COMPARISON OF PREDICTED AMINO ACID SEQUENCES OF ALLATOTROPIN/ALLATOSTATIN RECEPTORS FROM SOLITARY TO EUSOCIAL BEE SPECIES (HYMENOPTERA, APOIDEA)

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Abstract

An increasingly amount of evidence supports that the evolution of eusociality is accompanied by shifts in ancient molecular and physiological pathways. The juvenile hormone, one of the most important hormones in the post-embryonic development of insects, attracts the most attention in the context of social organization. Allatregulatory neuropeptides (Allatotropin, Allatostatin-A and Allatostatin-C) are known to regulate juvenile hormone synthesis and release in insects. In order to clarify the transitions of juvenile hormone synthesis involved in eusocial evolution, the substitutions of amino acid residues and the complexity of post-translational modifications in allatregulatory neuropeptide receptors were characterized. Both allatotropin and allatostatin receptors are identified in all examined bee species regardless if they are solitary or eusocial. Although the amino acid sequences are highly conserved, phylogenetic results are consistent with the eusocial status. The abundance of predicted post-translational modifications correlates with social complexity except for that in allatostatin-C receptors. Even though the consequences of these specific amino acid substitutions and various post-translational modification complexity have not been studied, they likely contribute to the localizing, binding and coupling characteristics of the receptor groups.

Keywords: allatostatin receptor, allatotropin receptor, bees, eusocial evolution, juvenile hormone, post-translational modifications

INTRODUCTION

Most bee species are solitary, but some exhibit diverse levels of social complexity, in which the colony size ranges from tens to thousands of individuals with intricate communication systems, morphological caste systems and labor division systems (Woodard et al., 2011; Kapheim et al., 2015). Based on the degree of social complexity, the bee species are sorted into advanced eusocial, primitively eusocial and solitary/subsocial groups (Cardinal & Danforth, 2011). Such diversity makes bee species an excellent model system for studying endocrine systems involved in the evolution of sociality (Woodard et al., 2011). Genomic signatures of evolutionary transitions indicate that the eusocial bee species have evolved independently due to independent genetic underpinnings. However, these road

maps of eusociality share general features, such as more constrained protein, less diversity and abundance of transposable elements, and more complicated gene regulation networks (Kapheim et al., 2015).

Juvenile hormone (JH) is one of the most important hormones during the post-embryonic development of insects (Areiza et al., 2014; Verlinden et al., 2015) but is also the most-studied endocrine signal associated with the evolution of eusociality (Amsalem et al., 2014; Bomtorin et al., 2014; Shpigler et al., 2014; Shpigler et al., 2016). It functions as a gonadotropin in solitary adult females and is in the regulation of vitellogenin production in fat body that accumulates in the developing oocyte (De Loof et al., 2001). However, the role of JH changes with the eusocial evolution and its relevance with vitellogenin weakens (Lattorff &

Moritz, 2013). The honey bee is one of the well-studied exceptions for this canonical model, in which low JH titer and high-level vitellogenin appear simultaneously in both fertile queens and workers (Bomtorin et al., 2014). It makes no difference whether corpus allatum (CA) is removed or not for egg-laying (Bomtorin et al., 2014). Moreover, a mutual repression model between JH and vitellogenin has been proposed, and the levels of JH and vitellogenin are relevant to the regulation of age-related division of labor and aggressive behavior (Bomtorin et al., 2014). JH synthesis is mainly regulated via allatoregulatory neuropeptides and the activation of the corresponding receptors (Veenstra, Rodriguez, & Weaver, 2012; Verlinden et al., 2015). Allatotropin (AT) is originally identified as a neuropeptide that stimulates JH synthesis and release (Kataoka et al., 1989; Kaneko & Hiruma, 2014; Kaneko & Hiruma, 2015). However, more and more physiological effects have been described in specific insect species, which are involved in cardiac rhythm, oviduct and hindgut contractions, nutrient absorption and circadian cycle (Hernández-Martínez et al., 2017). There are four allatostatins (AST-A, AST-B, AST-C and AST-CC) which act as inhibitors of JH synthesis (Stay & Tobe, 2007; Verlinden et al., 2015). Apart from the inhibition of JH synthesis, all of the ASTs are involved in various physiological processes in specific insect species or development stages (Verlinden et al., 2015; Dong, Wang, & Jing, 2017).

