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Quantitative Neuropeptidome Analysis Reveals Neuropeptides Are Correlated with Social Behavior Regulation of the Honeybee Workers

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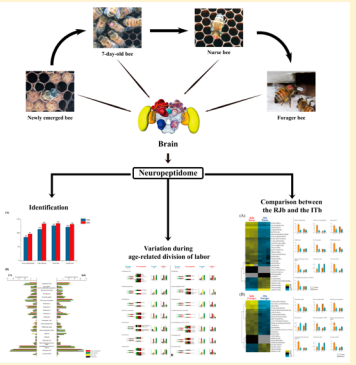
Supporting Information

ABSTRACT: Neuropeptides play vital roles in orchestrating neural communication and physiological modulation in organisms, acting as neurotransmitters, neuromodulators, and neurohormones. The highly evolved social structure of honeybees is a good system for understanding how neuropeptides regulate social behaviors; however, much knowledge on neuropeptidomic variation in the age-related division of labor remains unknown. An in-depth comparison of the brain neuropeptidomic dynamics over four time points of age-related polyethism was performed on two strains of honeybees, the Italian bee (*Apis mellifera ligustica*, ITb) and the high royal jelly producing bee (RJb, selected for increasing royal jelly production for almost four decades from the ITb in China). Among the 158 identified nonredundant neuropeptides, 77 were previously unreported, significantly expanding the coverage of the honeybee neuropeptidome. The fact that 14 identical neuropeptide precursors changed their expression levels during the division of labor in both the ITb and RJb indicates they are highly related to task transition of honeybee workers. These observations further suggest the two lines of bees employ a similar neuropeptidome modification to tune their respective physiology of age polyethism via regulating excretory system, circadian clock system, and so forth. Noticeably, the enhanced level of neuropeptides implicated in regulating water homeostasis, brood pheromone recognition, foraging capacity, and pollen collection in RJb signify the fact that neuropeptides are also involved in the regulation of RJ secretion. These findings gain novel understanding of honeybee neuropeptidome correlated with social behavior regulation, which is potentially important in neurobiology for honeybees and other insects.



KEYWORDS: *neuropeptidome, honeybee, age-related division of labor, social behavior, royal jelly*

1. INTRODUCTION

Over the past decades, the honeybee (*Apis mellifera*) has fascinated us as a powerful model to investigate different basic scientific principles, particularly at the behavioral, neural, and molecular levels.[1] Given the complex social behavioral repertoire of honeybees, a variety of insect sociological and behavioral studies have been performed, such as communication, navigation, colony defense, mating, and learning.[2−4] Such a complicated social network is controlled by the central nervous system (CNS) of the honeybees, with the brain being only 1 mm^3 in size and containing less than a million neurons.[5]

As is typical of eusocial insects, division of labor is considered as the distinguishing feature in the honeybee community, in which the primary division of labor is caste differentiation (female: reproductive queen and sterile worker; male: drone), and age-dependent polyethism of honeybee workers is at the secondary level.[6] In this three-caste system, the queen and drones take specific responsibility of reproduction, while the workers perform all the tiring tasks necessary for the colony survival, ranging from comb building and larvae feeding ("nursing") to field foraging and hive defending. Younger workers (<2 weeks of age) tend to perform tasks inside the hive, during which the major behaviors of newly emerged bees are cell cleaning and hive maintenance, while brood tending and queen caring are mainly performed between the first and second week of age. In the transition from the second to third week, the worker bees change their duties to forage for nectar and pollen outside the hive, and the foraging capacity of workers is at its height after 3 weeks.[7,8] However, this age-related polyethism is very plastic; both biological factors inside the hive and interaction of the colony with environmental clues may interfere with behavioral development, thereby changing or even reverting the timing of the transition from hive work to field foraging for the need of the entire colony.[9,10] Hence, age-related division of labor and its plasticity are the evolutionary strategies that benefit colonies through task specialization to increase operating efficiency or colony homeostasis.

Similar to neural and behavioral plasticity in vertebrates, such as learning, sexual behavior, and even drug addiction,[11,12] the age-related behavioral transition in the honeybee workers is closely associated with changes in structure, gene expression, and protein synthesis of the brain.[13] At the structural level, the remarkable volume increase in the mushroom body (MB) calyces driven by foraging experience has been observed, and Golgi impregnation research indicates that this change may be

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determined by outgrowth and branching of Kenyon cell (KC) dendrites.[14,15] At the genetic level, positive correlations between foraging behavior and molecular pathways related to the *foraging* (*for*, which encodes a cGMP-dependent protein kinase), *malvolio* (*mvl*), and *period* (*per*) genes have been documented, implying their potential roles in hive-bee−forager transition.[16−19] Furthermore, the dynamics of gene expression in the brain are strongly correlated with the age-related transition by adult honeybees from hive work to foraging activity.[20−22] With the fast development of proteomics, there is now the ability to unravel brain proteomic changes during honeybee behavioral maturation and in several protein functional categories that are differentially expressed between nurses and foragers;[13,23] however, previous surveys have generally focused on nurse and forager bees. The biological variation of the brain during the whole ontogeny and behavioral transition of adult worker bees still remains to be elucidated, and knowledge on the change of the neuropeptidome during the above process is still very limited.

In the nervous system, neuronal connections and neuron-to-neuron communication is achieved via signaling molecules, such as acetylcholine, glutamate, gamma-aminobutyric acid, biogenic amines, and peptides, among which the neuropeptides are the largest and most diverse category.[24] Neuropeptides are capable of performing functions via acting on G-protein-coupled receptors in multiple ways, including acting as direct neurotransmitters, neurons coexpress markers for classical neurotransmitters, autocrine or paracrine regulators in the local cellular microenvironment, and hormones on distal targets as well.[25] The biological activities of neuropeptides are involved in the regulation of neurons, neuronal circuits, organs, and ultimately behaviors.[26] In the insect kingdom, of seven holometabolous insects whose genomes have been sequenced, *Nasonia*, *Apis*, *Drosophila*, *Aedes*, *Bombyx*, *Tribolium*, and *Acyrthosiphon pisum*, a core set of 19 neuropeptide precursor genes have been conservatively distinguished in all genomes, while the existence of 26 other precursors vary among different species.[27] Approximately 42 neurohormone precursor genes, for instance, are found in the genome of *Drosophila*,[28] while in the hymenoptera *Nasonia vitripennis* (a parasitic wasp) the number decreases to only 30 neuropeptide genes.[27] The insect neuropeptides are functionally important in modulating development, metabolism, reproduction, sleep, courtship, and feeding-related behaviors.[28−31] In regard to honeybees, by the combination of homology and codon-scanning searches, a total of 36 putative neuropeptide genes in the honeybee brain have been annotated; meanwhile, qRT-PCR and mass spectrometry (MS) verifications have confirmed 100 peptides deriving from 20 precursors, laying the groundwork for honeybee neuropeptidomic research.[32] The follow-up study separately analyzed all ganglia of adult worker bees, depicting a distribution pattern of 67 neuropeptides in CNS.[33] Quantitative peptidomic techniques have been widely used as an efficient strategy to investigate connections between social behavior and bioactivities of neuropeptides, yielding several neuropeptides function in the regulation of foraging activity in honeybee;[34,35] however, the physiological and behavioral functions of most neuropeptides in the honeybee brain remain largely unknown. Noticeably, it is likely that a cocktail of neuropeptides is generally involved in a given brain physiological pathway or in the regulation of a specific behavior rather than by a single one.[34,36] In this research, brain neuropeptidomes of honeybees at the age of day 0 (newly emerged bees), day 7, day 14 (nurse bees), and day 21 (forager bees) were systematically analyzed by employing high-resolution and high-accuracy MS, and neuropeptidomic differences were compared between the Italian bee (ITb) and the high royal jelly producing bee (RJb, a line of bees selected from ITb with 5 times higher RJ production than its "wild type" counterpart).[37] Our unprecedented depth of neuropeptidome coverage characterizes the whole time scale of age-related task transition in honeybees and dissects the potential interaction between neuropeptide functions and honeybee social behaviors. This is of great importance for the better understanding of neurobiological activity in both the honeybee and other social insect communities.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

