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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.

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

precursor genes. By carefully comparing the neuropeptide gene sequences in our prediction with those from Veenstra's publication (2014), we found that more than twenty sequences of reported neuropeptide genes were incomplete with absent 5′ or 3′ open reading frame (ORF) encoding sequences. We finally revised 21 neuropeptide precursor genes by extending their 5′ or 3′ sequences, and filled the gaps in ORF encoding sequences of another 2 neuropeptide genes (*AST-A* and *DH-CRF*) (Table 1). The revised sequences were either supported by the transcriptome database or confirmed by RT-PCR. Detailed revision information for 23 neuropeptide genes was shown in Supplementary Fig. 1. We also summarized all identified neuropeptides and protein hormones in *L. migratoria* (a total of 62 neuropeptide genes, including alternative splicing) (Supplementary Table 1). Currently, nearly all of the identified precursors cover the corresponding mature peptides previously captured from peptidomimetics, except for four protein hormones, namely, Bursicon hormones (α and β) and Glycoprotein hormones (A2 and B5), because of the absence of their complete ORFs. Up to 23 precursor genes encoding >60 possible active peptides have never been experimentally characterized (Supplementary Table 1, marked with triangles). Neuropeptide precursors that have obvious expansion and alternative splicing in *L. migratoria* were analyzed as discussed below.

3.2. Evolutionarily related peptide families: AKH, corazonin and ACP

Among all insect species with sequenced genomes, *L. migratoria* contains the largest number of AKH precursors. Precursors encoding ACP and corazonin, both of which are considered evolutionarily related peptide families with AKH (Hansen et al., 2010), were also discovered in the *L. migratoria* genome. Comparing with *L. migratoria*, members of the three evolutionarily related neuropeptide families were selectively lost in other insect species, such as *D. melanogaster*, *Apis mellifera*, *Tribolium castaneum* and *Bombyx mori* (Table 2). Phylogenetic analysis suggested that all corazonin precursors in insects belonged to an independent group, which seemed evolutionarily divergent. Evolutionary relationship between insect AKHs and ACPs were closer. All AKH precursors from *L. migratoria*, *B. mori* and *T. castaneum* belonged to the same cluster distinct from Lom-ACP precursor. By contrast, AKH precursors in *Aedes aegypti* and *Anopheles gambiae* were evolutionarily divergent. AKH1 from the two mosquito species were much closer to insect AKH, whereas AKH2 from the two species were evolutionarily closer to the ACP precursors (Fig. 1).

Table 2
Evolutionary related neuropeptide precursors identified in *Locusta*, *Drosophila*, *Apis*, *Tribolium* and *Bombyx*.

Neuropeptide	*Locusta*	*Drosophila*	*Apis*	*Tribolium*	*Bombyx*
AKH	4	1	1	2	2
ACP	1	nd	nd	1	1
Corazonin	1	1	1	nd	1

3.3. Allatostatins

Four members of the allatostatin (AST) neuropeptide family, which were discovered as inhibitors of juvenile hormone synthesis, were predicted according to the locust genome sequence (Wang et al., 2014). Lom-AST-A precursor contained 10 possible mature peptides (Fig. 2A), and only Lom-AST-A4 and Lom-AST-A9 were found in the previous peptidomimetics (Clynen and Schoofs, 2009). Lom-AST-B precursor contain 11 possible active peptides, including three repeat sequences (Fig. 2B), seven of which were novel predicted peptides; only one was confirmed by MS (Schoofs et al., 1991). Both *Lom-AST-C* and *Lom-AST-CC* encode only one predicted peptide (Fig. 2C). However, the newly predicted Lom-AST-CC from its precursors was not consistent with the previously described AST-CC (Veenstra, 2009), hence requiring confirmation. AST peptide systems were also compared between two locust species, *L. migratoria* and *S. gregaria*. Alignment showed that AST-A peptides shared high similarity in the two locust species, especially in the conserved 'FG' sites and in the amidated C-terminus (Fig. 2A). Nearly all Lom-AST-A peptides exhibited an ortholog in *S. gregaria*. However, neither AST-B and AST-C/CC peptides nor their precursors were discovered in *S. gregaria* thus far.

3.4. Ion transport peptide, NPF1 and orcokinin

In addition to the previously described precursor genes, *Lom-neuroparsin* and *Lom-myosuppressin*, three neuropeptide genes

Table 1
Revised neuropeptides and protein hormones in *Locusta migratoria*.

