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Molecular characterization and expression profiles of neuropeptide precursors in the migratory locust

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ABSTRACT

Neuropeptides serve as the most important regulatory signals in insects. Many neuropeptides and their precursors have been identified in terms of the contig sequences of whole genome information of the migratory locust (*Locusta migratoria*), which exhibits a typical phenotypic plasticity in morphology, behavior and physiology. However, functions of these locust neuropeptides are largely unknown. In this study, we first revised the 23 reported neuropeptide precursor genes and identified almost all the neuropeptide precursors and corresponding products in *L. migratoria*. We further revealed the significant expansion profiles (such as AKH) and alternative splicing of neuropeptide genes (*Lom-ITP*, *Lom-OK* and *Lom-NPF1*). Transcriptomic analysis indicated that several neuropeptides, such as *Lom-ACP* and *Lom-OK*, displayed development-specific expression patterns. qRT-PCR data confirmed that most neuropeptide precursors were strongly expressed in the central nervous system. Fifteen neuropeptide genes displayed different expression levels between solitarious and gregarious locusts. These findings provide valuable clues to understand neuropeptide evolution and their functional roles in basic biology and phase transition in locusts.

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1. Introduction

Neuropeptides serve as the most diverse group of neural signaling molecules in insect physiology. Larger precursors of these signaling messengers are produced mainly in neurons and endocrine cells, after which they are cleaved and modified into mature peptides, then secreted into the extracellular parts (Veenstra, 2000). Alternative splicing often occurs in neuropeptide precursor genes to produce more mature peptides, thereby strongly enhancing neuropeptide diversity (Dircksen et al., 2011; Veenstra, 2014). Mature peptides bind to specific membrane receptors and stimulate intracellular molecules, thus performing their biological functions (Xu et al., 2010). A large number of neuropeptides and their precursors have been characterized in various insects with sequenced genomes (Baggerman et al., 2002; Broeck, 2001; Hummon et al., 2006; Li et al., 2008; Roller et al., 2008). Their significance, for example, in the development, reproduction, metabolism, feeding and locomotion of model insects has been widely reported (Brogiolo et al., 2001; Janssen et al., 2001; Nassel, 2002). However, studies on neuropeptide functions are seriously hindered by the limited number of identified precursor genes in other insects.

The migratory locust (*Locusta migratoria*) is an ideal model for neuropeptide research (Clynen and Schoofs, 2009) and displays remarkable density-dependent phase changes from harmless solitarious forms to destructive gregarious forms that cause serious losses in agriculture (Uvarov, 1977). The transition process is accompanied by changes in many aspects, including morphology, physiology and behavior (Verlinden et al., 2009). Various studies demonstrated that changes in neural network and several neuromodulators, such as dopamine (Ma et al., 2011), serotonin (Guo et al., 2013), octopamine and tyramine (Ma et al., 2015), play key regulatory roles in phase transition in *L. migratoria*. As important neuromodulators, neuropeptides are undoubtedly closely related to phase-specific characteristics. [His7]-corazonin was reported to induce dark color and morphometric changes in solitarious locusts in both *L. migratoria* and *Schistocerca gregaria* (Maeno et al., 2007; Tawfik et al., 1999). Adipokinetic hormone (AKH) displays

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different amounts in corpora cardiaca of crowded and isolated migratory locusts (Ayali et al., 1996b). In spite of these, it still remains largely unclear how neuropeptides participate in locust behavior and physiology.

Base on peptidomimetics, plenty of mature neuropeptides have been biochemically identified in two important locust species, *L. migratoria* and *S. gregaria* (Clynen and Schoofs, 2009; Homberg, 2002; Schoofs et al., 1997). In fact, identification of neuropeptide precursors does not progress because the limited availability of genome data hinders the implementation of functional studies on these neuropeptides in locusts. Only several neuropeptide precursors have been identified (Clynen et al., 2006) from our previously published Expressed Sequence Tag (EST) database of *L. migratoria* (Kang et al., 2004). Apparently, discovery of all precursor genes was difficult because of their small size and low transcription levels without the entire genome information. Until recently, dozens of genes encoding neuropeptide precursors have been reported (Veenstra, 2014) on the basis of the contig sequences of *L. migratoria* genome (Wang et al., 2014). Nevertheless, many of these neuropeptide genes are incomplete with missing 5′ or 3′ encoding sequences in the prediction, which should be further confirmed and corrected.

To elucidate the distinct characteristics and functions of neuropeptides and their precursors in locusts, we performed a lot of analysis and experiments in bioinformatics and molecular biology. In this study, we revised the gene sequences of the reported neuropeptide precursors by combining bioinformatics analyses based on the whole genome sequences and transcriptome sequences. We then analyzed the phylogenetic relationship of several neuropeptide families and represented alternative splicing of three neuropeptide genes. We further determined tissue-specific, development-related and phase-dependent expression patterns of neuropeptide precursors by transcriptomic and qRT-PCR analyses.