All of the chemicals used were analytical grade or better and were purchased from Sigma (St. Louis, MO).

2.2. Brain Sampling and Neuropeptide Extraction

The ITb (*Apis mellifera ligustica*) and the RJb (*Apis mellifera ligustica*) were kept in the apiary of Institute of Apicultural Research, Chinese Academy of Agricultural Sciences in Beijing. Five colonies of each bee strain with mated queens of identical age, sufficient brood, and similar colony strength were selected as the experimental colonies. Newly emerged worker bees (day 0) were obtained by taking frames containing old pupae and placing into an incubator (34 °C and 80% relative humidity) for their eclosion. Newly emerged worker bees (<2 h, $N > 1000$) were marked on their thoraxes and placed back into the parent colonies. Thereafter, the marked worker bees were collected on days 7, 14, and 21. In particular, on day 14 only marked workers with heads in cells containing small larvae were collected (identified as nurse bees), and on day 21 only marked bees flying into the hive with pollen loads were collected (recognized as foragers). For each one bee strain at each time point (day 0, 7, 14, and 21) we sampled ∼100 worker bees. The brain was carefully dissected from the head capsule according to the method described elsewhere,[38] the process was performed on ice, and the dissected brains were frozen at −80 °C. Three independent biological replicates per time point were produced; then, we pooled the replicates together (∼300 brains) and split them into two parts, one for LC−MS/MS analysis and the other for Western blot analysis.

The brains were homogenized and neuropeptides were extracted at 4 °C by using a 90:9:1 solution of methanol, H_2O, and acetic acid to extract the neuropeptides. Afterward, the homogenate was centrifuged at 12 000*g* for 10 min at 4 °C. The supernatant was collected and vacuum-dried using a SpeedVac system (RVC 2−18, Marin Christ, Osterod, Germany) as a neuropeptide sample; then, it was stored at −80 °C for further LC−MS/MS analysis.

2.3. LC−MS/MS Analysis

Neuropeptide pellets were redissolved in 0.1% formic acid in distilled water, and the final peptide concentration was quantified using a Bradford assay.[39] LC−MS/MS analysis was performed on the Easy-nLC 1000 (Thermo Fisher Scientific, Bremen, Germany) coupled LTQ-Orbitrap Elite (Thermo Fisher Scientific) hybrid mass spectrometer with three replicates. Samples were loaded onto a 2 cm long, 100 μm inner diameter fused silica trap column containing 5.0 μm Aqua C18 beads (Thermo Fisher Scientific) for 2 min in buffer A (0.1% formic acid) at a flow rate of 5 μL/min prior to analytical separation. Peptides were separated on a column packed with 2 μm C18

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(100 Å, 75 μm x 50 cm, Thermo Fisher Scientific) at a flow rate of 350 nL/min using the following gradients: from 3 to 8% buffer B in 5 min, from 8 to 20% buffer B in 80 min, from 20 to 30% buffer B in 20 min, from 30 to 90% buffer B in 5 min, and 90% buffer B in 10 min. In data-dependent acquisition mode (range from m/z 300−1800 with a resolution of 70 000 at m/z 400), the 10 most abundant precursor ions with charge state greater than +1 were fragmented and previously acquired precursor ions (repeat count 1, repeat duration: 30 s; exclusion duration 45 s) were dynamically excluded. MS/MS spectra were acquired in higher energy collisional dissociation (HCD) mode with a resolution of 17 500 at m/z 400 and started from m/z 100 using a normalized energy of 30. The MS/MS data were acquired in raw files using the Xcalibur software (version 2.2, Thermo Fisher Scientific).

2.4. Neuropeptide Identification and Label-Free Abundance Quantitation

The extracted MS/MS spectra were searched against a composite database containing 21 763 protein sequences of *Apis mellifera* (released in January 2015) using in-house PEAKS software (version 7.0, Bioinformatics Solutions, Waterloo, Canada). The following modifications were applied: C-terminal amidation (A, −0.98) and pyroglutamination from Q (P, −17.03), variable modifications. The other parameters used were the following: parent ion mass tolerance, 15.0 ppm; fragment ion mass tolerance, 0.05 Da; enzyme, none; and maximum allowed variable post translational modification (PTM) per peptide, 2. A fusion target-decoy approach was used for the estimation of the false discovery rate (FDR) and controlled at ≤1.0% ($-10 \log P \geq 20.0$) at both protein and peptide levels. Neuropeptide identifications were used only if at least two spectra were identified in one sample.

Relative quantification of the brain neuropeptidome was performed by the label-free approach in PEAKS Q module. Feature detection was performed separately on each sample by using the expectation−maximization algorithm. The features of the same peptide from different samples were reliably aligned together using a high-performance retention time alignment algorithm.[40] The identification results were chosen to attach as the last step of the label-free quantification. Peptide features and proteins were considered to be significantly changed between different brain samples using statistical p value <0.01 and a fold change ≥2. The peptides used for identification statistical analysis across all samples are included in Supplementary Tables S1 and S2.

2.5. Neuropeptide Prediction

Before neuropeptide prediction, SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide of identified neuropeptide precursors, using $a > 0.5$ probability value cutoff.[41] Then NeuroPred application (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) was applied to provide predicted cleavage sites and neuropeptides with PTMs from their precursor sequences.[42] Apis was selected as a model; trim C-terminal K and R, amidation, and pyroglutamination were selected as PTMs; and the rest settings were used as default values.