Neuropeptide names	Peptide acronym	Accession number	Revised sequence	Predicted peptides
Allatostatin-A	Lom-AST-A	KP895542	middle	10
Allatostatin-B	Lom-AST-B	KP895543	5′-sequence	8
Allatotropin	Lom-AT	KP895541	5′-sequence	1
Bursicon-alpha	Lom-Burα	KP895534	5′-sequence	1
Bursicon-beta	Lom-Burβ	KP895535	5′-sequence	1
Corazonin	Lom-CRZ	KP895544	3′-sequence	1
CCHamide1	Lom-CCH1	KP895537	5′and 3′	1
CCHamide2	Lom-CCH2	KP895536	5′and 3′	1
Calcitonin1	Lom-Calc1	absent	5′-sequence	3
Diuretic hormone 31	Lom-DH31	KP895538	3′-sequence	1
Diuretic hormone-CRF	Lom-DH-CRF	P23465	middle	1
Elevenin	Lom-Elevenin	KP895548	3′-sequence	1
FMRFamide	Lom-FMRF-like 1	KP895545	5′ and 3′	6
Glycoprotein hormone A2	Lom-GPA2	KP895540	5′-sequence	1
Kinin (Leucokinin)	Lom-KIN	KP895546	5′-sequence	4
Natalisin	Lom-Natalisin	KP895550	5′ and 3′	6
Neuropeptide F2	Lom-NPF2	absent	3′-sequence	1
Neuropeptide like protein	Lom-NPLP	KP895547	5′ and 3′	5
Pigment-dispersing factor	Lom-PDF	KP895553	5′-sequence	1
Periviscerokinins (CAPA)	Lom-PVK	KP895549	3′-sequence	3
Tachykinins	Lom-TK	KP895539	3′-sequence	8
Trissin	Lom-Trissin	KP895551	5′ and 3′	1
Trypopyrokinin4	Lom-TPVK4	KP895552	3′-sequence	1

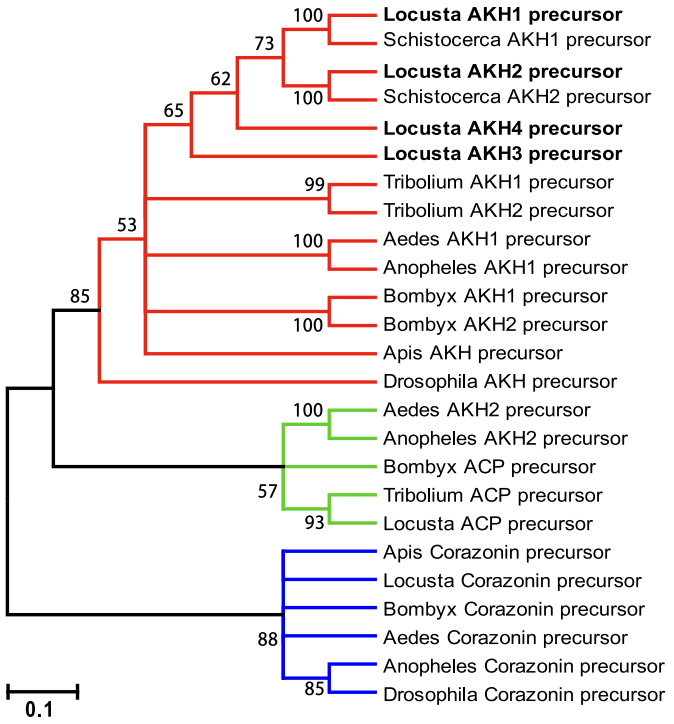

Fig. 1. Phylogenetic analysis of AKH, ACP, and corazonin precursors from several representative insect species, including *A. aegypti*, *A. gambiae*, *A. mellifera*, *B. mori*, *D. melanogaster*, *T. castaneum*, *S. gregaria* and *L. migratoria*. Four AKH members from *L. migratoria* are highlighted in bold font.

(*Lom-ITP*, *Lom-NPF1* and *Lom-OK*) were alternatively spliced into two transcripts encoding different neuropeptide precursors. *Lom-ITP* consists of three exons and two introns. The first exon shared by *Lom-ITP-A* and *Lom-ITP-B* encoded a 33 aa signal peptide. Lom-ITP-A was derived from coding regions of e1 and e3. By contrast, Lom-ITP-B was encoded by coding regions of e1 and e2. Consequently, each of these regions produced a mature neuropeptide with 95 aa or 87 aa, respectively (Fig. 3A). *Lom-NPF1* included five exons and encoded two different products by using a splicing pattern similar to that of *Lom-ITP*. Lom-NPF1a transcript was encoded by e1, e2, e3 and e4, thus resulting in a 36 aa neuropeptide. Lom-NPF1b mRNA was derived from all five exons and produced a much longer product with 86 aa (Fig. 3B). Splicing of *Lom-OK* not only altered the resulting peptide sequences but also strongly increased number of products. *Lom-OK* contained five exons and produced two different transcripts, namely Lom-OK-A and Lom-OK-B. Coding regions of e1 and e2 together with that in e3 encoded Lom-OK-B was further modified to 31 neuropeptides with six different types. Lom-OK-A was made up of e1, e2, e4 and e5, thus resulting in eight different putative neuropeptides. Among all five exons, e3 was the largest one, and even much longer than the total length of e4 and e5. Therefore, Lom-OK-B transcript was relative longer than Lom-OK-A and produced larger number of mature peptides. A signal peptide with 21 aa was shared by Lom-OK-A and Lom-OK-B (Fig. 3C).

