptides are significantly higher expressed in the resistant population. To our knowledge, this is the first report to associate differential neuropeptide expression with insecticide resistance. The information provided here contributes to creating conditions to widely extend functional and genetic studies involving neuropeptides in triatomines.

1. Introduction

Neglected tropical diseases (NTDs) are a group of transmissible diseases prevailing in tropical and subtropical regions, mainly affecting populations living in poverty, in close contact with infectious vectors (Rassi et al., 2010). Chagas' disease is one of the most prevalent NTDs, affecting 6–7 million people worldwide. Even though Chagas' disease is more prevalent in the Americas, the movement of human populations has spread the disease to other regions in the world (Rassi et al., 2010). The causative agent of Chagas' disease is the protozoan parasite *Trypanosoma cruzi*, transmitted to mammals by the feces of triatomine insects. *Triatoma infestans* is the most important insect vector species in the South Cone, whereas *Rhodnius prolixus* is distributed in Central America and northern regions of South America, *Triatoma dimidiata* goes from northern South America to Mexico, where *Triatoma pallidipennis* is also an important vector (Rassi et al., 2010).

Given the absence of vaccines and efficient treatments for the chronic stage, the preferred strategy to control Chagas' disease is the reduction of vector populations in human dwellings by spray-

Abbreviations: AKH, Adipokinetic hormone; AT, allatotropin; AST, allatostatin; CCHa, CCHamide; CAPA, capability; CNMa, CNMamide; CNS, central nervous system; CRF-DH, CRF-like diuretic hormone; CT-DH, calcitonin-like diuretic hormone; ESTs, expressed sequence tags; ETH, ecdysis-triggering hormone; ITP, ion transport peptide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NPF, long neuropeptide F; MIP, myoinhibitting peptide; MTGM, mesothoracic ganglionic mass; MS, myosuppressin; NPLP1, neuropeptide-like precursor 1; NPAA, neuroparsin A; NTDs, neglected tropical diseases; OK, orcokinin; ORF, open reading frame; PK, pyrokinin; Proc, proctolin; PRO, prothoracic ganglion; PTTH, prothoracicotropic hormone; RYa, RYamide; sNPF, short neuropeptide F; SOG, suboesophageal ganglion; TK, tachykinin.

* Corresponding author.
E-mail address: sheila.ons@presi.unlp.edu.ar (S. Ons).

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ing domiciles with neurotoxic compounds, mainly pyrethroids (Mougabure-Cueto and Picollo, 2015). The target site of pyrethroids is the voltage-gated sodium channel present in the membranes of excitable cells and centrally involved in the transmission of the action potential (Dong et al., 2014). Pyrethroids modify the normal sodium channel function provoking nervous overexcitation and, at lethal doses, the death of the insect (Dong et al., 2014). Sublethal effects of pyrethroids in triatomines also include the induction of secretion by Malpighian tubules (Maddrell and Casida, 1971), probably due to the release of diuretic factors from CNS that is caused by the nervous excitation. Apart from this, other sublethal effects of pyrethroids remain to be determined in triatomines.

Insecticide spraying of dwellings, along with the implementation of pretransfusional screening of blood banks and controls during pregnancy, has led to an important diminution in the prevalence of Chagas' disease in Latin America (Rassi et al., 2010). However, in the last decade the persistent presence of *T. infestans* after spraying with pyrethroids has been repeatedly reported in wide areas of the Argentinean and Bolivian Gran Chaco ecoregion. High levels of pyrethroid resistance in *T. infestans* from these areas were confirmed in experimental determinations in the laboratory (Mougabure-Cueto and Picollo, 2015). Even though it is a multicausal phenomenon, pyrethroid resistance associated with mutations in the sodium channel seems to be one of the main reasons for the failures in the elimination of *T. infestans* in Gran Chaco (Capriotti et al., 2014; Fabro et al., 2012; Roca Acevedo et al., 2015; Sierra et al., 2016).

Resistance to neurotoxic insecticides is one of the major challenges for the control of harmful insect species. Resistance management strategies include early detection and the rationally planned use of different compounds. Hence, a diversity of insecticide targets is essential for insect pest management. The study of resistance mechanisms and the search of targets for new insecticides are fundamental research fields for the improvement of pest management strategies.