2.6. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from the brains of 0, 7, 14, and 21 day old worker bees of the two bee strains using TRIzol regent (Takara Bio, Kyoto, Japan). A triplicate was produced of each sample. Sixteen differentially expressed proteins among the samples were examined to detect the corresponding mRNA levels. Gene names, accession numbers, and forward and reverse primer sequences are listed in Supplementary Table S2-5, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene to normalize expression data. Reverse transcription was performed using an RNA PCR kit (Takara Bio, Kyoto, Japan), according to the manufacturer's instructions. Real-time PCR amplification was conducted on iQ5Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA), as previously described.[43] After verifying amplification efficiency of the selected genes and GAPDH at approximately equal levels, the differences in gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The statistically significant difference of gene expression was considered only on an error probability of $p < 0.05$ by one-way ANOVA (SPSS version 16.0, SPSS) using Duncan's multiple-range test.

2.7. Western Blot Analysis

To further verify the variation tendencies of differentially expressed neuropeptide precursor proteins identified by our peptidomic approach, we selected diuretic hormone, pigment-dispersing hormone, and tachykinins for Western blot analysis with three replications. Polyclonal antibodies of these three proteins were developed in New Zealand female rabbits by ChinaPeptides (Shanghai, China) and were affinity purified. After purification, the specificity of the antibodies was verified by ELISA (tested by ChinaPeptides). Western blot analysis was performed by the method we previously described with some modifications.[44] In brief, equal amounts of a protein sample (12 μg/lane) were separated by stacking (4%) and separating (12%) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and then transferred to a nitrocellulose transfer membrane (0.2 μm pore size) (Invitrogen, Eugene, OR) using an iBlot apparatus (Invitrogen). After blocking, the membrane was incubated overnight at 4 °C with primary antibodies at a dilution of 1:500. Following three washes, the membrane was incubated with horseradish peroxidase-conjugated goat antirabbit secondary antibody at a dilution of 1:2000 for 2 h. Immunoreactive protein bands were detected using the ECL Western blotting substrate (Pierce, Rockford, IL) and quantified by densitometry using Quantity-One image analysis system (Bio-Rad). GAPDH was detected simultaneously as a loading control of the analysis.

3. RESULTS

3.1. In-Depth Profiling of Age-Related Brain Neuropeptidomes

To establish an in-depth map of honeybee brain neuropeptidomes during the entire course of age-related division of labor, a high-resolution and high-sensitivity MS (LTQ-Orbitrap Elite) was applied, and the neuropeptidomes were analyzed and compared between the two strains of honeybees, the ITb and RJb. In total, 158 nonredundant neuropeptides derived from 22 precursor proteins were identified in the brain of the two strains of honeybee workers. Among them, tachykinin was the neuropeptide precursor protein containing the highest number of identified neuropeptides (24 neuropeptides), while neuropeptide Y-like and allatotropin had only one neuropeptide each. Among all of the 158 identified neuropeptides, 81 of them were overlapped with previously identified in the honeybee brain neuropeptidomes, while the remaining 77 had not been previously identified (Supplementary Table S1-1, Supplementary Figure S1).

In regard to ITb workers, among the 138 nonredundant neuropeptides, 85, 114, 127, and 122 were identified in 0, 7, 14,

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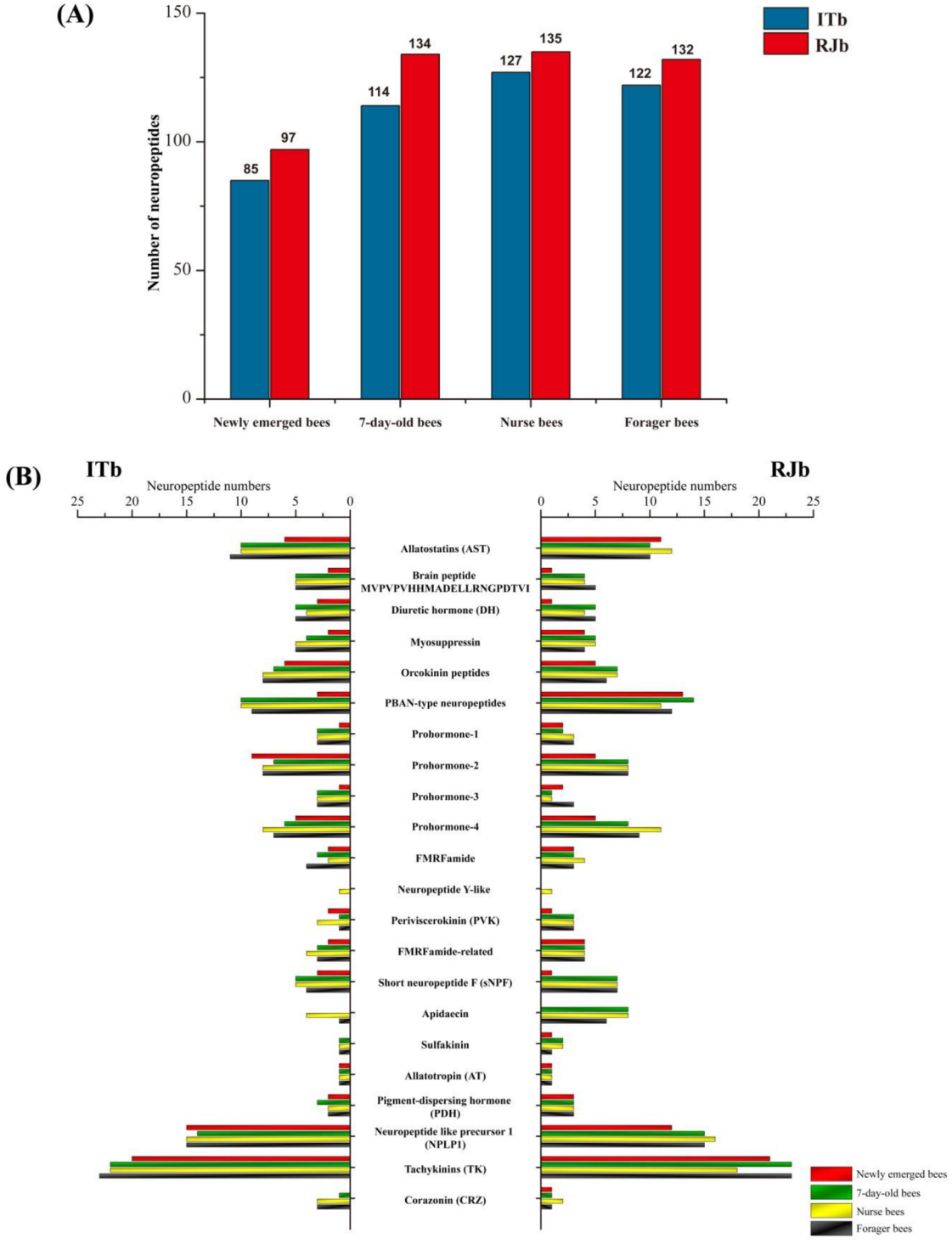

Figure 1. Comparison of brain neuropeptides identified in newly emerged bees (day 0), 7 day old bees, nurse bees (day 14), and forager bees (day 21) of both Italian bee (ITb) and high royal jelly producing bee (RJb). (A) Total number of neuropeptides identified in each time point. (B) Number of neuropeptides identified in each neuropeptide precursor proteins. The *x* axis is the number of neuropeptides under each precursor protein and is represented by the bars.

and 21 day old worker bees, respectively. Likewise, of the 149 neuropeptides in the brain of RJb workers, 97, 134, 135, and 132 neuropeptides were identified at each time point in the ITb (Figure 1A). Apparently, the highest number of neuropeptides was identified in the nurse bees (day 14), and the numbers of neuropeptides derived from all precursor proteins at each time point in the two honeybee strains were quite similar (Figure 1B).