3.5. Development-specific expression profiles of neuropeptide precursors

To investigate the functions of neuropeptides in the developmental process, we examined the expression profiles of neuropeptide precursor genes during locust development from egg to adult by analyzing our previous transcriptome data (Chen et al., 2010). Totally, RPKM values for 17 neuropeptide genes (including alternative splicing forms) were obtained. The average RPKM from gregarious and solitarious locusts was used to present the expression level for each neuropeptide gene. During the entire developmental process, *Lom-AST-C*, *Lom-GPB5*, *Lom-ILP*, *Lom-MS2*, *Lom-AT* and *Lom-AST-B* were mainly expressed in the middle nymph stages. By contrast, *Lom-ACP* and *Lom-OK-A* expression levels sharply increased from the fifth-nymph stage and remained at high levels until adult stage. *Lom-GPA2*, *Lom-ITP-A*, *Lom-ITP-B*, *Lom-AST-CC*, *Lom-MS1*, *Lom-DH-Calc*, *Lom-OK-B* and *Lom-Bur-β* were highly expressed in embryonic and early nymph stages. No expression of these genes was detected in the adult stage. Whereas *Lom-EH* displayed abundant expression level in the early nymph and adult stages rather than in embryonic stages (Fig. 4).

3.6. Tissue-specific expression patterns of neuropeptide precursors

The qRT-PCR experiment and analysis were employed to examine the tissue-specific expression patterns of 22 neuropeptide

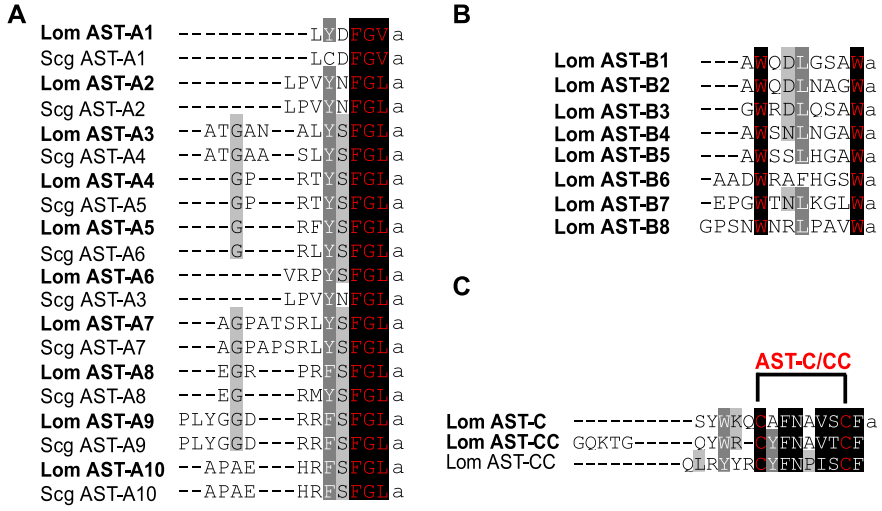

Fig. 2. Sequence alignment of AST neuropeptides in *L. migratoria* and *S. gregaria*. (A) Alignment of AST-A in the two locust species; (B) Alignment of eight AST-B peptides in *L. migratoria*; (C) Comparison of AST-C and AST-CC in *L. migratoria*. Red fonts show the conserved amino acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

