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Omics approaches to study juvenile hormone synthesis

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Abstract

The juvenile hormones (JHs) are a family of insect acyclic sesquiterpenoids produced by the *corpora allata* (CA), a pair of endocrine glands connected to the brain. They are involved in the regulation of development, reproduction, behavior, caste determination, diapause, stress response, and numerous polyphenisms. In the post-genomics era, comprehensive analyses using functional ‘omics’ technologies such as transcriptomics, proteomics and metabolomics have increased our understanding of the activity of the minute CA. This review attempts to summarize some of the ‘omics’ studies that have contributed to further understand JH synthesis in insects, with an emphasis on our own research on the mosquito *Aedes aegypti*.

Introduction

‘Omics’ is a general term used to describe several rapidly growing fields of science that study large sets of biological molecules [1]. ‘Omics’ sciences are characterized by their comprehensive unbiased goals (do it all), as well as by their high throughput approaches (do it in a single experiment). In the post-genomics era, all-inclusive analyses using functional ‘omics’ technologies such as transcriptomics, proteomics and metabolomics have increased our understanding of insect endocrinology [2]. The juvenile hormones (JHs) are a family of insect acyclic sesquiterpenoids synthesized by the *corpora allata* (CA), a pair of endocrine glands connected to the brain [3]. Juvenile hormones have pleiotropic effects and participate in the regulation of development, reproduction, behavior, caste determination, diapause, stress response, and numerous polyphenisms [4]. Juvenile hormone titer is mostly determined by the rate of JH synthesis [5]. In the past, the study of JH synthesis was hindered by the small size of the CA, which prevented the use of classical biochemical and molecular approaches. In the post-genomic era, important progresses have been made in our understanding of the regulation of JH synthesis. Among them, we can highlight the identification of all the enzymes involved in the JH synthesis pathway, as well as the measurement of changes in transcripts, JH precursor metabolites and enzymatic activities in the minute CA of insects [6,7]. ‘Omics’ approaches also helped in the identification of new allatoregulatory molecules and their receptors, as well as provided insights into their

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mechanisms of action. This review attempts to summarize some of the ‘omics’ studies that have contributed to further understand JH synthesis in insects, with an emphasis on our own research on the mosquito *Aedes aegypti*.

Genomics and transcriptomics studies

The release of the whole-genome assembly of *Drosophila melanogaster* in early 2000 was an historic event for insect research [8]. It was followed by the sequencing of the genomes of additional important insect models, such as *Anopheles gambiae* [9], *Bombyx mori* [10] and *Tribolium castaneum* [11], and the implementation of ‘omics’ studies in multiple insect species [2]. Even before the complete sequence of the *Ae. aegypti* genome was released [12], a comparative analysis of ESTs from CA of *Ae. aegypti* (821 individual transcripts) and the cockroach *Diploptera punctata* (478 individual transcripts) was reported [13]. This pre-next generation sequencing (NGS) pioneer work, although technologically limited, still remains as the only published transcriptome analysis of the CA of an insect, and provided some early insights into CA-specific molecular pathways. Later, a follow up study used Illumina sequencing to generate a new transcriptome analysis of the CA of *Ae. aegypti*. This study detected 11627 genes that were expressed in the CA with a minimum of 10 reads (Nouzova et al., unpublished).

The main compounds synthesized by the CA are several types of sesquiterpenes (JHs and MF). The biosynthetic pathway of JH III includes 13 enzymatic reactions, and it is commonly divided into early and late steps [6,4]. The early steps follow the mevalonate pathway (MVAP) to form farnesyl pyrophosphate (FPP) [14]. The late steps involve the hydrolysis of FPP to farnesol (FOL) [15], followed by oxidation to farnesal (FAL) [16] and farnesoic acid (FA) [17]. In mosquitoes, FA is finally converted to JH III by the activity of a methyl transferase (JHAMT) [18] and a P450 epoxidase (EPOX) [19]. The enzymes of the MVAP are well conserved in eukaryotes. In insects, all the MVAP enzymes seem to be encoded by single-copy genes, and identification of predicted amino acid sequences in insect genomes was possible based on sequence homology [13,20,6,21,22,23]. The late steps of JH biosynthesis (JH-branch) were generally considered to be JH-specific [4], all the genes encoding these enzymes have now been identified in several insects using molecular approaches that included EST sequencing [19,13], mRNA differential display [18] or homology to orthologue enzymes [15,17,22].

The identification of all the genes encoding the JH biosynthetic enzymes opened the door for more profound studies on the regulation of CA activity. Comprehensive studies on the expression of transcripts have been implemented in several holometabola and hemimetabola insects, including *B. mori* [20,24], *Ae. aegypti* [6,7], *D. punctata* [13,23] and *Schistocerca gregaria* [21]. The transcripts for most JH biosynthetic enzymes are highly enriched or exclusively expressed in the CA, and their expressions are often coordinated with JH titers [20,7,23]. In addition, transcriptomes from whole body of dipteran and coleopteran species have shown differential expression of genes encoding JH biosynthetic enzymes during diapause [25,26].