3.2. Quantitative Comparison of Brain Neuropeptidomes

To reveal abundance change of neuropeptide functions in regulating worker bee behaviors during age-related task transition, a label-free quantitative strategy was employed to compare the level of neuropeptide abundance at the four time points in each honeybee strain. Overall, the shared 14 precursor proteins showed significant level of differences during the four

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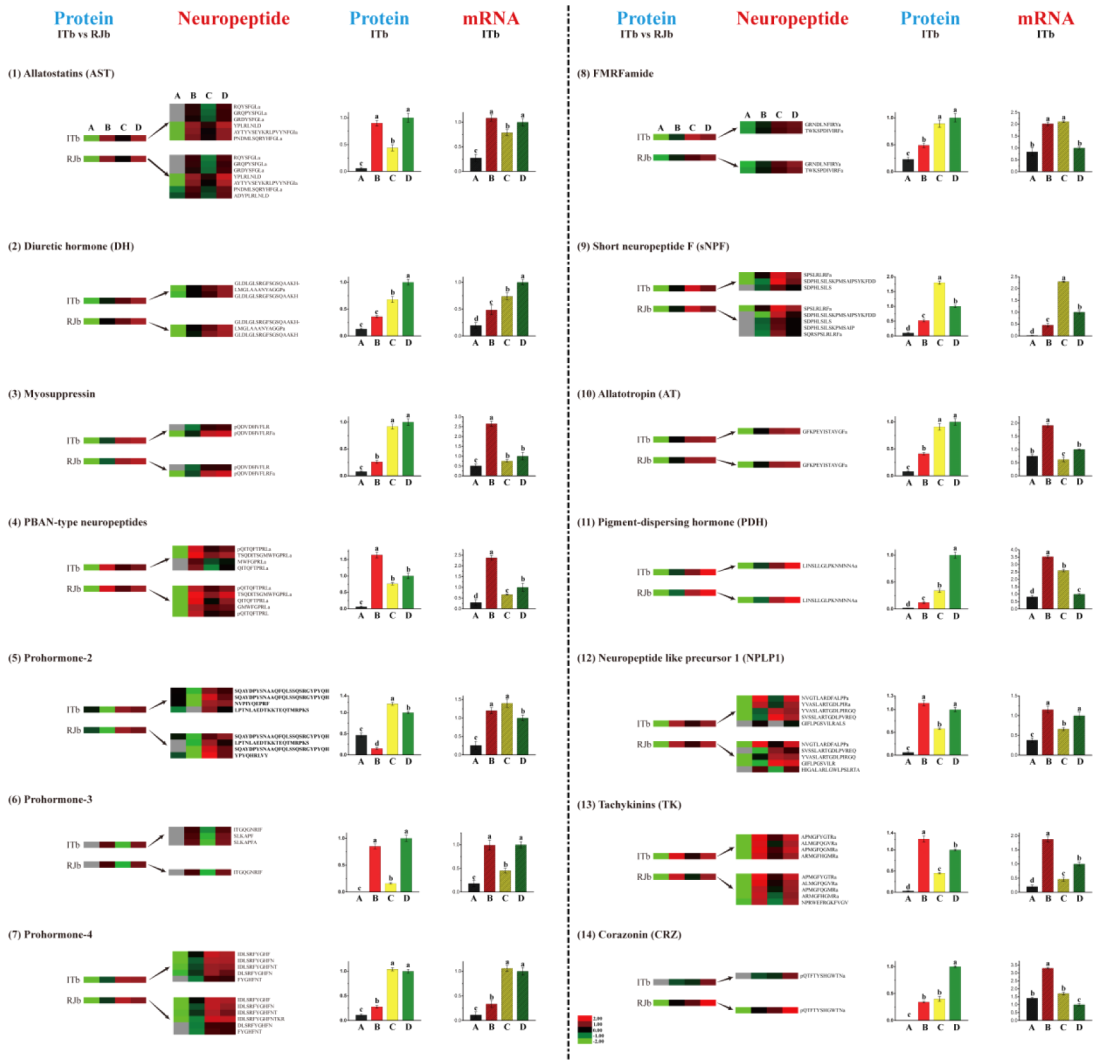

Figure 2. Quantitative comparison of brain neuropeptides expression during age-related polyethism in honeybee workers of both the Italian bee (ITb) and the high royal jelly producing bee (RJb). The relative abundance of precursor proteins and neuropeptides in A (newly emerged bees, day 0), B (7 day old bees), C (nurse bees, day 14), and D (forager bees, day 21) are represented as a heat map; the up- or down-regulated proteins are indicated by red and green color code, respectively. The color intensity changes with the protein expressional level, as noted on the key bar. The histograms denote the expression trend of the precursor proteins and their mRNA in the ITb. (a) is significantly higher than (b), (c), and (d); (b) is significantly higher than (c) and (d); and (c) is significantly higher than (d). Error bar is standard deviation.

development stages in both ITb and RJb, that is, allatostatins (AST), diuretic hormone (DH), myosuppressin, PBAN-type neuropeptides, prohormone-2, prohormone-3, prohormone-4, FMRFamide, short neuropeptide F (sNPF), allatotropin (AT), pigment-dispersing hormone (PDH), neuropeptide like precursor 1 (NPLP1), tachykinins (TK), and corazonin (CRZ). After statistical analysis, on the basis of the expression profiles, these differentially expressed neuropeptide precursor proteins were classified into five categories: (1) the expression level gradually increased throughout the development, including DH, PDH and CRZ; (2) highly expressed in 7 day old bees and foragers (no significant difference between them), including AST, prohormone-3, and NPLP1; (3) highly expressed in the nurse bees and foragers (no significant difference between them), including myosuppressin, prohormone-4, allatotropin, and FMRFamide; (4) most abundant in the 7 day old bees, that is, PBAN-type neuropeptides and TK; and (5) most abundant in the nurse bees, that is, sNPF and prohormone-2 (Figure 2).

Furthermore, to investigate the neuropeptides that are potentially associated with nursing behavior and eventually influence RJ production, we compared the abundance level of neuropeptides in ITb and RJb at each time point. Intriguingly, no significant difference was found in newly emerged bees and 7 day old bees, but significant variations were observed in nurse and forager bees (Figure 3). For nurse bees, 31 neuropeptides from 10 precursor proteins were found significantly altered their expression, of which, 9 precursor proteins, DH, PBAN-type neuropeptides, prohormone-3, FMRFamide, periviscerokinin (PVK), FMRFamide-related, PDH, NPLP1, and CRZ, were up-regulated in RJb nurse bees, but only TK was up-regulated in the ITb (Figure 3A). In foragers, of the 24 differentially regulated neuropeptides from 9 precursor proteins, 7 were significantly up-regulated in RJb foragers, AST, DH, PBAN-type neuropeptides, sNPF, PDH, NPLP1, and CRZ, while prohormone-4 and TK were up-regulated in ITb foragers (Figure 3B).