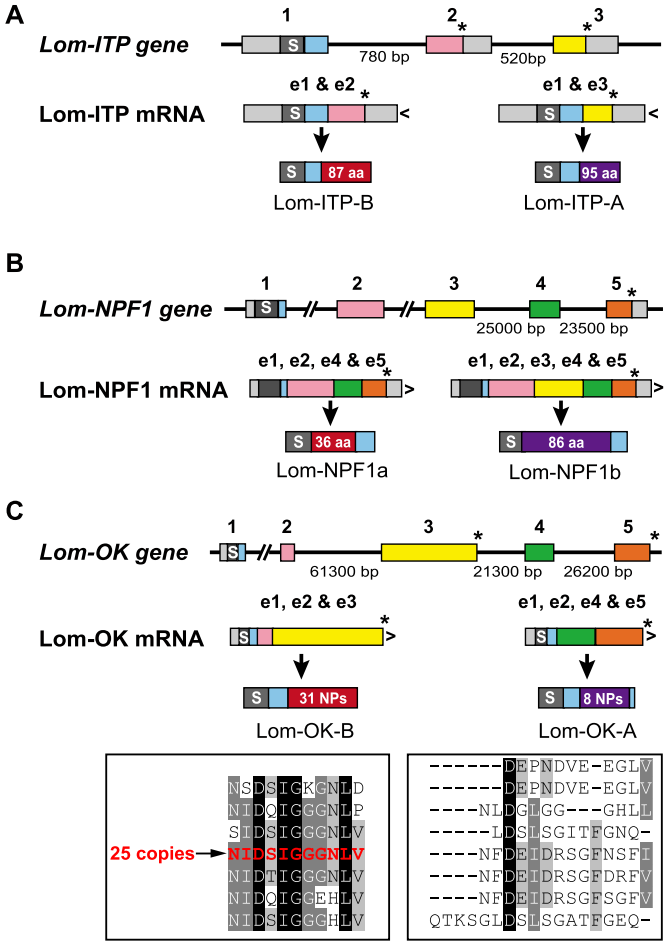

Fig. 3. Schematic of alternative splicing of *ion transport peptide* (*ITP*), *NPF1* and *orcokinin* (*OK*) in *L. migratoria*. Gene structures and alternative splicing forms of neuropeptide genes (A) *Lom-ITP*; (B) *Lom-NPF1*; (C) *Lom-OK*. Exons are boxed in different colors. Double slash indicates a long gap in the genome sequence. Numbers between the exons indicate approximate number nucleotides separating them. Putative products derived from alternative splicing forms are colored in red and purple. Light-gray shading indicate untranslated regions; S indicates signal peptide; e is exon; asterisk indicates stop codon; >, < means plus and minus strand reading direction, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

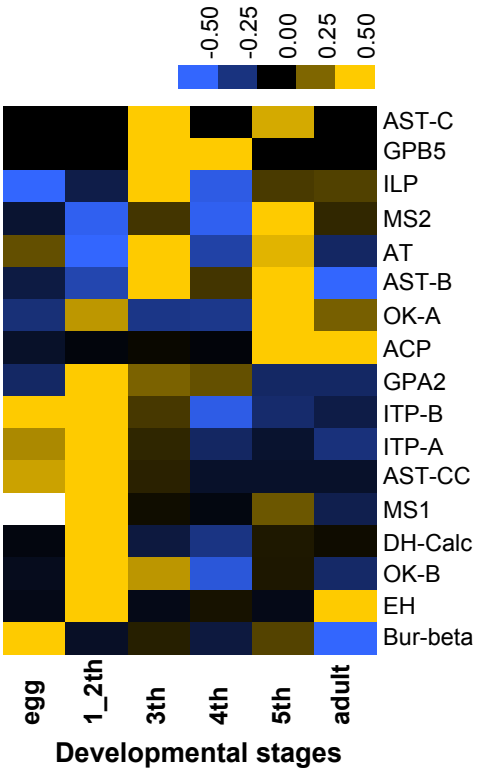

Fig. 4. Expression patterns of 17 neuropeptide genes in different developmental stages from egg to adult in locusts. Data were obtained from our previous transcriptome data (Chen et al., 2010) and presented in average RPKM from gregarious and solitarious locusts using Cluster software. Values for total reads were normalized by multiplying with normalization factors. Yellow indicates high expression patterns, whereas blue indicates low expression levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

precursors in the fourth-instar nymph stage. Six tissues, including the brain (Br), thoracic ganglia (Tg) and peripheral tissues, such as pronotum (Pn), antenna (An), hind leg (HL) and midgut (Mg) were investigated. The results proved that most of the precursor genes were restrained to the central nervous system (CNS), including those in Br and Tg. Several genes, such as NPF family members (*Lom-NPF1a*, *Lom-NPF2* and *Lom-sNPF*), *Lom-OK-A*, *Lom-OK-B*, *Lom-NPP1* and *Lom-TK*, also presented in the midgut compare with other peripheral tissues. Additionally, *Lom-NPF1b* and *Lom-NPP2* showed detectable transcript levels in Pn. Particularly, only *Lom-NPP1* was detected in HL (Fig. 5).