Recently, AT, AST-A, AST-C, AST-CC and the corresponding receptors - ATR, AST-A-R, AST-C-R, have been determined in all examined bee species (Kapheim et al., 2015). All of the receptors belong to the rhodopsin G protein-coupled receptor family and transform the extracellular signal carried by AT or ASTs into kinase activation and gene expression in the cytosol side, which will regulate the synthesis of JH (Verlinden et al., 2015).

Activation and signal transduction depend on the post-translational-modification of receptor, such as glycosylation, myristoylation and palmitoylation for subcellular localization and phosphorylation for signal transduction (Zheng et

al., 2012). The activation and desensitization of specific signal pathways depend on the patterns of several phosphorylation sites in the intracellular domains (Tobin, 2008). The analysis of Adipokinetic hormone receptors (AKHRs) of bee species indicates that the eusocial bee species possess more potential phosphorylation sites and modification patterns in the intracellular domains (Yang et al., 2018).

In the current study, the amino acid sequences of ATR, AST-A-R and AST-C-R were extracted from the Genbank database. The substitutions of amino acid sequences and the complexity of post-translational modifications (PTMs) were analyzed firstly via sequence alignment, motifs analysis and prediction of post-translational modifications. Furthermore, the correlation between these transitions and social complexity were carried out, so as to display the changes of potential sequences and PTMs involved in the eusocial evolution of bee species.

MATERIAL AND METHODS

Sequences collection and phylogenetic analysis

The sequence collection and phylogenetic analysis were performed with the use of a method reported previously by Yang et al. (2018) but with slight modifications. With the sequences of ATR (XP_026295776.1), AST-A-R (XP_006560262.1) and AST-C-R (XP_006560939.1) in *Apis mellifera* as the queries, BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was implemented for all available amino acid sequences of ATR, AST-A-R and AST-C-R of bee species based on sequence similarity (Supplementary file). The involved species were sorted into three groups according to the degree of social complexity: *Apis mellifera* (*A_mel*), *Apis cerana* (*A_cer*), *Apis florea* (*A_flo*), *Apis dorsata* (*A_dor*) and *Melipona quadrifasciata* (*M_qua*) for the advanced eusocial group; *Bombus terrestris* (*B_ter*), *Bombus impatiens* (*B_imp*) and *Bombus ignitus* (*B_ign*) for the primitively eusocial group; and *Eufriesea mexicana* (*E_mex*), *Habropoda laboriosa* (*H_lab*), *Megachile rotundata* (*M_rot*), *Dufourea novaeangliae* (*D_nov*) and *Ceratina calcarata* (*C_cal*) for the solitary/subsocial group

Fig. 1. The phylogeny of ATR (A), AST-A-R (B) and AST-C-R (C) families in bee species established by *MEGA* software version 6. The social complexity of their corresponding species was labeled on the right side, and the *A_mel_AKHR* was taken as the outgroup. *A_mel*: *Apis mellifera*; *A_cer*: *Apis cerana*; *A_flo*: *Apis florea*; *A_dor*: *Apis dorsata*; *M_qua*: *Melipona quadrifasciata*; *B_ter*: *Bombus terrestris*; *B_imp*: *Bombus impatiens*; *B_ign*: *Bombus ignitus*; *E_mex*: *Eufriesea mexicana*; *H_lab*: *Habropoda laboriosa*; *M_rot*: *Megachile rotundata*; *D_nov*: *Dufourea novaeangliae*; *C_cal*: *Ceratina calcarata*.

(Fig. 1).