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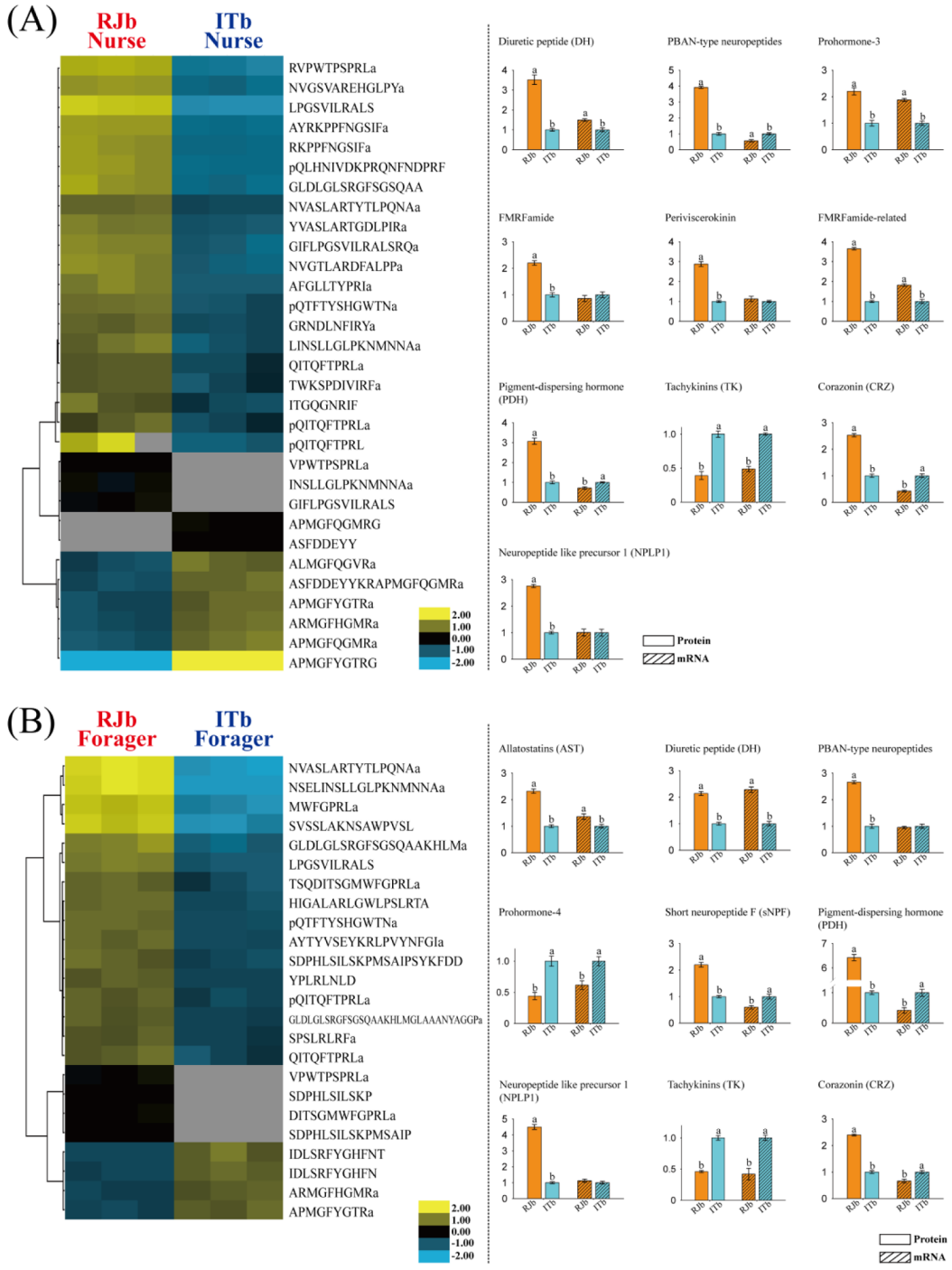

Figure 3. Quantitative comparison of brain neuropeptides expression between the Italian bee (ITb) and the high royal jelly producing bee (RJb) in (A) the nurse bees and (B) the forager bees. Figures on the left are unsupervised hierarchical clustering of the differentially expressed (fold change >2 and $p <$ 0. 05) neuropeptides, the columns represent the two honeybee stains, and the rows represent the individual neuropeptides. The up- or down-regulated proteins are indicated by yellow and blue color code, respectively. The color intensity changes with the protein expressional level, as noted on the key bar. The histograms on the right are the quantitative comparison of the expression trend of the precursor proteins and their mRNA between the ITb and the RJb. (a) is significantly higher than (b), and error bar is standard deviation.

3.3. Neuropeptide Prediction

To confirm the identified neuropeptides, we employed SignalP 4.1 and NeuroPred to predicate neuropeptides from their precursors (Supplementary Table S3). Among all of the 158 identified neuropeptides in this research, 80 neuropeptides from all 22 precursors were consistent with the predication result (Supplementary Table S1-1), of which 22 were overlapped with 77 novelly identified neuropeptides, while the remaining 58 neuropeptides were included in neuropeptidomes confirmed here and before.

3.4. Comparative Analysis of Neuropeptide Precursor Proteins at Transcription Level

To test expression concordance between the neuropeptide precursor proteins and their encoding genes, we selected all of the differentially expressed neuropeptide precursors for qRT-