3.7. Expression patterns between gregarious and solitarious phases

To identify candidate neuropeptide genes involved in locust polyphenism, we compared expression profiles of 17 obtained neuropeptide genes between gregarious and solitarious phases based on transcriptome data. The analysis indicated that several genes displayed apparent phase-dependent expression patterns. *Lom-ACP* and *Lom-GPB5* showed significantly higher transcription levels in gregarious locusts, whereas *Lom-MS1*, *Lom-MS2*, *Lom-OK-B*, *Lom-ITP-A*, *Lom-ITP-B*, *Lom-AST-CC*, *Lom-EH* and *Lom-DH-Calc* were abundantly expressed in solitarious locusts. *Lom-OK-A* did not show obvious difference in expression levels between gregarious and solitarious locusts in the first-to fourth-instar nymph stages, but showed considerably higher transcription levels in gregarious locusts in the fifth-instar nymph until adult stage. Similarly, *Lom-ILP* displayed a higher expression level in gregarious locusts in the third-to fourth-instar nymph stages, whereas higher transcription level was observed in the solitarious phase in the fifth-instar nymph and adult stages (Fig. 6). No obvious difference was observed for other neuropeptide gene expressions (*Lom-AST-B*, *Lom-AST-C*, *Lom-AT*, *Lom-Bur-beta* and *Lom-GPA2*) (Supplementary Fig. 2).

Given that most of the neuropeptide genes were highly expressed in the CNS (Fig. 5), we determined the differences in neuropeptide gene expression in the CNS of gregarious and solitarious locusts. In details, we investigated expression patterns of 9 neuropeptide genes that have higher transcription levels in the transcriptome. Meanwhile, several neuropeptide precursors with essential functions on locust physiology and behavior were also analyzed. Varying transcription levels in the brain were observed among 15 genes. Eight genes (*Lom-ACP*, *Lom-AT*, *Lom-ILP*, *Lom-ITP-A*, *Lom-sNPF*, *Lom-NPP2*, *Lom-OK-A* and *Lom-TK*) displayed relatively higher transcription levels, whereas the other seven genes (*Lom-DH-Calc*, *Lom-MS1*, *Lom-NPF1a*, *Lom-NPF2*, *Lom-NPP4*, *Lom-NPP5* and *Lom-PDF*) showed lower expression levels in gregarious locusts (Fig. 7A). In the thoracic ganglia, five genes (*Lom-ACP*, *Lom-DH-Calc*,

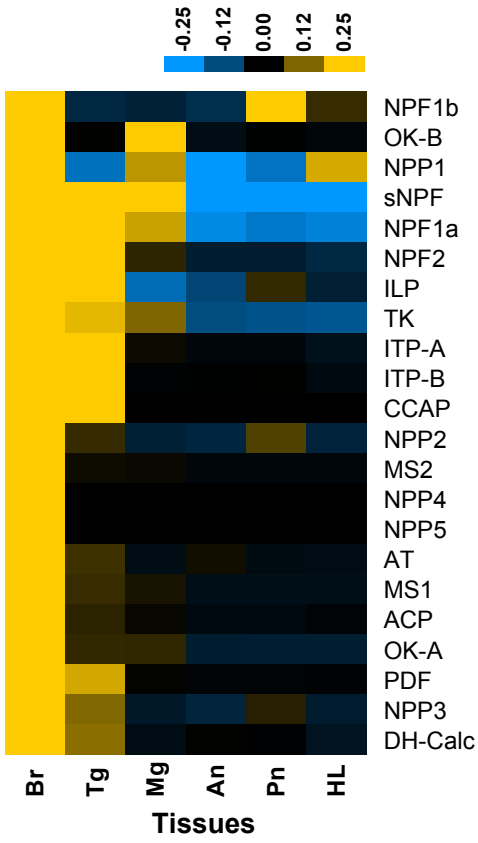

Fig. 5. Tissue-specific expression patterns of 22 neuropeptide genes in the fourth-instar gregarious nymphs analyzed by qRT-PCR. Hot-map indicates expression patterns of neuropeptide genes in six locust tissues including brain (Br), thoracic ganglia (Tg), and peripheral tissues antenna (An), pronotum (Pn), hind leg (HL) and midgut (Mg). Yellow indicates high expression patterns, whereas blue indicates low expression levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lom-CCAP, *Lom-sNPF* and *Lom-NPP3*) were differentially expressed between gregarious and solitarious locusts. *Lom-CCAP* and *Lom-NPP3* displayed higher transcription levels in solitarious locusts, whereas both *Lom-ACP* and *Lom-sNPF* were abundantly transcribed in gregarious locusts, similar to the pattern observed in the brain. By contrast, *Lom-DH-Calc* was highly transcribed in the thoracic ganglia of gregarious locusts with an opposite trend to that in the brain (Fig. 7B).

