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Molecular characterization of a short neuropeptide F signaling system in the tsetse fly, *Glossina morsitans morsitans*

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ABSTRACT

Neuropeptides of the short neuropeptide F (sNPF) family are widespread among arthropods and found in every sequenced insect genome so far. Functional studies have mainly focused on the regulatory role of sNPF in feeding behavior, although this neuropeptide family has pleiotropic effects including in the control of locomotion, osmotic homeostasis, sleep, learning and memory. Here, we set out to characterize and determine possible roles of sNPF signaling in the haematophagous tsetse fly *Glossina morsitans morsitans*, a vector of African *Trypanosoma* parasites causing human and animal African trypanosomiasis. We cloned the *G. m. morsitans* cDNA sequences of an sNPF-like receptor (Glomo-sNPFR) and precursor protein encoding four Glomo-sNPF neuropeptides. All four Glomo-sNPF peptides concentration-dependently activated Glomo-sNPFR in a cell-based calcium mobilization assay, with EC_{50} values in the nanomolar range. Gene expression profiles in adult female tsetse flies indicate that the Glomo-sNPF system is mainly restricted to the nervous system. *Glomo-snpfr* transcripts were also detected in the hindgut of adult females. In contrast to the *Drosophila* sNPF system, tsetse larvae lack expression of *Glomo-snpf* and *Glomo-snpfr* genes. While *Glomo-snpf* transcript levels are upregulated in pupae, the onset of *Glomo-snpfr* expression is delayed to adulthood. Expression profiles in adult tissues are similar to those in other insects suggesting that the tsetse sNPF system may have similar functions such as a regulatory role in feeding behavior, together with a possible involvement of sNPFR signaling in osmotic homeostasis. Our molecular data will enable further investigations into the functions of sNPF signaling in tsetse flies.

1. Introduction

Tsetse flies (genus *Glossina*) are the transmitters of African trypanosomes throughout sub-Saharan Africa, thereby causing human and animal African trypanosomiasis with impact on human health and local economy. One of the best trypanosomiasis control strategies has proven to be the reduction of tsetse fly populations (Attardo et al., 2010). The success of this vector control resides in the low reproduction potential of tsetse flies stemming from their viviparous reproductive strategy, which is rather unique in insects. Female tsetse flies can generate only 8–12 individuals during their lifetime (Tobe and Langley, 1978), which are nourished during larvagenesis via intrauterine milk secretions from the maternal accessory gland (Denlinger and Ma, 1974). Similarly to tsetse reproduction physiology, their feeding behavior is also rather unique because both males and females are obligate blood feeders and rely exclusively on proline as major energy source (Bursell, 1977). However, little is known about the regulation of this blood feeding behavior (Lehane, 2005). Studying the involved signaling systems can point towards interesting targets that may decrease fly fitness and lead to the development of new vector control strategies.

Neuropeptide systems are essential signaling systems that are well conserved during evolution and engaged in many physiological functions, including feeding behavior. Amongst these, the short

Abbreviations: sNPF, short neuropeptide F; sNPFR, short neuropeptide F receptor; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; NPF, neuropeptide F.

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neuropeptide F (sNPF) system has been widely linked to feeding behavior. So far, it has been exclusively identified in Arthropoda, wherein sNPFs are characterized by an xPxLRLRFamide consensus sequence (Nässel and Wegener, 2011). From transcript analyses, peptide administration and proteomics studies, it has become evident that sNPFs have opposing effects on feeding depending on the insect species. While it promotes food intake in some species, such as *Drosophila*, it negatively correlates with food intake in other species such as the silk moth *Bombyx mori* (Nagata et al., 2012, reviewed by Spit et al., 2012).

Besides a well-described role in the regulation of feeding behavior, sNPF has been implicated in other physiological processes. In *Drosophila*, sNPF signaling is involved in locomotion (Kahsai et al., 2010b), metabolism and stress resistance (Kahsai et al., 2010; Kapan et al., 2012). In addition, sNPFs are thought to regulate hormone release (Nässel et al., 2008), circadian rhythm (Johard et al., 2009), osmotic homeostasis (Kahsai et al., 2010) and learning and memory (Dillen et al., 2015; Johard et al., 2008). In *B. mori*, sNPF has been associated with suppression of juvenile hormone synthesis (Kaneko and Hiruma, 2014; Yamanaka et al., 2008).

With the publication of its genome in 2014, we identified several neuropeptide systems in the tsetse fly *Glossina morsitans morsitans*, including an sNPF system (International Glossina Genome Initiative, 2014). *In silico* studies predicted a single *G. m. morsitans snpf* (*Glomo-snpf*) gene with four putative Glomo-sNPF peptides (International Glossina Genome Initiative, 2014). This is in accordance with findings in other insect species in which only one *snpf* gene is present encoding one to five sNPFs, with the exception of *Aedes aegypti* which has two *snpf* genes (Predel et al., 2010). In addition, the *Glossina* Genome Initiative predicted a *G. m. morsitans snpfr* (*Glomo-snpfr*) gene that encodes a single Glomo-sNPFR of the rhodopsin G-protein coupled receptor (GPCR) family. Besides sNPF, over 30 other neuropeptides are conserved in tsetse flies including a single gene for neuropeptide F (Glomo-NPF) and the neuropeptide F receptor Glomo-NPFR (International Glossina Genome Initiative, 2014).