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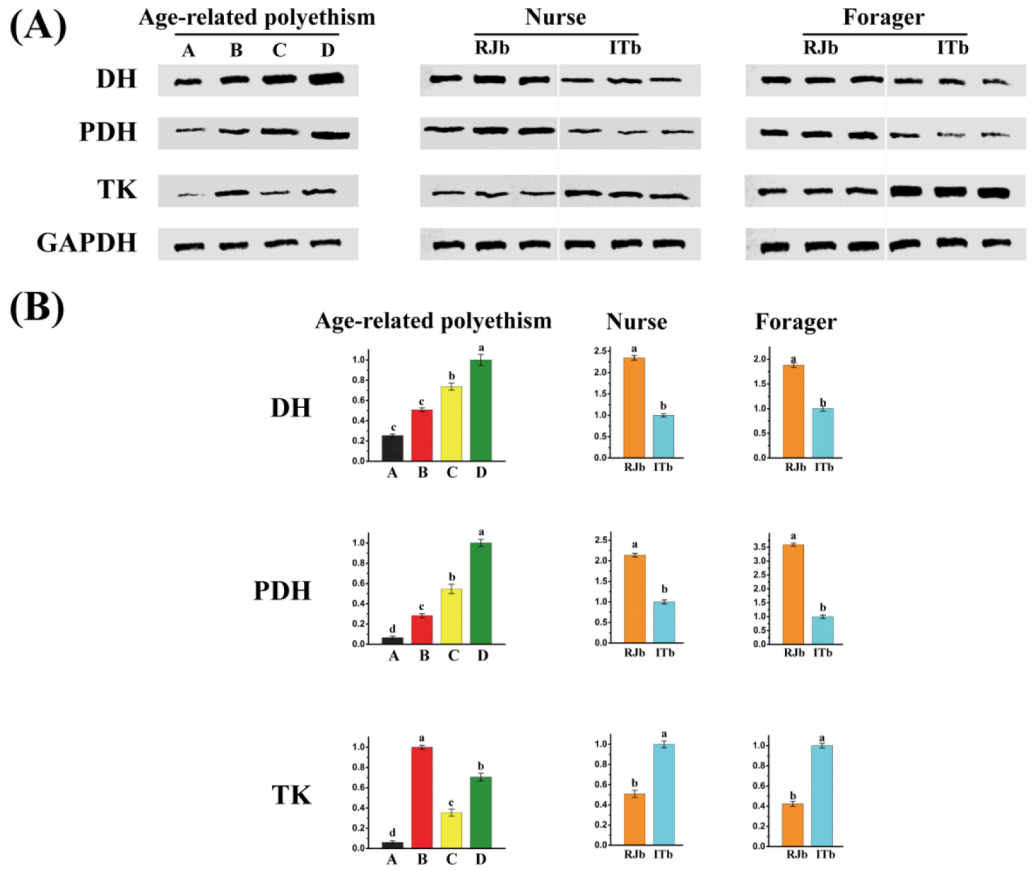

Figure 4. Western blot analysis of diuretic hormone (DH), pigment-dispersing hormone (PDH), and tachykinins (TK). The protein samples from the brain of the Italian bee (ITb) during age-related polyethism (A, newly emerged bees; B, 7 day old bees; C, nurse bees; and D, forager bees) were subjected to SDS-PAGE followed by Western blot analysis, and Western blot analysis was also used for the comparison between the ITb and the high royal jelly producing bee (RJb) in the nurse bees and foragers. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference control. (A) Western blot bands of DH, PDH, TK, and GAPDH. (B) Normalized fold change of DH, PDH, and TK during age-related polyethism in the brain of the Italian bee (ITb) and between the ITb and the RJb in the nurse bees and foragers. (a) is significantly higher than (b), (c) and (d); (b) is significantly higher than (c) and (d); and (c) is significantly higher than (d). Error bar is standard deviation.

PCR analysis. Given that the independent data set of protein species and their expression profiles were consistent between ITb and RJb over various time points, only ITb was selected for gene expression analysis. The mRNA expressions of AST, DH, prohormone-3, prohormone-4, sNPF, NPLP1, and TK were consistent with their protein expressions; however, seven other genes encoding myosuppressin, PBAN-type neuropeptides, prohormone-2, FMRFamide, allatotropin, PDH, and CRZ were observed to have inconsistent tendencies between mRNA−protein expressions (Figure 2). For example, allatotropin, PDH, and CRZ were expressed at the highest abundance level in forager brains, but their mRNA levels peaked in 7 day old workers. In the comparison between ITb and RJb, to further reveal the expression tendency between proteins and genes, we also selected all of the differentially expressed proteins for analyzing. Among the 10 validated genes in nurse bees, the expression trend of four genes was in line with protein expression, that is, DH, prohomone-3, FMRFamide-related, and TK, whereas PBAN-type neuropeptides, PDH and CRZ, showed opposite expression tendencies between proteins and mRNA; no significant difference was found in the mRNA expression of FMRFamide, PVK, and NPLP1 between ITb and RJb. In foragers, the mRNA expression of AST, DH, prohomone-3, and TK was in agreement with the protein expression, while the opposite tendencies were observed in sNPF, PDH, and CRZ. Two genes encoding PBAN-type neuropeptides and NPLP1 showed no significant differential expression between ITb and RJb. Taken together, more than half of the mRNA expression of the neuropeptide precursors was not in line with protein expression, which probably was caused by releasing peptides from the brain to other parts of the nervous system or the circulatory system or suggesting that PTMs contribute to this discordance.

3.5. Western Blot Analysis

To validate the expression of neuropeptide precursor proteins, we selected several important proteins playing key roles in division of labor for Western blot analysis, including DH in modulating the excretory system, PDH in governing the circadian clock system, and TK in regulating olfactory recognition. Likewise, to further confirm the expression of the neuropeptides that related to regulating RJ production, differentially expressed neuropeptide precursors between ITb and RJb were also verified. The expression of DH and PDH was significantly increased with the progress of age development, while TK was more abundant in 7 day old workers and foragers. On the contrary, comparing the nurse bees and foragers between ITb and RJb, DH and PDH were up-regulated in RJb, while TK was down-regulated (Figure 4). In all, the results of Western blot analysis were consistent with the proteomic data.

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4. DISCUSSION

The elaborate modulations of social behaviors demand an intricate controlling nervous system, in which specialized neuropeptidergic signals function as an efficient code. To unveil the neurobiological linkages between neuropeptides and behavior mediation, special attention has been paid to the honeybees due to the multifaceted patterns of their social behaviors. Here the in-depth brain neuropeptidomes of the honeybee were analyzed over four time points throughout adult development, representing distinct age-related division of labor. Moreover, the neuropeptidome of two strains of honeybee with significant difference in RJ production, ITb and RJb, was compared to elucidate how neuropeptidic signals were involved in the regulation of RJ secretion-related behaviors. Consequently, 158 nonredundant neuropeptides (derived from 22 protein precursors) were identified in the two lines of bees, expanding the honeybee neuropeptidome coverage to an unprecedented depth. Task-dependent expression profile of neuropeptidome manifests the fact that neuropeptide signals deeply participate in regulating honeybee physiological transitions during the age-related polyethism. The down-regulation of almost all of the neuropeptides in the brain of newly emerged bees indicates that the neuron signal networks are still immature and neuromodulators have not been fully recruited before the onset of social works. With the development of age, radical changes in tasks require the bees to modify their physiology, and thus up-regulated neuropeptides such as FMRFamide, DH, PDH, and AT are thought to tune biological functions in the excretory system, muscular system, circadian clock system, and juvenile hormone synthesis. Noticeably, the up−down-regulated neuropeptides in nurse and forager of RJb reveal the fact that neuropeptidic modulations are also correlated with the elevated RJ secretion.