The sequence alignment and phylogenetic analysis were conducted using the *MEGA* version 6 software package by a method reported previously but with slight modifications (Tamura et al., 2013; Hauser & Grimmelhuijzen, 2014). In detail, all the available protein sequences were aligned by CLUSTALW using the BLOSUM62 protein weight matrix. The neighbour-joining tree was established based on a model selected with the use of the Maximum likelihood method,

and the phylogenetic analysis was calculated with 10000 bootstrap replicates using the best-fit substitution model (Models with the lowest BIC scores). The JTT matrix-based method was applied to assess the evolutionary distances. The rate variation among the sites was modelled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated (Tamura et al., 2013). The *A_mel_AKHR* (NP001035354.1) was taken as the outgroup.

Fig. 2. Sequence alignment results of ATRs. The name of each sequence was listed on the left. The conserved domains were labeled at the bottom, while the highly conserved key residues were marked by black dots (●) on the top. TM: Transmembrane; ICL: Intracellular loop; ECL: Extracellular loop.

Prediction of Signal peptides and post-translational modifications (PTMs)

The signal peptides were predicted by SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>), and then the subcellular location analysis were predicted by TargetP 1.1 Server (<http://www.cbs.dtu.dk/services/TargetP/>) based on the amino acid sequences of predicted signal peptides. The MEME analysis (<http://meme-suite.org/tools/meme>) was performed based on the top eight of all the signal peptides to identify the amino acid residues contributed to the secretory pathway. The potential phosphorylation sites of the serine, threonine and tyrosine residues of the intracellular domain were calculated via the NetPhos3.1 server to predict the PTMs (<http://www.cbs.dtu.dk/>

[services/NetPhos/](http://www.cbs.dtu.dk/services/NetPhos/)) (Blom, Gammeltoft, & Brunak, 1999; Blom et al., 2004). The Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was taken to predict the glycosylation and myristoylation.

Statistics

The Spearman correlation coefficients with a two-tailed test of significance were calculated via *PASW statistics* version 18.0.0 software, and the charts were drawn by *GraphPad prism* software version 5.02.

RESULTS

Sequence alignment and phylogenetic analysis

Fig. 3. Sequence alignment results of AST-A-Rs. The name of each sequence was listed at the left. The conserved domains were labeled at the bottom, while the highly conserved key residues were marked by black dots (●) on the top. TM: Transmembrane; ICL: Intracellular loop; ECL: Extracellular loop.

The sequence alignment and phylogenetic analysis were conducted to characterize the amino acid substitutions and their consistency with the social complexity. All the domains for ATRs were highly conserved except for the N-terminal and C-terminal domains. The conserved DRW motif occurred at the beginning of the intracellular loop (ICL) 2 domain. The W amino acid residue was conserved in the TM4 domain, while it was substituted for Y in the TM6 domain. Notably, the *H_lab*_ATR possessed the shortest N-terminal domain and the longest ICL3 domain (Fig. 2).

As to AST-A-Rs and AST-C-Rs, the N-terminal domain was the least conserved. The consistent DRY motif and two W amino acid residues occurred at the beginning of ICL2, TM4 and

TM6 domains, respectively. Similar to the ATRs, the *H_lab*_AST-A-R and AST-C-R possessed the shortest N-terminal domain, while the *M_qua*_AST-C-R possessed the longest N-terminal and C-Terminal domains (Fig. 3 and 4). The proline residue in the TM7 domain and the cysteine residue for palmitoylation in the C-terminal domain were conserved in all three receptor groups (Fig. 2, 3 and 4).

The results of the phylogenetic analysis showed that all the sequences were mainly sorted into three branches, which were consistent with the social complexity (the solitary/subsocial, the primitively eusocial and the advanced eusocial group), except for the *M_qua* receptors which were excluded from the advanced eusocial group (Fig. 1).

Fig. 4. Sequence alignment results of AST-C-Rs. The name of each sequence was listed on the left. The conserved domains were labeled at the bottom, while the highly conserved key residues were marked by black dots (●) on the top. TM: Transmembrane; ICL: Intracellular loop; ECL: Extracellular loop.