MicroRNAs (miRNAs) are small non-coding RNAs that play key roles in the regulation of gene expression at the post-transcriptional level, adding a new layer of control to the complex pathways that exist in cells [27]. Recently, it has been completed the first transcriptome study of the miRNA repertoire of the CA of an insect. High-throughput small RNA sequencing of CA from *Ae. aegypti* early pupa (non-detectable JH synthesis), sugar-fed female (high JH synthesis), and blood-fed female (very low JH synthesis), showed dramatic alterations of miRNA profiles among the three developmental stages, in particular between the pupal and the adult stages [28]. In *D. melanogaster*, loss of function of *bantam* miRNA increases JHAMT transcripts, while overexpression of *bantam* miRNA represses JHAMT expression, decreases JHB3 and JH III titers, and causes pupal lethality; suggesting that miRNA *bantam* plays a role on the regulation of JH titers [29].

Proteomics and peptidomics studies.

The small size of the endocrine gland precluded the purification and characterization of CA proteins; consequently, most of the information we have on JH biosynthetic enzymes is based on the analysis of recombinant protein activities, or homology modeling and docking simulations [30]. Biochemical characterization of purified or recombinant enzymes of the MVAP and JH-branch in insects have been reported for acetoacetyl-CoA thiolase [31], HMG-CoA synthase [32,33], mevalonate kinase [34] isopentenyl-PP isomerase [35,36], farnesyl-PP synthase [37,38,39,40,41,42] farnesyl pyrophosphatase [15,43], farnesol dehydrogenase [16], farnesal dehydrogenase [17], JHAMT [18,44,45,46,47], and EPOX [19,48,49]. In addition, the activities of 8 of the 13 JH biosynthetic enzymes have now been monitored *in vitro* in CA extracts of mosquitoes under 5 different developmental and physiological conditions [7]. The catalytic activities of the enzymes of the MVAP and JH-branch changed in a coordinated fashion in the “active” and “inactive” CA. As seen with transcript levels, changes in enzymatic activities were generally concurrent with increases or decreases in JH synthesis [7].

A number of regulatory factors have been described that can modulate JH synthesis in different insect species and at different stages of development. They include three types of inhibitory allatostatins (AST) [50], as well as several stimulatory compounds, such as allatotropin (AT)[51], insulin-like peptides (ILP)[52,53,54], ecdysis triggering hormone (ETH)[55,56,57] and 20-hydroxyecdysone (20E)[58]. A seminal study reported the comprehensive cloning of neuropeptide G protein-coupled receptors (GPCR) from *B. mori*, as well as the systematic analyses of their expression [59]. Six receptors were highly expressed in the CC-CA complexes, including receptors for AST-C and AT [59]. In the CC-CA of mosquitoes the expression of the following receptors has been detected: ETH A and B, ecdysone A and B, ILP, ultraspiracle A and B, allatotropin, AST-C, -A and -B, and short neuropeptide F (sNPF) [60]. It is possible that signals from all these modulators are integrated in the CA, which suggests that the regulation of JH synthesis is extremely complex [61]. Each of the three structurally unrelated types of allatostatins (A, B and C) are associated with a unique GPCR family that includes vertebrate orthologues [62,63,64]. The AT receptor is also a GPCR and shows homology to the vertebrate orexin/hypocretin receptors [59,65,66,67].

Little is still known about the targets and mechanisms of action of allatregulatory factors. In mosquitoes, AST-C exerts a strong, rapid and reversible inhibition of JH synthesis that can be overridden by addition of any of the 13 JH precursors, indicating that the AST-C target is located before entry of acetyl-CoA into the JH biosynthetic pathway [68]. Stimulation experiments using different sources of carbon (glucose, pyruvate, acetate and citrate) revealed that AST-C acts after pyruvate is converted to citrate in the mitochondria [68]. AST-C inhibits JH synthesis by blocking the citrate carrier (CIC) that transports citrate from the mitochondria to the cytosol, obstructing the production of cytoplasmic acetyl-CoA that sustains JH synthesis in the CA of mosquitoes. Similar results were reported for the AST-A inhibition of JH synthesis in cockroaches [69]. Most studies on the activities of JH synthesis regulators have been done *in vitro*; validation of these results *in vivo* will be facilitated by the development of CA-specific expression system that could enable a tissue-specific and time dependent manipulation of gene expression. *Corpora allata* specific promoters have been identified and used to modify JH synthesis, or to study different aspects of CA physiology in *D. melanogaster* [70]. In recent studies, two different genotypes Aug21-Gal4 > UAS-GCaMP3 and JHMT-Gal4 > UASGCaMP5 were used to detect calcium mobilization inside the CA as a result of ETH treatment [56,57].