4. Discussion

Up to 62 precursors encoding over 140 putative peptides have been identified in *L. migratoria*. Combination of the whole genome and various transcriptome has improved the sequences of 23 neuropeptide precursor genes reported in a previous prediction (Veenstra, 2014). In fact, the numbers of neuropeptide precursor genes and their products may be even larger because some *Locusta*-specific neuropeptide genes cannot be identified by homolog searching. Nearly all the reported neuropeptides are covered by currently identified precursors except in several protein hormones owing to their incomplete precursors. Nevertheless, about 60 active peptides have been experimentally characterized in *L. migratoria* (Clynen et al., 2006; Clynen and Schoofs, 2009). Over half of the predicted peptides need to be confirmed by other methods in the future, especially collaborative efforts of bioinformatics and peptidomimtics.

The *L. migratoria* genome contains more neuropeptide genes than other insect species, such as *D. melanogaster*, *A. aegypti*, *A. mellifera* and *T. castaneum* (with 31, 31, 36 and 41 precursor genes, respectively) (Hewes and Taghert, 2001; Hummon et al., 2006; Li et al., 2008; Predel et al., 2010), probably because of less loss events and possible expansion of neuropeptide genes in the locust species. *L. migratoria* retains all AKH-related neuropeptide precursors (ACP and corazonin) and AST family members, whereas these precursors are all selectively lost in other insect species (Table 2). Moreover, the AKH neuropeptide family with four members expands further in *L. migratoria* compared with any other insect species. All AKH members in *L. migratoria* belong to the same evolutionary cluster (Fig. 1), suggesting they arose by duplication from the common ancestor. AKH can exert pleiotropic effects comprising metabolism, homeostasis, antioxidant defense and foraging behavior (Sajwan et al., 2015). Thus, the AKH expansion implies that this peptide family significantly participates in the evolutionary success of locusts, which can form huge aggregation and migration in long distances. Furthermore, AKH displays high expression levels in the gregarious phase of *L. migratoria* (Ayali et al., 1996b), further supporting its significant role in locust phase change and aggregation formation.

We also found several neuropeptide genes (*Lom-ITP*, *Lom-NPF1* and *Lom-OK*) that exhibited alternative splicing in *L. migratoria* (Fig. 3). Splicing events in neuropeptide genes have also been reported in several arthropod species, including *D. melanogaster* (Veenstra and Ida, 2014), *A. mellifera* (Pascual et al., 2004), *Zootermopsis nevadensis* (Veenstra, 2014) and water flea *Daphnia pulex* (Dircksen et al., 2011), suggesting a possible conserved mechanism underlying the generation of proteomic and functional diversity of neuropeptide genes in arthropods. Each alternative splicing form of *Lom-ITP*, *Lom-MS* and *Lom-NPP* presents distinct tissue- and phase-specific expression pattern, suggesting that they may undertake diverse biological significances. Moreover, alternative splicing of *Lom-OK* gene can produce a total of 39 mature neuropeptides with 15 different sequences, giving rise not only to different transcription products but also to an increased number of mature products. *Lom-OK-A* has much higher expression level than *Lom-OK-B* and shows gregarious-dependent transcription pattern in locust Br and Tg (Fig. 7), whereas *Lom-OK-B* is highly expressed in the whole body of solitarious locusts, imply their diverse significances in phase-related physiology. OK has been reported to serve as a prothoracicotropic regulator in the CNS of *B. mori* (Yamanaka et al., 2011) and modulate circadian locomotor activity in the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006). Thus, the phase-specific expression profiles of *Lom-OK-A* and *Lom-OK-B* may contribute to the regulation of the reported differential circadian rhythmicity of locomotion activity between gregarious and solitarious locusts (Ayali et al., 1996a).

Neuropeptide precursors, such as *Lom-ACP* and *Lom-ILP*, exhibited significant phase-related expression patterns during the developmental process in *L. migratoria*. *Lom-ACP* showed gregarious-upregulated transcription during the whole developmental stage (Fig. 4). This finding is also supported by the higher expression levels of *Lom-ACP* in the CNS of gregarious nymphs (Fig. 5). Although ACP shares high structural similarity with AKH and corazonin, ACP distribution and physiological effects seems totally distinct from the other two peptides. ACP showed no effect on either lipid levels or heart beat frequency compared with AKH and corazonin (Patel et al., 2014). Thus, the exact functional roles of ACP in locust phase change should be explored. ILP had been demonstrated to act as a vital growth and reproduction regulator in insects (Badisco et al., 2011; Sim and Denlinger, 2013). Multiple ILP members are discovered in *D. melanogaster*, *A. gambiae* and *T. castanetum* (Riehle et al., 2006), and even 40 ILP genes have also been