Prediction of signal peptides

The signal peptide is critical for PTMs and subcellular localization. The predicted results showed that a signal peptide only occurred in the ATRs, except for the *H_lab*_ATR (Supplementary file). The prediction of subcellular localization directed by the signal peptides indicated that ATRs would be guided into the secretory pathway (Fig. 5A). The length of signal peptides correlated with the social complexity (Spearman's rho correla-

tion coefficient=0.693, P value=0.026) and the probability of secretory (Spearman's rho correlation coefficient=0.811, P value=0.004) (Tab. 1). The conserved amino acid residues, which were contributed to the secretory pathway, were described in Figure 5B.

Post-translational modifications

PTMs are crucial for the receptor localization, structure maintenance and accurate signal

Table 1

Spearman correlation with two-tailed test significance of ATR

	Social complexity	Number of glycosylation sites	Number of myristoylation sites	Number of phosphorylation sites	Length of signal peptides	Probability of secretory
Social complexity	1	0.387	-0.128	-0.315	0.693*	0.618
<i>P</i> value		0.240	0.707	0.346	0.026	0.057
Number of glycosylation sites		1	0.137	0.258	0.721*	0.808**
<i>P</i> value			0.687	0.444	0.019	0.005
Number of myristoylation sites			1	-0.047	0.09	0.045
<i>P</i> value				0.890	0.805	0.902
Number of phosphorylation sites				1	0.192	0.130
<i>P</i> value					0.595	0.719
Length of signal peptides					1	0.811**
<i>P</i> value						0.004

Table 2

Spearman correlation with two-tailed test significance of AST-A-R

	Social complexity	Number of glycosylation sites	Number of myristoylation sites	Number of phosphorylation sites
Social complexity	1	-0.175	-0.580*	0.674*
<i>P</i> value		0.568	0.038	0.012
Number of glycosylation sites		1	0.193	0.027
<i>P</i> value			0.528	0.930
Number of myristoylation sites			1	-0.801**
<i>P</i> value				0.001

transduction. Based on the prediction of amino acid residues for glycosylation, myristoylation and phosphorylation, the potential relationship between PTMs switches and eusocial status could be investigated (Supplementary file). The phosphorylation sites localized in the cytosol domains (ICL1, ICL2, ICL3 and C terminus) of all three receptor families were mostly distributed in the ICL3 and C terminal domains (Fig. 6). The number of glycosylation sites in the ATRs correlated with the length of the signal peptide

(Spearman's rho correlation coefficient=0.721, *P* value=0.019) and the probability of secretory (Spearman's rho correlation coefficient=0.808, *P* value=0.005) (Tab. 1). In the AST-A-Rs, the social complexity negatively correlated with the number of myristoylation sites (Spearman's rho correlation coefficient=-0.580, *P* value=0.038), and positively correlated with the number of phosphorylation sites (Spearman's rho correlation coefficient=0.674, *P* value=0.012). Meanwhile, the number of glycosylation sites

Fig. 5. The length and their secretory probability (A) and the conserved motif (B) of signal peptides in ATRs.

Table 3

Spearman correlation with two-tailed test significance of AST-C-R

	Social complexity	Number of glycosylation sites	Number of myristoylation sites	Number of phosphorylation sites
Social complexity	1	-0.259	0.557	-0.434
<i>P</i> value		0.416	0.060	0.159
Number of glycosylation sites		1	0.165	0.680*
<i>P</i> value			0.607	0.015
Number of myristoylation sites			1	-0.160
<i>P</i> value				0.618

negatively correlated with the number of phosphorylation sites (Spearman's rho correlation coefficient=-0.801, *P* value=0.001) (Tab. 2). The number of glycosylation sites in the AST-C-Rs positively correlated with the number of phosphorylation sites (Spearman's rho correlation coefficient=0.680, *P* value=0.015) (Tab. 3).