Neuropeptides are encoded in precursors that are post-translationally cleaved and modified in specific residues, giving rise to the biologically active mature molecules. Usually, the different mature bioactive peptides encoded in a precursor possess a conserved core domain that is also conserved among orthologues (Nassel and Winther, 2010). Neuropeptide precursors also encode "spacer peptides", i.e., predicted peptides that lack the core characteristic sequence (Wegener and Gorbashov, 2008). The biological function of spacer peptides has not been well studied to date, but evidence suggested that they could modulate the activity of bioactive core peptides (Brezden et al., 1999). However, it is assumed that biological activity resides in the highly conserved peptides.

Neuropeptides have fundamental roles in regulating vital physiological processes such as osmoregulation, development, reproduction, and behavior. Neuropeptidergic systems have been well studied in *R. prolixus* for many years, using physiological, molecular, genomic, transcriptomic and peptidomic approaches (see Ons, 2016 for a recent review).

Given their relevance in physiological regulation, molecules from insect neuroendocrine system, i.e., neuropeptides and their receptors, have been proposed as attractive pesticide targets (Audsley and Down, 2015; Verlinden et al., 2014). Furthermore, neuroendocrine signaling could contribute to tolerance or resistance to neurotoxics by different mechanisms, reducing the impact of the stress caused by the insecticides, given that neuropeptides are involved in coping with stressful stimuli in insects (Plavsin et al., 2015; Veenstra, 2009). The involvement of neuropeptides in the stress response to insecticides, or in any other mechanisms for insecticide metabolism and resistance, is a poorly explored research field to date.

Datamining in genomes and transcriptomes for novel insecticide target identification was proposed as the first step in the "genome-to-lead" approach for developing new insecticides (Meyer et al., 2012). Transcriptomic sequencing from insect vectors will provide information for further physiological studies, and eventually for the discovery of new insecticide targets. Besides, peptidomic information is an important complement of transcriptomics, in particular for the analysis of neuropeptides, given that these molecules undergo cleavages and other post-translational modifications to produce the mature bioactive products (Nassel, 2002). In this work, the identification of neuropeptide precursors in *T. infestans* transcriptomes is presented and further compared with *R. prolixus*, *T. dimidiata* and *T. pallidipennis* orthologues. In order to complete neuropeptide characterization, we performed liquid chromatography-tandem mass spectrometry (LC-MS/MS) peptidomic assays to identify a large set of neuropeptides in brain and ganglia of *T. infestans* central nervous system (CNS). Finally, expression levels of selected neuropeptide precursor genes are compared between *T. infestans* from sensitive and high pyrethroid resistant populations, with the aim to providing a proof of concept to explore the possible involvement of the neuroendocrine system in xenobiotic detoxification. The results shed light on the structure of the neuroendocrine system in Chagas' disease vectors, and provide a genetic and molecular background for future functional studies in these medically relevant species.

2. Material and methods

2.1. Neuropeptide precursor gene identification in Triatoma spp. databases

The dataset used here is fully accessible at NCBI Sequence Read Archives (SRA) under the Bioproject ID number PRJNA304741 (SRA numbers: SAMN04317639 for *T. dimidiata* SAMN04317638 for *T. infestans*, and SAMN04317640 for *T. pallidipennis*). The normalized libraries were generated by Martinez-Barnetche, Lavore et al. (unpublished results) using RNA of *T. dimidiata*, *T. infestans* and *T. pallidipennis* from all the stages of their life cycle, both sexes and different feeding conditions (starved and fed), sequenced on the platforms GS FLX+(454-ROCHE) and assembled with the GS DeNovo assembler v.2.8 software.

For neuropeptide precursor gene identification, iterative BLASTX searches were performed using local BLAST (Altschul et al., 1990) on a *R. prolixus* neuropeptide precursor gene dataset described earlier (Ons, 2016) and including orthologues from *Bombyx mori*, *Drosophila melanogaster* and *Tribolium castaneum* for molecules not detected or detected as incomplete precursors in the *R. prolixus* genome. The normalized transcriptome for each *Triatoma* sp. was used as query. Furthermore, an online TBLASTN search was performed in the salivary gland transcriptome from *T. infestans* available in vectorbase (www.vectorbase.org), using the neuropeptide precursor dataset described above as query. For the structural analysis of the neuropeptide precursors, SignalP3 (identification of signal peptide) (Bendtsen et al., 2004) and the rules previously proposed for the prediction of convertase cleavage sites (Veenstra, 2000) were used. Sequence alignments were performed using Clustal Ω (Sievers and Higgins, 2014) and manually corrected.

2.2. Neuropeptidomics in T. infestans

2.2.1. Peptide extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS)

CNSs from *T. infestans* were dissected and separated in brain, suboesophageal ganglion (SOG), prothoracic ganglion (PRO) and

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