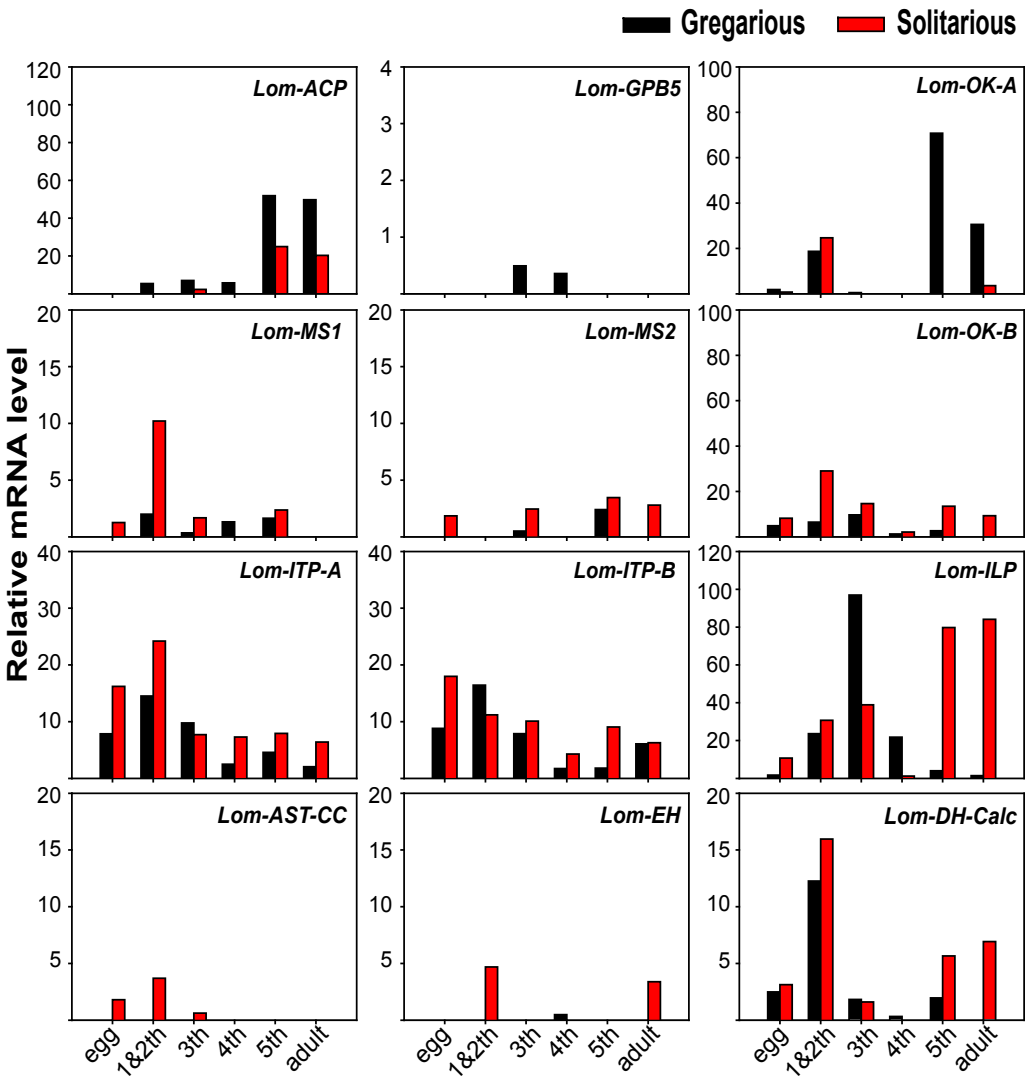

Fig. 6. Expression patterns of 12 neuropeptide genes between gregarious and solitarious locusts in different developmental stages. Gene expression levels were calculated using RPKM reads based on our previous transcriptome data.

predicted in *C. elegans*. Differential expression patterns of these ILP genes are reported to mediate the switch between the *C. elegans* developmental processes of reproductive growth and dauer arrest (Cornils et al., 2011). By contrast, only a single ILP gene is found in *L. migratoria*. Previous studies have suggested significant differences in the developmental and reproductive properties between gregarious and solitarious locusts (Chen et al., 2015). Therefore, the diverse expression patterns of *Lom-ILP* during locust development imply that the genes undertake regulatory roles in developmental rate in nymph or adult fertility. Furthermore, we found several solitarious-dependent neuropeptide genes (*Lom-MS*, *Lom-ITP*, *Lom-EH*, *Lom-DH-Calc* and *Lom-AST-CC*). As suggested by earlier studies, these genes participate in endocrine regulation on either metabolism or ecdysis behavior (Apone et al., 2014; Arakane et al., 2008; Zandawala, 2012). The coincident expression patterns these genes in the first- and second-stadium solitarious locusts may imply their coordinative regulation on phase-related growth and development at early nymph stage, as affected by their parent phase states.

