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Brain Membrane Proteome and Phosphoproteome Reveal Molecular Basis Associating with Nursing and Foraging Behaviors of Honeybee Workers

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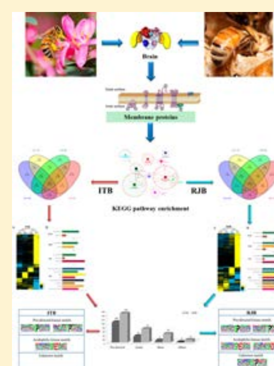
Supporting Information

ABSTRACT: The brain is a vital organ in regulating complex social behaviors of honeybees including learning and memory. Knowledge of how brain membrane proteins and their phosphorylation underlie the age-related behavioral polyethism is still lacking. A hitherto age-resolved brain membrane proteome and phosphoproteome were reported in adult worker bees from two strains of honeybee (*Apis mellifera ligustica*): Italian bee (ITB) and Royal Jelly bee (RJB), a line selected from ITB for increased RJ outputs over four decades. There were 1079 membrane protein groups identified, and 417 unique phosphosites were located in 179 membrane protein groups mainly phosphorylated by kinase families of MAPKs, CDKs, and CK2. Age-resolved dynamics of brain membrane proteome and phosphoproteome are indicative of their correlation with the neurobiological requirements during the adult life of honeybee workers. To stimulate immature brain cell development in newly emerged bees (NEBs), the enriched functional classes associated with metabolism of carbohydrates, nucleosides, and lipids by the up-regulated proteins suggest their enhanced role in driving cell maturity of the brain. In nurse bees (NBs) and forager bees (FBs), a higher number of membrane proteins and phosphoproteins were expressed as compared with in the young stages, and the enriched signal-transduction-related pathways by the up-regulated proteins suggest their significances in sustaining the intensive information processing during nursing and foraging activities. Notably, RJB has shaped unique membrane proteome and phosphoproteome settings to consolidate nursing and foraging behaviors in response to decades of selection underpinning the elevated RJ yields. In RJB NBs, the enriched pathways of phosphatidylinositol signaling and arachidonic acid metabolism indicate a stronger olfaction sensation in response to larval pheromone stimulation. In RJB FBs, the enriched pathways related to signal processing such as SNARE interactions in vesicular transport, wnt signaling, TGF-beta signaling, and taurine and hypotaurine metabolism suggest an enhanced nerve sensitivity to prime the stronger tendency to pollen collection. Our data gain a novel insight into membrane proteome and phosphoproteome-driven cerebral regulation of honeybee behaviors, which is potentially useful for further neurobiological investigation in both honeybees and other social insects.



KEYWORDS: *brain, membrane proteome, phosphoproteome, honeybee, behavior, royal jelly*

1. INTRODUCTION

In the central nervous system (CNS) of living organisms, the brain plays fundamental roles in processing sensory clues and alters its morphology and functional dynamics to fulfill behavioral demands. The well-established honeybee social structure is supported by the complex cognitive capacity of individuals to ensure the behavioral polymorphism, such as larva feeding, foraging, recognition of color and olfactory clues, dance communication, and colony defense. The honeybee brain is a highly efficient headquarters, offering an easy and direct access to the electrophysiology and optical recording of single-marked cells.[1] Although the structural difference between insect brains and mammalian brains is evident, the basic requirements for life bear a very intimate resemblance, thus having general principles that apply to both classes of animals. Furthermore, neurons of the honeybee brain largely employ the same neurotransmitters as those of the mammalian brain.[2] Therefore, the honeybee brain serves as an excellent model system to facilitate novel understanding of neural networks and their connection to learning, memory, and social behavior.[3]

The evolutionary success of eusocial insects is achieved by division of labor among the colony members, which is characterized by different groups of individuals performing distinct tasks and their abilities to respond to the signals that come from internally and externally of the colony.[4] For instance, task specialization of honeybee workers is strongly associated with their age, also known as age-related polyethism, which means that the younger worker bees perform the in-hive tasks and the older ones forage food in the field. This age-dependent polyethism of honeybees is regulated in a sophisticated and multifaceted manner, associated with social influences, pher-

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omones, environmental perturbations, and other physiological conditions such as endocrine regulation vitellogenin titer and nutritional state.[5−8] The honeybee brain is, however, strongly engaged in this behavioral transition, showing striking variations ranging from morphology to gene expression and protein synthesis. The forager bees increase the volume of mushroom body (MB) calyces,[9] mediated by rearrangement of cytoskeletal elements.[10] The expressions of several genes in the brain are related to behavioral maturation of honeybee workers, including the period gene (*per*, a circadian rhythms related gene), the foraging gene (*for*), the acetylcholinesterase gene (*ache*), and the malvolio gene (*mvl*).[11−14] Gene expression changes in the honeybee brain can be used as an indicator of naturally occurring behavior.[15,16] At the same time, the brain proteome of honeybees is strongly associated with the age-related transition from hive work to foraging.[17,18] Moreover, we have established that the dynamic of the neuropeptidome in honeybee brain is correlated with social behavior regulation.[19] Membrane proteins function in a wide cascade of roles, especially as signaling molecules transferring information via recognition of receptor proteins located in the cell membrane.[20] Despite intensive investigations of brain membrane proteome in other organisms so far,[21−23] knowledge of how membrane proteins in the honeybee brain are implicated in regulating the behavioral transition is still lacking.

