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The short neuropeptide F modulates olfactory sensitivity of *Bactrocera dorsalis* upon starvation

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ABSTRACT

The insect short neuropeptide F (sNPF) family has been shown to modulate diverse physiological processes, such as feeding, appetitive olfactory behavior, locomotion, sleep homeostasis and hormone release. In this study, we identified the sNPF (BdsNPF) and its receptor (BdsNPFR) in an important agricultural pest, the oriental fruit fly *Bactrocera dorsalis* (Hendel). Afterwards, the receptor cDNA was functionally expressed in Chinese hamster ovary cell lines. Activation of BdsNPFR by sNPF peptides caused an increase in intracellular calcium ions, with a 50% effective concentration values at the nanomolar level. As indicated by qPCR, the *BdsNPF* and *BdsNPFR* transcripts were mainly detected in the central nervous system and antennae, and they showed significantly starvation-induced expression patterns. Furthermore, we found that the starved flies had an increased electroantennogram response compared to the normally fed flies. However, this enhanced olfactory sensitivity was reversed when we decreased the expression of *BdsNPF* by double-stranded RNA injection in adults. We concluded that sNPF plays an important role in modulating the olfactory sensitivity of *B. dorsalis* upon starvation. Our results will facilitate the understanding of the regulation of early olfactory processing in *B. dorsalis*.

1. Introduction

In insects, neuropeptides are essential signaling molecules that regulate nearly all physiological processes, such as reproduction, growth, metabolism and behavior (Altstein and Nassel, 2010). One of these insect neuropeptides is the short neuropeptide F (sNPF), whose functions draw extensive attention in many insects, especially in *Drosophila* (Nassel and Wegener, 2011). sNPF serves as neuro-regulator with a key role in a variety of physiological processes such as feeding (Dillen et al., 2014; Lee et al., 2008, 2004), regulation of locomotion (Kahsai et al., 2010), sleep homeostasis (Chen et al., 2013; Shang et al., 2013), control of hormone release (Nassel et al., 2008) and olfaction (Ko et al., 2015; Root et al., 2011). Unfortunately, the studies on sNPF functions in other insect species are still very limited (Brown et al., 1994; Dillen et al., 2014; Mikani et al., 2012).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a destructive agricultural pest in tropical and subtropical areas of the world. It attacks over 250 host plants by ovipositing inside them, where the larvae feed until pupation. Its wide distribution and polyphagy feature cause severe economic loss in many countries (Clarke et al., 2005; Stephens et al., 2007). Recently, we found that oriental fruit flies showed hyperactivity and enhanced olfactory sensitivity when they are starved. Similarly, starvation-induced hyperactivity was also observed in *Drosophila*. Further investigation demonstrated that octopamine, the insect counterpart of the vertebrate norepinephrine, as well as the neurons expressing octopamine, were both necessary and sufficient for starvation-induced vigorous locomotion (Yang et al., 2015). Furthermore, another study found that starved *Drosophila* exhibited an enhanced sensitivity to attractive odors due to an elevated sNPF signaling in the antennal lobe glomeruli wired for early olfactory processing (Ko et al., 2015; Root et al., 2011).

Therefore, in order to know whether the sNPF is playing the same role in *B. dorsalis* as in *Drosophila* and to have a better understanding of the olfaction processing in this agriculturally important pest fruit fly under starvation stress, we investigated the molecular characteristics of *B. dorsalis* sNPF (BdsNPF) and its receptor. Subsequently, a calcium reporter assay was performed to further confirm that the BdsNPF receptor (BdsNPFR) is functional with specific binding of sNPF peptide. In addition, we investigated the effects of BdsNPF on the olfactory sensitivity in the antennae of *B. dorsalis* flies by the injection of BdsNPF-

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dsRNA, when they encountered starvation.

2. Materials and methods

2.1. Test insects

The oriental fruit flies were reared as described previously (Shen et al., 2013). Briefly, *B. dorsalis* were reared in a nylon mesh covered cage placed in a growth chamber under constant temperature of 27 ± 1 °C, with a relative humidity of 70 ± 5%, and under a 14:10 (L:D) photoperiod.

2.2. Primers, peptides, plasmids and chemicals

Primers used in this study were synthesized by Invitrogen (Shanghai, China). The putative mature peptides of sNPF from *B. dorsalis* and *Tribolium castaneum* were synthesized by Genescript (Nanjing, China). The plasmids with high purity for transfection were prepared using a QIAGEN Plasmid *Plus* Midi kit (Valencia, CA). DMEM/F12 medium, fetal bovine serum (FBS), fungizone and penicillin/streptomycin for cell culture, as well as coelenterazine for incubation with the cells prior to the functional assays were all purchased from Gibco cell culture at Life Technologies (Grand Island, NY). The transfection reagent (TransIt) was purchased from Mirus Bio (Madison, WI).

2.3. Identification, cloning and sequence analysis of BdsNPF and BdsNPFR

A BlastP was conducted using the *Drosophila sNPF* and *sNPFR* against the MAKER predicted protein database of *B. dorsalis* on the i5k website https://i5k.nal.usda.gov/webapp/blast/. Primers used for the cloning of the full open reading frame (ORF) were designed based on the *B. dorsalis* genome data (Table S1). For *BdsNPF*, we started with the MAKER prediction ID 155900. Meanwhile, we started with MAKER prediction ID 217498 for *BdsNPFR*. Noticeably, the predicted *BdsNPFR* possessed 8 rather than 7 transmembrane domains (it was 76 amino acids longer at the N terminus) compared to the functional *BdsNPFR*, which was later experimentally confirmed.