Most of the present neuropeptide precursor genes exhibited abundant transcripts in the CNS of *L. migratoria* (Fig. 5), which is consistent with other insect species (Fontana and Crews, 2012; Herbert et al., 2010). Surprisingly, we also found that several neuropeptide precursors (*Lom-NPF1a*, *Lom-NPF2*, *Lom-sNPF*, *Lom-TK*, *Lom-OK-B* and *Lom-NPP1*) showed detectable expression levels in locust midgut. *OK*, *TK* and *ITP* are conserved brain-gut peptides, with functions including myotropic effects and regulation of ion and fluid transport (Audsley et al., 2013; Begum et al., 2009; Pascual et al., 2004; Predel et al., 2005). They may act as important factors to regulate peristalsis of digestive tract and food digestion in locust midgut. NPF members in invertebrates, as well as vertebrates, have been revealed as central regulators in feeding (Lee et al., 2004; Van Wielendaele et al., 2013). Few studies have reported the expression and functions of NPF family members in insect midgut. Our findings support the possibility that this peptide family plays significant roles in the digestive system.

We revealed that more phase-related genes are expressed in the brain than in the thoracic ganglia (Fig. 7), In spite of *Lom-ILP*, other fourteen novel phase-related neuronal markers were identified in the brain of migratory locusts. Gregarious-biased genes are *Lom-ACP*, *Lom-AT*, *Lom-ITP-A*, *Lom-sNPF*, *Lom-NPP2*, *Lom-OK-A* and *Lom-TK*, whereas solitarious-biased neuropeptide genes consist of *Lom-DH-Calc*, *Lom-MS1*, *Lom-NPF1a*, *Lom-NPF2*, *Lom-NPP4*, *Lom-NPP5* along with *Lom-PDF*. Studies on these neuropeptides revealed their distinct localizations in the CNS and their diverse biological

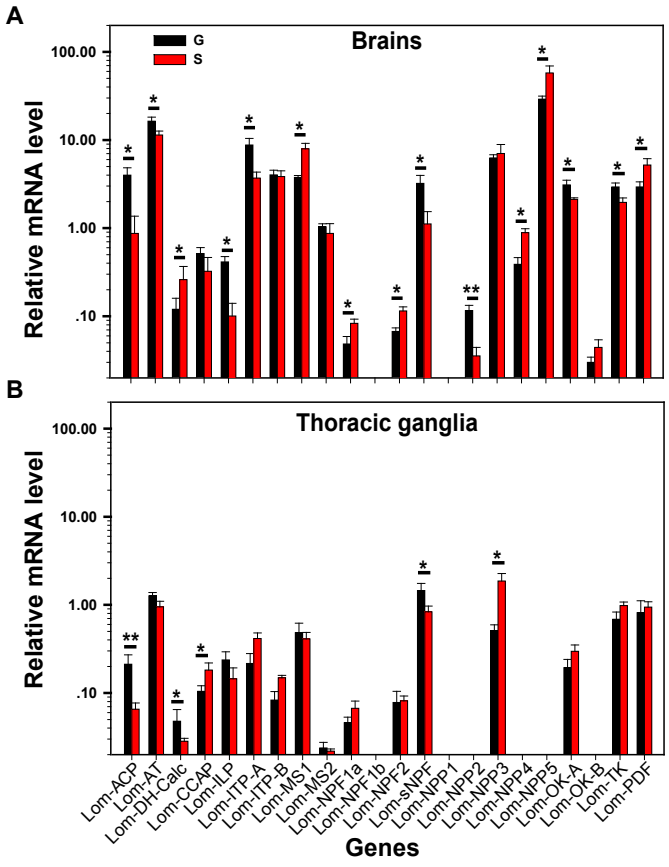

Fig. 7. Expression patterns of 22 neuropeptide genes in the CNS of fourth-instar gregarious and solitarious nymphs. (A) Expression levels of neuropeptide genes in brains between gregarious and solitarious nymphs; (B) Expression levels of neuropeptide genes in thoracic ganglia between gregarious and solitarious nymphs. Data were analyzed by Student's *t*-test and described as mean ± SEM ($n = 4$). *P < 0.05; **p < 0.01.

functions in multiple physiological processes (Homberg, 2002; Nassel, 2002). Single neuromodulator can influence certain phase-related traits only (Ernst et al., 2015; Ma et al., 2011; Wang and Kang, 2014). However, the locust phase traits include many aspects, such as body coloration, food selection, energy metabolism, developmental rate, reproductive physiology and behavior (Verlinden et al., 2009). Therefore, a coordinate regulatory mechanism of these phase-specific markers is indispensable for locust phase transition.

In summary, our results elucidated the special characteristics and expression profiles of neuropeptide precursors in *L. migratoria*, as well as provided fundamental information and clues to better understand the neuro-hormone mechanisms on basic biology and polyphenism of the locust species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.05.014.

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