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Functional characterization of mosquito short neuropeptide F receptors

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ABSTRACT

Mosquito blood feeding transiently inhibits sugar- and host seeking through neuropeptide signaling. Short neuropeptide F (sNPF) is one of the neuromodulators involved in this regulation. Here, we identified the genes for the sNPF precursor and the sNPF receptor in the southern house mosquito, *Culex quinquefasciatus*. Comparative analyses are made with the genes of the sNPF precursor and receptor from two other important vectors, *Aedes aegypti* and *Anopheles coluzzii*. We functionally characterized the receptors in all three species using endogenous neuropeptides, and quantified their transcript expression following a blood meal and a sugar meal. Our analysis reveals several *Cx. quinquefasciatus*-specific duplications of the sNPF-3 isoform on the sNPF precursor, which are not reflected in the precursors of the other two species. In contrast, the structure of the sNPF receptors is highly conserved within mosquitoes, and a putative ligand binding region is proposed and discussed. Reflecting the high structural conservation, the sNPF receptor sensitivity to endogenous sNPF isoforms is conserved across mosquito species. Using quantitative real time PCR, we demonstrate that transcript abundance of the sNPF receptor and precursor is regulated following feeding, only in *Cx. quinquefasciatus*. We discuss our findings in relation to previous work on sNPF signaling and its role in feeding regulation.

1. Introduction

Short neuropeptide F (sNPF) regulates feeding in insects, including mosquitoes [1–10]. Blood feeding to completion transiently inhibits the odor-mediated host seeking behavior in mosquitoes [1,7,11–13] in two phases through distinct mechanisms, as described in the yellow fever mosquito, *Aedes aegypti*. An initial abdominal distension inhibits host attraction for 24 h [14], followed by a delayed inhibition, regulated by a humoral factor, acting for up to 72 h post-blood meal [2]. Coincident with the second phase of behavioral inhibition, the hemolymph titer of Head Peptide-I, an *Aedes*-specific sNPF-like humoral factor, increases [2]. Systemic injection of Head Peptide-I and sNPFs in non-blood fed mosquitoes inhibits host seeking [1,2,7], suggesting that sNPFs act as satiety factors, in line with that described in other insects [4,9,15,16]. Interestingly, the level of one of the isoforms generated from the sNPF precursor, sNPF-2, changes in the primary olfactory center during this phase [7]. This indicates that sNPF signaling in *Ae. aegypti* may modulate the olfactory processing which mediates foraging directly, similar to that observed in *Drosophila melanogaster* [5,6].

While all *bona fide* sNPFs (RLRF/W-amide peptides) in *Ae. aegypti*, are detected by the neuropeptide Y-like receptor 1, NPYLR1 (VectorBase ID: AAEL013505), the receptor has a lower affinity to Head Peptide-I (pERPhPSLKTRFamide) [1]. The expression of NPYLR1 significantly increases following blood feeding in *Ae. aegypti*. However, neither host seeking nor feeding is affected in *npylr1* knock out mutants [1]. This suggests that short neuropeptide F signaling may not be the sole regulator of the host-odor refractory behavior post-blood meal [1]. Rather, there appears to be redundancy in the regulation of the signaling pathways regulating satiety, as previously suggested [7].

In both *Ae. aegypti* and the African malaria mosquito, *Anopheles coluzzii,* the *sNPF* gene encodes a single prepropeptide from which four sNPFs are processed [17,18]. In *Ae. aegypti* and *An. coluzzi*, the expressed sNPFs terminate in either RLFRa (Aedae/Anoco-sNPF-1 and −2, with sNPF-2 being duplicated; see 2.2 about the nomenclature for the isoforms) or RLRWa (Aedae-sNPF-3; Anoco-sNPF-4, −5). This is consistent with *D. melanogaster*, which expresses four sNPF copies ending with either RLRFa (Drome-sNPF-1 and 2) or RLRWa (Drome-sNPF-3 and 4), and with other dipterans [19–21]. In contrast, non-