RNA extraction was conducted from 2 PM to 5 PM of the day. Total RNA was extracted from four pooled adult flies (2 males and 2 females) using TRIzol reagent (Invitrogen, Carlsbad, CA), with a genomic DNA elimination by an RQ1 DNase I (Promega, Madison, WI) treatment. The RNA was further purified by a phenol-chloroform extraction prior to the reverse transcription. First-strand cDNA was synthesized by GoScript Reverse Transcription System (Promega) with random hexamers in a total volume of 20 µl, according to the user's manual. The ORF of *BdsNPF* and the *BdsNPFR* were amplified by a nested PCR using high fidelity DNA polymerase PrimeSTAR (Takara, Dalian, China). PCR conditions were set as follows: initial denaturation at 98 °C for 2 min, followed by 35 cycles of 15 s at 98 °C, 15 s at 58 °C, and 1.5 min at 72 °C, and final extension of 10 min at 72 °C. The PCR reaction was 50 µl including 24 µl of ultrapure water, 20 µl of 2 × PrimeSTAR Max Premix (TaKaRa), 2 µl of each primer (10 µM), and 2 µl of the cDNA template. PCR products were purified and cloned into a pGEMT Easy vector (Promega) and sequenced (BGI, Beijing, China).

Sequence alignment of BdsNPFR with several sNPFRs from other insect species was produced in JalView 2.9 (Waterhouse et al., 2009). Transmembrane helices were predicted using a TMHMM server (http://www.cbs.dtu.dk/services/TMHMM). Signal peptide of BdsNPF was predicted using the SignalP server (http://www.cbs.dtu.dk/services/SignalP), and sequence logos for the C-terminal motifs of BdsNPF were generated by Weblogo (Crooks et al., 2004). A phylogenetic tree was constructed using and the neighbor-joining method with 1000 bootstrap tests in MEGA 5.0 (Tamura et al., 2011).

2.4. Heterologous expression and calcium reporter assay

After the sequence confirmation including Sanger sequencing (BGI, Beijing, China), the ORF of the *BdsNPFR* was inserted into the mammalian expression vector pcDNA3.1(+) with the CMV promoter. The high-quality plasmid was transfected in Chinese Hamster Ovary (CHO-WTA11) cells supplemented with aequorin and Gα16 using TransIt transfection reagent for heterologous expression. Thirty hours post-transfection, cells were collected and followed by an incubation with the coelenterazine (Invitrogen) for 3 h prior to the functional assays according to published methods (Aikins et al., 2008; Jiang et al., 2013). Luminescence caused by the intracellular calcium mobilization was measured using a TriStar^2 LB 942 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany). Briefly, 50 µl test ligands were firstly added to a well in the microplate, another 50 µl of pre-incubated cell suspension were injected into the wells containing the test ligands by the automatic injector on the TriStar^2 reader. The luminescence was measured in continuous for 20 s at half-second intervals. In our assay, 2 putative mature peptides (BdsNPF2 and BdsNPF4), neuropeptide F (BdNPF, YGDRARARFamide) as predicted from the genome of *B. dorsalis*, and TcsNPF (SPSLRLRFamide, based on the genome of *T. castaneum*) were tested. Ten-fold serial dilutions (ranging from 0.01 nM to 10 µM) of the tested peptides were applied to the cells. Concentration-response curves of the receptor to the ligands were generated using logistic fitting in Origin 8.6 (OriginLab), based on luminescent values. All experiments were conducted in three biological replicates.

2.5. Quantitative real-time PCR

Samples were prepared to investigate developmental and spatial transcription patterns of *BdsNPF* and *BdsNPFR*, according to a previously established protocol (Gui et al., 2017). For different developmental stages, we collected samples including eggs, larvae (1-, 4- and 7-day), pupae (1-, 4- and 7-day), and adults (1-, 5- and 9-day). Meanwhile, the central nervous system (CNS), Malpighian tubules, midgut, hindgut, antennae, female ovary and male testes were dissected from 5-day-old adults in chilled PBS (pH 7.4). Total RNA extraction and synthesis of the first strand cDNA were conducted as mentioned above.

The qRT-PCR was performed on an Mx3000P thermal cycler (Stratagene, La Jolla, CA). The reaction system contained 5 µl of GoTaq qPCR Master Mix (Promega), 0.5 µl of cDNA samples (approximately 200 ng/µl), 0.3 µl of each primer (10 µM), and 3.9 µl of nuclease-free water. The thermal cycling condition was set as follows: an initial denaturation at 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. A melting curve analysis from 55 to 95 °C was performed for all reactions to ensure the specificity and consistency of all generated products. Primers used in qRT-PCR are shown in Table S1. The previously evaluated gene *α-tubulin* (GenBank accession number GU269902) served as the internal reference gene (Shen et al., 2010). All experiments were performed in three biological replicates. For the relative expression calculation, target transcript expression in 7-day-old pupae was set as a calibrator for developmental transcription, while target transcript transcription in the CNS was a calibrator for tissue specific transcription of *BdsNPF* and *BdsNPFR*.

2.6. Design of different feeding conditions

For different feeding conditions, the mRNA transcript levels and electroantennogram (EAG) responses were determined with starved and fed adults by qRT-PCR and EAG recording separately. The design of different feeding conditions were performed as previously described with slight modification (Van Wielendaele et al., 2013). Five-day-old flies (both sexes) were employed in this study. Briefly, we removed the food for 6 h to ensure all flies were in the same "feeding state". Subsequently, these flies were allowed to have one meal. Afterwards, flies under the "starved" condition did not receive food while the flies