DISCUSSION

Recently, the clarified correlation between vitellogenin and JH in bumblebees, a primi-

tively eusocial bee species, has enhanced the hypothesis that the eusocial evolution is accompanied by shifts in ancient molecular and physiological pathways, especially in JH signalling (Amsalem et al., 2014; Shpigler et al., 2014; Jedlička et al., 2016; Shpigler et al., 2016). The allatoregulatory neuropeptides are known for their regulation of JH synthesis and release in insects (Verlinden et al., 2015). As the underpinning of functions, the substitutions of amino acid sequences and PTMs of allatoregulatory neuropeptide receptors were character-

Fig. 6. The distribution of phosphorylation sites in intracellular domain of ATRs (A), AST-A-Rs (B) and AST-C-Rs (C). ICL: Intracellular loop; C-ter: C-terminus.

ized. AST-A-Rs and AST-C-Rs were identified in all of the examined bee species regardless of they were solitary or eusocial. Although the amino acid sequences were highly conserved, the phylogenetic results were consistent with the eusocial status except for those of the *M_qua* receptors. Furthermore, the abundance of predicted PTMs correlated with the social complexity.

Based on the model-based ancestral state reconstructions, stingless bees and honey bees independently evolved advanced eusocial behavior from the same primitive eusocial ancestor, but their swarming, communication and rearing behavior differed (Cardinal & Danforth, 2011). This might result in the exclusion of *M_qua* (stingless bee) receptors from the *Apis* groups. The results of functionally characterization and tissue-specific transcription of *B_ter* ATR display high ATR expression in the male accessory gland (Verlinden et al., 2013). Our results show that all of the ATRs, except for *H_lab* ATR, possess a signal peptide. Moreover, the length of signal peptides and their probability of secretion positively correlate with the social complexity. The presence of a signal peptide only means that the protein enters the secretory pathway, but it is not sufficient for the protein to be secreted to the extracellular environment. In contrast, the proteins with one or more transmembrane helices downstream of the signal peptide is retained in the membrane of all kinds of organisms (Nielsen, 2017). Hence, a higher secretory probability indicates that the ATRs of eusocial bee species are more likely to localize to the cytomembrane. Nevertheless, the absence of a signal peptide in *H_lab* ATR is still not understood.

Furthermore, the correlation between social complexity and glycosylation, a critical PTMs for protein subcellular localization (Norskov-Lauritsen, Jorgensen, & Brauner-Osborne, 2015), was consistent with the length and secretory probability of signal peptide. The question remains whether the different secretory probability and glycosylation is correlated with the regulation of JH biosynthesis.

ASTs, the inhibitors, also play crucial roles in the

accurate regulation of JH synthesis in response to the physiological switches. Three ASTs (AST-A, AST-C, AST-CC) and two AST receptors (AST-A-R, AST-C-R) have been identified in all of the examined bee species (Kapheim et al., 2015). The results of the sequence alignment of AST-Rs display that the substitutions mostly occur in the N and C terminus. Furthermore, all of the conserved motifs of the rhodopsin GPCR family are uniform regardless of AST-A-R or AST-C-R. The correlation analysis of AST-A-Rs reveals that the social complexity positively correlates with the number of phosphorylation sites, while negatively correlates with the number of myristoylation sites. Since phosphorylation significantly affects receptor signal transduction (Tobin, 2008), the results indicate that the more social complexity, the more regulation complexity to get more required functions. However, the decreased tendency of myristoylation, which contributes to the stability of the receptor structure (Torrecilla & Tobin, 2006), might endow receptors with a greater variety of conformational changes. No correlation happened to the social complexity of the AST-C-R, while only the number of phosphorylation sites positively correlates with the number of glycosylation sites, which affects surface targeting and second messenger production of GPCR (Norskov-Lauritsen, Jorgensen, & Brauner-Osborne, 2015). Pleiotropic ASTs also participate in other physiological processes, such as myoinhibition of visceral muscles (Veenstra, 2009). The ASTs have been characterized as a learning modulator in *Apis mellifera* (Urlacher et al., 2016). Hence, the changes in AST-C-Rs might be not related with JH synthesis but the details still need to be determined.

In conclusion, the sequence alignment, phylogenetic and PTMs analysis of the ATRs and ASTRs demonstrate that transitions potentially occur during eusocial evolution. Although the consequences of these specific amino acid substitutions and various PTM complexity have not been studied for these receptors, one can assume that they contribute to the specific localizing, binding and coupling characteristics of the receptor groups.

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Supplementary files

The sequence information and the original predicted results of signal peptides and PTMs are supplied in a supplementary file.

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