Here we further characterized the predicted sNPF system of the tsetse fly and studied the activation of Glomo-sNPFR by its neuropeptide ligands as well as the spatiotemporal expression of *Glomo-snpf* and *Glomo-snpfr* genes in female tsetse flies.

2. Materials and methods

2.1. Animal rearing conditions

G. m. morsitans flies were obtained from the Institute of Tropical Medicine in Antwerp (Elsen et al., 1993) and reared at 26 °C and 65% relative humidity. Flies were fed three times a week using an artificial membrane with defibrinated and gamma-irradiated bovine blood.

2.2. Cloning of Glomo-snpf and Glomo-snpfr cDNAs

The open reading frames (ORFs) encoding the predicted *G. m. morsitans* sNPFR (*Glomo-snpfr*. Vectorbase ID: (GMOY006636) and sNPF (*Glomo-snpf*. Vectorbase ID: (GMOY012142) precursor were amplified from female *G. m. morsitans* cDNA using Advantage 2 Polymerase (Clontech) and the following primers (Sigma-Aldrich) for *Glomo-snpfr*: Fw 5′-CACCATGCCCAACTTCAATCTCACCAAGAC-3′ and Rev 5′-TCAGTTGCAGCTTTGTTTGTTTCGCTC-3′ and *Glomo-snpf*: Fw 5′-CATGCATTTTCGCAGTCG-3′ and Rev 5′-CATTCGTG CAGCGCATTTAG-3′. The *Glomo-snpfr* forward primer was designed with a partial Kozak sequence preceding the authentic start codon to optimize initiation of translation in mammalian cells which were used in the calcium mobilization assay (Kozak, 1987). PCR products were purified and subsequently extended with a single 3′ A-overhang by making use of dATP and *Taq* polymerase before cloning to enable T/A cloning into the pcDNA3.1/V5-His-TOPO® TA expression vector (*Glomo-snpfr*) or the pCR4-TOPO® TA sequencing vector (*Glomo-snpf*) (Invitrogen). Cloned *Glomo-snpfr* and *Glomo-snpf* constructs were transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen) and plasmids were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). Cloned cDNA sequences were determined on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

2.3. Sequence analyses of Glomo-sNPFR and Glomo-sNPF precursor

The protein sequence of the cloned Glomo-sNPFR ORF was analyzed for the presence of putative transmembrane regions with the software programs PSIPRED and MEMSAT3 (bioinf.cs.ucl.ac.uk/psipred) (Jones, 2007). Phosphorylation, glycosylation and palmytoylation sites were respectively predicted with NetPhos 2.0, NetNGlyc 1.0 and GSS-PALM 4.0 (www.cbs.dtu.dk/services/NetPhos, www.cbs.dtu.dk/services/NetNGlyc and csspalm.biocuckoo.org/online.php) (Blom et al., 1999; Gupta and Brunak, 2002; Ren et al., 2008). Protein sequence alignment of Glomo-sNPFR with other dipteran sNPFRs was performed with Clustal Omega (www.clustal.org) (Sievers et al., 2011) and included *Drosophila melanogaster* sNPFR (Drome-sNPFR: CG7395) (Mertens et al., 2002), *Musca domestica* sNPFR (Musdo-sNPFR: MDOA011011), *Anopheles gambiae* sNPFR (Anoga-sNPFR: AGAP012378) (Garczynski et al., 2007), and *Aedes aegypti* sNPFR (Aedae-sNPFR: AGX84996.1). The protein sequence of the cloned sNPF precursor was analyzed for the presence of an N-terminal signal peptide using SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

2.4. Spatial and temporal transcript profiles of Glomo-snpf and Glomo-snpfr

Transcript profiles of *Glomo-snpf* and *Glomo-snpfr* were determined by real-time quantitative PCR (RTqPCR) in different tissues of female tsetse flies and at different developmental stages for which sex could not be distinguished. Tissues were dissected under a binocular microscope, collected in ice-cold phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.76 mM; pH 7.4) and immediately frozen in liquid nitrogen. Three biological repeats were assembled for each tissue. For each biological repeat, the following tissues were collected: a single female head, thorax and abdomen; an 18-day-old pupal head, thorax and abdomen; a complete 18-day-old pupa; an 11-day-old pupa; and first, second and third larval instars. Additionally, tissues from five flies were pooled in one biological repeat for: the female reproductive system including the uterus and ovaries; the flight muscles; the fat body including the milk gland; the anterior midgut; the posterior midgut; the Malpighian tubules; and the entire brain including the optical lobes, antennal lobes and suboesophageal ganglion. For the thoracic ganglion, corpora cardiaca and corpora allata, salivary glands, and hindgut, tissues from 10 females were pooled for one biological repeat. The dissected tissues were subsequently transferred to MagNA Lyser Green Beads tubes (Roche) and homogenized with the MagNa Lyser® (Roche). Total RNA isolation was performed using the RNeasy® Lipid Tissue Mini Kit (Qiagen) in combination with a DNase digestion (RNase-free DNase Set, Qiagen). The PrimeScript™ RT Reagent Kit (Perfect Real Time; TaKaRa Bio Inc.) was used to generate cDNA that was diluted 10-fold. For every tissue sample, a negative control sample was generated to control for the presence of unwanted genomic DNA in which reverse