4.1. Significantly Extended Neuropeptidome Profile of the Honeybee Brain

In neurons, neuropeptide precursor proteins are first synthesized in the endoplasmic reticulum, then enzymatically digested by prohormone convertases, and the propeptides are finally processed into functional peptides by further modifications, such as the removal of basic residues from the C-terminal by carboxypeptidases E and D,[45] C-terminal amidation. These modifications can protect the C-terminus from degradation by proteases,[46] N-terminal glutamine, glycosylation, and phosphorylation.[47] Because neuropeptides were uncovered as chemical messagers in the nervous system over half a century ago,[48] 36 putative genes coding for neuropeptides have been annotated in the honeybee genome, and 20 of them are experimentally confirmed at the peptide level.[32] In this study, among the identified neuropeptide precursor proteins, two precursors, allatotropin and neuropeptide Y-like, were found in honeybees for the first time. Allatotropin is a juvenile hormone stimulatory neuropeptide produced in neurosecretory cells of the brain and acts on corpora allata.[49] Despite the fact that allatotropin isolated from tobacco hawkmoth (*Manduca sexta*) is able to stimulate honeybees juvenile hormone synthesis[50] and its encoding gene has been found in the honeybee genome,[51] there is no information available on the identification of allatotropin peptide in honeybees yet. In addition, neuropeptide Y (NPY) is one of the most abundant peptides in the CNS of mammals associated with depression and stress.[52] In *Drosophila*, as the homologue of NPY-like peptides, neuropeptide F is functionally important in regulating larvae food searching and food intake.[53] In honeybees, the NPY-like gene is reported to be located in the brain's neurosecretory cells;[54] however, it has not been detected at the peptide level until now. Therefore, our data provide solid experimental evidence of two new neuropeptide precursors in the brain and expand the neuropeptide precursors to 22 in honeybees.

In honeybee brains, exactly 100 neuropeptides have been sequenced by MS. In the present research, of the identified 158 neuropeptides, 81 were consistent with previous identifications (Figure 5). The unidentified 19 neuropeptides might be caused by our stricter FDR threshold ($P \leq 1.0\%$) and qualification control of peptide identification. (Neuropeptide identifications were only used if at least two spectra were identified in one sample.) By neuropeptide predication, 80 neuropeptides were found in accordance with the MS result. As for the unpredicted neuropeptides, they were mainly truncated neuropeptides and propeptides. In the case of truncated neuropeptides, if one terminal of neuropeptide represents receptor binding activity the neuropeptide truncated in the other end can still interact with the receptor, indicating the truncated neuropeptides likely have biological functions other than artifacts. For propeptides, they can be converted into mature peptides by intricate enzymatic processing. For example, ASFDDEYYKRAPMGFQGMramide can possibly be cleaved to the tachykinin APMGFQGMRamide, and pQDVDHVFLRF from the myosuppressin might be further modified into pQDVDHVFLRFamide. Therefore, although the functions of less-conserved peptides from polypeptide precursors, C- and/or N-terminal truncated neuropeptides and propeptides are not well addressed yet,[55] and their existence has been documented here and before.[32,33] Overall, our data significantly extend the neuropeptidome coverage of the honeybee brain, and this is of vital importance for functional investigation of these novel neuropeptides that regulate the honeybee neural activity.

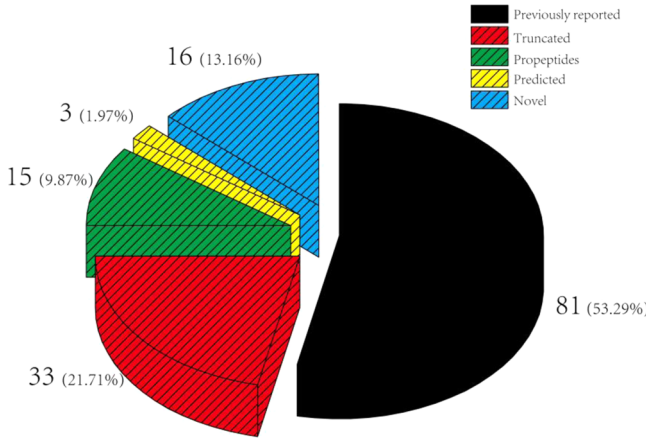

Figure 5. Classification of all the identified nonredundant brain neuropeptides. Color codes represent different neuropeptide groups.

Despite in-depth neuropeptidome analysis of the honeybee here, several precursor proteins were still not detected, such as adipokinetic hormone, insulin-like peptide, bursicon, eclosion hormone, and leucokinin, which is in concordance with previous findings.[32,33] This may suggest that the functionality of neuropeptides is under disparate evolutionary pressures in different species, and thus certain neuropeptides may have been lost and are not always in all organisms.[28,33]

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4.2. Neuropeptidome Variations Tune Physiological Transition during Age-Related Division of Labor in Honeybee Workers

The peptidergic signaling pathway is crucially important in the regulation of physiological and behavioral traits in the living organisms.[56,57] In multicellular animals the neuropeptide signaling has evolved into a dramatic diversity modulating communication between neurons, the nervous system, and other physiological systems and finally exhibits integrated regulations in development, reproduction, and behavior.[58−60] The similar task-related neuropeptidome profile in ITb and RJb suggests that the division of labor in honeybee workers is a brain-controlled and multicondition-effected transition, in which the neuropeptide signaling system is intensively implicated. This also indicates that the ITb and RJb employ a similar strategy in regulating physiological adaptations during labor division, even though they have quite different capacity in RJ production.

In honeybees, JH is vitally important in regulating caste determination and behavioral changes and especially associated with age-related division of labor.[61] The level of JH in honeybee workers increases with the shift from performing hive activities to foraging outside the hive, and an artificial change in JH levels can accelerate or delay this transition.[46,62] Two neuropeptides, allatotropin (AT) and allatostatin (AST), are directly associated with production and release of JH. The function of AT in stimulating JH biosynthesis has been observed in several insects.[63−65] In this research, the newly identified AT with a significantly increased level of expression during the task-related progress indicates that AT is likely a neuropeptidergic regulator through stimulating JH secretion to modulate age-dependent polyethism in honeybees. On the contrary, AST displays the adverse effect of AT by inhibiting the secretion of JH in the corpora allata;[66] however, the high abundance of AST in 7 day old workers and foragers at both peptide and mRNA levels is not in line with JH fluctuation, suggesting that JH synthesis is not influenced by AST in honeybees as in locusts and flies.[67,68]

In the life-history progression of adult honeybee workers, one of the major changes in social behavior is the shift from preforming hive tasks to intense flight bouts in the field (foraging). Accordingly, honeybee flight muscles undergo remarkable alterations in both structure and metabolism for the adaptation of increased flight activity.[69] In our data, two myotropic neuromodulators, FMRFamide and myosuppressin, were up-regulated during this life transition. The FMRFamides have roles in regulating skeletal muscle and tuning muscle of the heart and intestine.[70−72] Likewise, the myosuppressin potentiates the contraction in several types of skeletal muscle.[73] Thus, the elevated expressions of FMRFamide and myosuppressins across the development stages with peak level in the forager stage demonstrate neuropeptidic regulations in the enhancement of musculature governing to solidify the foraging works in the field.