Abbreviations: N-terminus, amino-terminus; C-terminu, scarboxy-terminus; cDNA, complementary deoxyribonucleic acid; CHO, Chinese hamster oocyte; cAMP, cyclic adenosine monophosphate; ECL, extracellular loop; EC_{50}, half maximal effective concentration; HEK cells, human embryonic kidney cells; ICL, intracellular loop; NA, not active; NPYLR1, neuropeptide Y-like receptor; PCR, polymerase chain reaction; sNPF, short neuropeptide; sNPFR, short neuropeptide F receptor; TM, transmembrane

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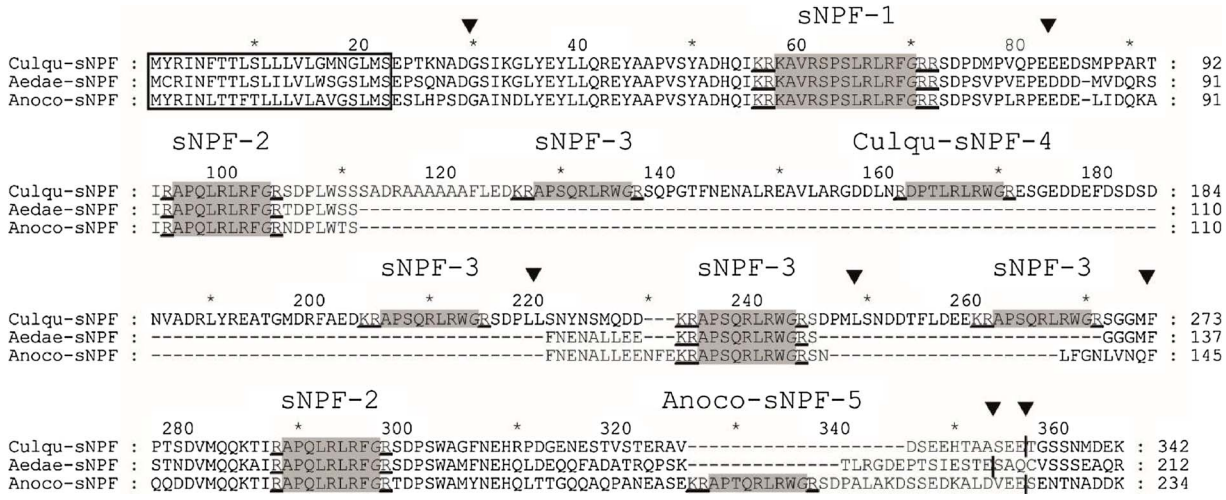

Fig. 1. Sequence of mosquito sNPF precursors. The deduced sequences were obtained for *Aedes aegypti* (Aedae; AAEL012542) and *Anopheles coluzzii* (Anoco; DQ437578.1). The sequence for *Culex quinquefasciatus* was obtained by comparison with the sequences of the other species and cDNA sequencing (Culqu; Supplementary Table 3). The signal peptide was predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP; [40]) and is highlighted in a box (black). Introns are indicated by arrowheads. Predicted mature neuropeptides are highlighted in gray. Glycine residues that encode the C-terminal post-translational modification to an amide are indicated in italics, and the basic recognition sites for neuropeptide cleavage are underlined.

dipteran insects only have one or two unique sNPFs encoded by the precursor [21].