2. Material and methods

2.1. Animals

Both gregarious and solitarious locusts were raised in the Institute of Zoology, Chinese Academy of Sciences, Beijing. The gregarious locusts were maintained in large cages (40 cm × 40 cm × 40 cm) at a density of 400–500 insects per cage. The solitarious locusts were reared separately in metal boxes (10 cm × 10 cm × 25 cm) supplied with charcoal-filtered compressed air. Both colonies were maintained at 30 ± 2 °C, under a 14:10 light/dark photocycle regime, and a diet of fresh wheat seedling and bran.

2.2. Gene prediction and identification

The whole genome sequences of *L. migratoria* (Wang et al., 2014), predicted 17,307 genes, were used for homology searching in the locust genome database. Gene structure and open reading frame (ORF) were predicted in GENBOREE (http://www.genboree.org). Short-matching sequences were obtained using known neuropeptide (precursor) sequences from *L. migratoria*, *S. gregaria*, and *Drosophila melanogaster.* Subsequently, gene structure prediction was performed. Resulting neuropeptide (precursor) gene sequences were then searched in published EST or transcriptome data for further completion of ORFs (Chen et al., 2010; Kang et al., 2004). RT-PCR was performed using specific primers, and the PCR products were sequenced to confirm the predicted gene sequences. Detailed gene sequences were individually compared with the recently published neuropeptide precursors by using DNAMAN software. Multiple alignments and phylogenetic analysis were performed using Genedoc program and MEGA software, respectively. Signal peptides were predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Putative cleavage sites in the precursors were analyzed as described by Veenstra (2000) or using web-based NeuroPred program (http://neuroproteomics.scs.illinois.edu/cgi-bin/neuropred.py).

2.3. Preparation of samples

Locust tissues, including antenna, brain, pronotum, thoracic ganglia, midgut and hind leg from both gregarious and solitarious fourth-instar nymphs (i.e., 48 h after ecdysis), were collected and immediately kept in liquid nitrogen. For pronotum, midgut and hind leg, each sample contained four individuals (i.e., two males and two females). For brain and thoracic ganglia, eight individuals (i.e., four males and four females) were collected for each sample. At least four independent biological replicates were prepared for further experiments.

2.4. PCR procedure, product sequencing, and qRT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. The Qiagen DNase I Set was used to remove the residual genome DNA. RNA purity (A260/A280) was measured between 1.8 and 2.2. cDNA was reverse-transcribed from 2 μg of total RNA using MMLV reverse transcriptase (Promega). Obtained cDNA was diluted with ddH_2O in 1:4 ratio for further use. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with Light Cycle 480 SYBR Green I Master kit (Roche). *Rp49* was used as internal reference. For alternative splicing genes (*Lom-ITP*, *Lom-MS*, *Lom-NPF1*, *Lom-OK* and *Lom-NPP*), specific primers for each isoform were designed to detect their distinct transcription levels. PCR products sequencing and melting curve analysis were performed to confirm the specific amplification of each alternative splicing forms. Gene expression levels were presented with $2^{-\Delta Ct}$ values ($\Delta Ct = Ct_{target} - Ct_{reference}$).

For normal RT-PCR, cDNA was obtained using the same method as that for qRT-PCR. Gene-specific primers were designed from the predicted precursor gene sequences and synthesized commercially (SANGON, Beijing, China) (Supplementary Table 2). PCR procedures were performed as follows: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 55 °C–60 °C for 30 s (depending on respective genes), 72 °C for 50 s; and 72 °C for 1 min. PCR products were sequenced to validate the predicted neuropeptide gene sequences.

2.5. Statistical analysis

Data from qRT-PCR were analyzed using Student's *t*-test in SPSS software. Values from four independent experiments were presented as mean ± standard error (SE). Clustal W analysis of tissue distribution was performed using Cluster 3.1 and TreeView software.

For transcriptomic analysis, RNA-seq reads in different developmental stages from Chen's work were mapped to the genome sequence using TopHat, as described previously (Wang et al., 2014). Briefly, gene expression levels were calculated using the reads per kb million mapped (RPKM) reads criteria. The total number of reads was normalized by multiplying with normalization factors. RPKM values of neuropeptide genes during locust development were presented using SigmaPlot software.

3. Results

3.1. Revision of neuropeptide precursor genes

On the basis of the genome sequences of *L. migratoria*, we independently conducted a prediction work for neuropeptide

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