Mass spectrometry peptidomics studies revealed the presence of AST-A, sNPF and pyrokinin in the CA of *Locusta migratoria* and *Schistocerca gregaria* [71,72]. A study of the CA-CC of the beetle, *Zophobas atratus* described the identification of two typical CC peptides, adipokinetic hormone (AKH) and crustacean cardioactive peptide (CCAP), as well as pyrokinin [73]. An analysis of *B. mori* brain-CA-CC complexes described the identification of 59 mature peptides that are expressed differentially across developmental stages [74]. Twelve peptides were identified in CC-CA complexes of larvae, including AST-A, AKH and corazonin [74]. Studies on neuropeptides expressed in the CA-CC of the Tsetse fly *Glossina morsitans* also revealed the presence of AKH and sNPF [75]. A proteomics study of the CC-CA in *Agrotis ipsilon* moths described the identification of AKH, AST-A, corazonin and sNPF [76]. The presence and function of these peptides in the CA is debatable for two main reasons. First, most of these studies analyzed the whole CA-CC complexes, consequently many of the peptides might be present only in the CC. Second, most likely some of these peptides were located in the nerve fibers that innervate the CA and not in the intrinsic cells of the gland [71]. Nevertheless, peptidomics and transcriptomics analysis of insect CA-CC peptides are advancing our understanding of the regulation of CA activity.

Metabolomics and pathway modeling

Juvenile hormone synthesis is controlled by the rate of flux of isoprenoids, therefore JH precursor pool concentrations and fluxes (which are flows into and out of pools) are critical variables in JH synthesis regulation [6]. Rivera-Perez et al [7] reported the only comprehensive metabolomics analysis of the changes in the concentrations of all JH precursors in the CA of an insect. In mosquitoes, global fluctuations in the intermediate pool sizes in the MVAP and JH-branch are not functioning as a unit, but behave inversely, when MVAP precursors are high, JH-branch metabolites are low, and vice versa [7]. Principal component analysis (PCA) of the metabolic pools indicated that in reproductive female

mosquitoes, at least four developmental switches alter JH synthesis by modulating the flux of isoprenoids at distinct points. Metabolic analysis established four distinct CA physiological conditions that alter JH synthesis by modulating the flux of metabolites at different points in the pathway [7].

A key step toward further understanding the regulation of CA activity is the establishment of numerical models. A recent study demonstrated the ability of two different quantitative approaches to describe and predict how changes in the individual metabolic reactions in the pathway affect JH synthesis [77]. Generalized additive models (GAMs) described the association between changes in specific metabolite concentrations with changes in enzymatic activities and substrate concentrations. Addition of information on enzymatic activities usually improved the fitness of GAMs built solely based on substrate concentrations. In addition, a system of ordinary differential equations (ODE) was developed to describe the instantaneous changes in metabolites as a function of the levels of enzymatic catalytic activities. ODEs underscored that in the active CA enzymatic activities were not limiting [77]. Stimulation of JH synthesis with exogenous precursors has been reported for the CA of many insect species [68,69], and it seems that having an excess of enzymes is common in most insects studied.

Concluding remarks

Figure 1 summarizes our current knowledge on the roles and effects of allatregulatory factors in the CA of *Ae. aegypti*. Genomics, transcriptomics, proteomics and metabolomics are reshaping the field of JH synthesis research. Functional studies using reverse genetic approaches such as RNA interference (RNAi)[17,53] or generation of knock-down or gain of function mutants using CRISPR-Cas9 technology [78,79] should validate molecules described in *in vitro* studies or discover new key players in the regulation of CA activity. Post-transcriptional regulation mechanisms including silencing of gene expression by miRNA most likely play important roles in the remarkable ability of the gland to adjust JH synthesis rates to environmental clues such as nutrition and photoperiod (80,81). Epigenetic factors might play roles on JH synthesis in social insects and other species (82,83,84). Early work in each omics area is beginning to illustrate that there is still much more to learn about the intricate regulation of JH synthesis.

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Figure 1. Regulation of juvenile hormone synthesis in mosquitoes.

Schematic representation of tissues and molecules (peptides and receptors) involved in the regulation of JH biosynthesis in pupa and adult mosquitoes. The activity of the CA is regulated by factors produced by the brain and ovaries in adult mosquitoes, as well as by factors from Inka cells and body wall in pupa. Allatregulatory factors stimulate (green) or inhibit (red) JH biosynthesis through receptors in the CA. 20E and ETH increase JHAMT activity. ATC and ILP modulate JH precursor pool sizes. CC: *corpora cardiaca*, CA: *corpora allata*, 20E: 20 hydroxyecdysone, AST-C: allatostatin-C, AT: allatotropin, ILP: insulin-like peptides, R: receptor, FAL: farnesal, FA: farnesoic acid, MF: methyl farnesoate, JHAMT: juvenile hormone acid methyl transferase, JH: juvenile hormone.