The sNPF receptors (sNPFRs) have been identified and functionally characterized in both *Ae. aegypti* (NPYLR1; [1]) and *An. coluzzii* (Anoco-sNPFR; [22]). Garczynski and colleagues [22,23] also investigated the structure-function relationship between the neuropeptide sequence and the receptor affinity in *An. coluzzii* and *D. melanogaster*. These studies revealed a higher affinity of both receptors for long sNPFs, and for sNPFs with a carboxy (C)-terminal motif of RLRFa rather than RLRWa, supporting preceding studies in *D. melanogaster* [24,25]. In contrast, Mertens et al. [26] showed an equipotent affinity of the *D. melanogaster* receptor for endogenous isoforms that was independent of length or C-terminus. Similarly, neither the *Ae. aegypti* NPYLR1, nor the sNPFRs of other insects, demonstrate a functional affinity that correlates with the endogenous sNPF isoforms sequences [1,16,27]. Using receptor modelling, Bass et al. [28] predicted the conserved residues involved in the ligand interaction with the *D. melanogaster* RFamide receptors, including the sNPFR. Three of the eight identified residue motifs were found in extracellular loops (ECLs; [28]). Another four residues were identified in the transmembrane domains (TMs), oriented to face into a putative ligand-binding pocket [28]. This suggests that these sites confer the distinct binding characteristics of the sNPF and other FRamide receptors [28]. The eighth residue, while conserved, was not available for ligand interaction.

To further characterize the role of neuropeptide signaling in regulating host seeking and feeding in mosquitoes, we identified the *sNPF* receptor and precursor genes in the southern house mosquito, *Culex quinquefasciatus*, a competent arbovirus vector. The functional characteristics of the sNPF receptors from *Cx. quinquefasciatus*, *Ae. aegypti,* and *An. coluzzii* are compared and discussed in the context of precursor and receptor gene expression at two time points following blood and sugar feeding.

2. Material and methods

2.1. Animal rearing

Aedes aegypti (Rockefeller strain), *An. coluzzii* (Suakoko strain; formerly *An. gambiae* M-molecular form) and *Cx. quinquefasciatus* (Thai strain) were reared at a 12 h:12 h light: dark cycle, and 27 ± 1 °C, 70% relative humidity, as previously described [29].

2.2. Nomenclature

The nomenclature used here was adopted from Yeoh et al. [30], with a few modifications. A five-letter code was used for interspecific isoforms, and for the intraspecific isoforms, the numbering was based on the order of the isoforms in the precursor. For neuropeptide isoforms conserved in all three species, we omitted the five-letter code. In addition, isoforms with identical amino acid sequence are only referred to using the name with the lowest number. The two isoforms without duplicates in the other species were named using the species-specific nomenclature (Culqu-sNPF-4 and Anoco-sNPF-5).

2.3. Database mining and sequence comparisons

The genes for the *Ae. aegypti* and *An. coluzzii sNPFRs* and *sNPF* precursors were identified in the genomes available at VectorBase (https://www.vectorbase.org; AaegL3.3; AgamP4.3) and compared with those already published ([1,17,22]; Supplementary Table 1). The *Cx. quinquefasciatus sNPFR* was identified following comparison with the other mosquito sequences (https://www.vectorbase.org; CpipJ2.2). The mosquito *sNPFR* genes were amplified from cDNA, using specific primers (Supplementary Table 2) based on the available sequences, cloned (see 2.4) and their identity confirmed by sequencing. The sequence of the *Cx. quinquefasciatus sNPF* precursor was derived from transcript assemblies based on transcriptome data (SRR1005577; [31]), and identified by direct sequencing from PCR products generated from cDNA using gene specific primers (Fig. 1; Supplementary Table 2). For cDNA generation and PCR protocols see 2.4.

For structural comparison of the mosquito sNPFRs, the amino acid sequences were aligned and conserved amino acids determined in GeneDoc [32]. Transmembrane domains were determined based on the TMs described in [33], and modified according to [28] and motifs identified in PROSITE [34]. In addition, two-dimensional representations of the receptor three-dimensional structure were created in TOPO2 (Johns S.J., TOPO2, transmembrane protein display software, http://www.sacs.ucsf.edu/TOPO2). Sequence similarities between sNPFRs were determined using the sequence identity and similarity tool (SIAS; http://imed.med.ucm.es/Tools/sias.html). A phylogenetic tree was constructed using MrBayes 3.2.3 (http://www.phylogeny.fr/) [35–37], based on a CLUSTALW alignment of full length predicted sNPFR protein sequences, including the Pacific oyster, *Crassostrea gigas*, as an outgroup. The likelihood model used was Blosum62, and the