Another significant physiological transition between hive bees and foragers is the dramatically changed metabolic rate. The 10 to 100 times higher metabolic rate in foragers than in nurse bees is a physiological demand to drive the accelerated locomotory activity but, in turn, generates more metabolic waste.[74,75] Also, the high metabolic water production during flight and nectar diet of foragers accumulates excess water in the circulatory system.[76] To maintain a high functioning physiological system, it is essential to strictly control the water and ion balance within the hemolymph and promptly excrete harmful wastes.[77] Modulation factors DH and PVK are the only ones identified in honeybees so far.[33,63] The peptides of DH exert diuretic activities in stimulating fluid secretion of MTs in several insects.[65,78−80] Therefore, the increased expression of DH (validated by both peptidomic quantitation and Western blot analysis) during the transition from nurse bees to foragers proves its role in the promotion of excreting extra water and waste produced by the accelerated metabolism in the foraging activity.

As is well known, the formation of age-related circadian rhythms occurs with the division of labor in honeybees: The younger bees have no daily rhythms when performing tasks inside hives around the clock, whereas forager bees have a remarkable internal diurnal rhythm that uses the sun as a compass for navigation, dance communication, and for maximum food rewards via synchronizing flower visits.[17,81] In the brain, there are neuropils in charge of organizing circadian activities generated by clock neurons, and the neuropeptide PDH is the first neurotransmitter reported in insect clock neurons. In honeybees, the neuropeptide PDH is localized in the optic lobes and brains.[33,82] Therefore, the low level of PDH in hive bees and dramatically increased expression in foragers here emphasize its importance in the regulation of the circadian clock during age-related polyethism.

In addition to the neuropeptides discussed above, the expression of some other neuropeptides, such as NPLP1, prohormone-2, prohormone-3, and prohormone-4, was also varied with the transition from each labor division, implying that a wide cascade of neuropeptides are involved in the modulation of honeybee physiological functions for the adaptation of labor division.

4.3. Neuropeptides Are Related to the Regulation of RJ Secretion Behavior

RJ plays important roles in honeybee nutrition,[83] defense,[84] and caste determination,[85] and growing evidence demonstrates that RJ has pharmacological activities, such as antibacterial,[44] antioxidative,[86] antihypertensive, and anticancer effects.[87] Brood care behavior of nurse bees has been artificially used to produce RJ for human consumption. The RJb is the most important RJ producer that is now being used on a large scale in China, which contributes >90% of global RJ output.[37] Although efforts have been made to elucidate the mechanisms underlying high RJ production,[37,88] knowledge at the neuropeptidome level is still missing. The significant difference of the brain neuropeptide abundance level between the RJb and the ITb in nurse and forager stages suggests that genetic selection has reshaped the neuropeptidome setting in RJb to fit the enhanced RJ production.

Fresh RJ is a gelatinous substance with water content >60%, and thus the water consumption of the honeybee colony is increased during the brood-rearing period.[76] Given that the RJ secreted by RJb is 5 times higher than that by ITb[37] and the genetics has changed in RJb to sustain the increased RJ secretion,[89] it is believed that RJb's need to adjust their circulatory systems to keep water and ion homeostasis. Because DH and PVK are neuropeptide hormones associated with maintaining a water and ion balance, the increased expression of these two neurohormones in the RJb is supposed to serve this purpose, which is also supported by our qPCR and Western blot results. On the contrary, by recognizing brood pheromones the honeybee workers can identify the presence and state of the larvae in deciding their feeding strategies.[90] Therefore, given the inhibitory activity of TK to olfactory recognition,[91,92] its decreased expression in the RJb indicates the enhanced sensitivity to brood pheromones of nurse bees to feed the larvae,

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which is in agreement with the observation that the higher larval acceptance is found in the queen cells in RJb compared with ITb.[93]

In regards to the forager bees, of the seven neuromodulators up-regulated in the brain of the RJb, DH is implicated in regulating osmotic equilibrium, PDH exerts a controlling role in biological rhythm, and several other neuropeptides are associated with foraging behavior. The elevated expressions of these neuropeptides in foragers of the RJb are believed to be linked to the enhanced foraging capacity to support the colony's demand of RJ secretion. This is in line with the report that the RJb has developed a stronger biological merit in foraging pollen.[94] It is worth noting that there is a negative correlation between TK expression and olfaction sensitivity of honeybees to sense brood pheromones, and pollen foragers are more responsive than nectar foragers to brood pheromone stimuli.[91,34] Hence, the low abundance of these two neuromodulators in the RJb foragers indicates a strong tendency for pollen collection to prime the protein demands of RJ secretion. This is in concordance with the notion that the down-regulation of prohormone-4 and TK in the bees has the predisposition of preferential pollen collection;[34] however, this causality needs further experimental verification, such as gene knock out or neuropeptide receptor blocking.

In conclusion, our data significantly add new insight into neuropeptidome changes during social behavior transition using the brain of honeybees as a model system. The reported novel neuropeptide mediators cover the honeybee neuropeptidome to an unprecedented depth, and quantified neuropeptidome changes related to age polyethism help us to build a bridge between neuropeptidomic regulation of honeybee physiology and age-related division of labor. In general, the RJb and the ITb undergo a similar neuropeptidome variation during the age polyethism; however, the genetic selection has reprogrammed neuropeptidome settings of the RJb to enhance its RJ production. Because of the great biological significance, deepening the identification of the neuropeptidome is a prerequisite for a detailed functional understanding in honeybee neurobiology. The reported data serve as a valuable resource for further investigation of neuropeptide functions in the central and peripheral nervous system; this is important for the honeybee and other insect communities.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00632.

Supplementary Figure S1: Annotated tandem mass spectra of the neuropeptides identified. (PDF)

Supplementary Table S1: Neuropeptide identification information. Table S1-1. The brain neuropeptides identified in both Italian bee (ITb) and high royal jelly producing bee (RJb) Table S1-2. Neuropeptides identified in the Italian bees (*Apis mellifera ligustica*, ITb). Table S1-3. Neuropeptides identified in the high royal jelly producing bees (RJb). (XLSX)

Supplementary Table S2: Neuropeptide quantitation information. Table S2-1. Quantitative comparison of brain neuropeptides during age-related division of labor. Table S2-2. Quantitative comparison of brain neuropeptide precursor proteins during age-related division of labor. Table S2-3. Quantitative comparison of brain neuropeptides between the Italian bees (*Apis mellifera ligustica*, ITb) and the high royal jelly producing bees (RJb). Table S2-4. Quantitative comparison of brain neuropeptide precursors between the Italian bees (*Apis mellifera ligustica*, ITb) and the high royal jelly producing bees (RJb). Table S2-5. Primer sequences used for qRT-PCR analysis. Table S2-6. Gene expression analysis of the differentially expressed neuropeptide precursor proteins by qRT-PCR. (XLSX)

Supplementary Table S3: Predicted of cleavage sites and peptides from neuropeptide precursors. (PDF)

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Notes

The authors declare no competing financial interest.

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