As an essential barrier between living cells and their external environments, biological membranes serve to maintain cellular homeostasis and compartmentalize intracellular organelles within eukaryotes.[24] Despite the fact that biological membranes are mainly composed of both a phospholipid bilayer and protein molecules, the most vital functions of the membrane system are achieved by proteins. Normally, about one-third of all genes code for membrane proteins in an organism,[25] and their functions are primarily related to cell shape maintenance, signal transduction, cell trafficking, material transportation (e.g., plasma membrane proteins), and energy production for the cell (e.g., mitochondria inner membrane proteins). Moreover, the special functions of membrane proteins are frequently regulated by reversible phosphorylation on the cytoplasmic domains. Phosphorylation plays key roles in regulating functions of membrane-associated receptors and kinases, such as binding affinity for ligands, interaction ability with biochemical effecters, and catalysis activities.[26] Furthermore, the subcellular location of receptors and kinases can be orchestrated by its phosphorylation status.[27] Recently, we have defined that phosphorylation in the worker bee brains is involved in regulating different pathways to tune cerebral functions corresponding to the respective biological duties of the bees, such as the glycolysis/gluconeogenesis pathway in nurse bees and the ATP metabolic process in forager bees.[28] However, although membrane-associated proteins are important in a wide range of biological processes, they are notoriously difficult to detect in general proteomic studies due to the low-stoichiometry, heterogeneity, and hydrophobicity of their natures,[29] and no research has been focused on the honeybee membrane proteome and membrane phosphoproteome. To bridge the knowledge gap of membrane proteome and phosphoproteome driven task preferences in different ages of honeybee workers, in-depth membrane proteome and phosphoproteome of honeybee brains were profiled in this study. Further comparisons between the ITB and RJB were carried out to reveal the regulatory mechanisms of membrane proteins and their phosphorylation in accommodating worker bee behaviors to facilitate RJ secretion. The findings will be potentially important in gaining novel insight into neural modulation of honeybee worker behaviors.

2. MATERIALS AND METHODS

2.1. Chemical Reagents

Mem-PER Plus Membrane protein extraction Kit was purchased from Thermo Scientific (Rockford, IL). Ti^{4+}-IMAC material was purchased from the Dalian Institute of Chemical Physics (Chinese Academy of Sciences). Other chemicals used were analytical grade or better and were purchased from Sigma (St. Louis, MO).

2.2. Honeybee Brain Sampling and Membrane Protein Extraction

Two strains of honeybees (*Apis mellifera ligustica*) were used: the Italian bee (ITB) and the Royal Jelly bee (RJB). RJB is a line of bees selected from ITB since the 1980s to increase RJ yields and can produce 10 times higher yields of RJ than ITB.[30] The honeybee colonies were managed at the apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences in Beijing. Five colonies of each bee strain were headed by open mated queens of identical age, sufficient brood, and similar colony strength. Brain sampling was done at four ages. The frames containing old pupae were placed into an incubator (34 °C and 80% relative humidity) for their eclosion. Newly emerged bees (NEBs, day 0) were obtained after emerging (<2 h). The NEBs were marked on the thoraxes and placed back into the parent colonies. The marked worker bees were collected on days 7 (7DBs), 14, and 21. In particular, on day 14, only marked workers with head extension into cells containing little larvae were collected and identified as nurse bees (NBs), and on day 21 only marked bees flying into the hive with pollen loads were recognized as forager bees (FBs). For each bee strain at each time point (day 0, 7, 14 and 21), ~100 worker bees were sampled from five colonies. The brains were carefully dissected from the head capsule according to the established method.[39] The process of brain dissection was performed on ice, and the dissected brains were immediately frozen at −80 °C. Three independent biological replicates per time point were prepared. The samples were then pooled together (~300 brains) for further analysis, which could ensure both the representative of the samples and minimize the variance during sample preparation.

Membrane protein was extracted using Mem-PER Plus Membrane Protein Extraction Kit according to the manufacturer's instructions with minor modifications. In brief, 20−40 mg of brain sample was washed by cell wash solution, followed by homogenization in permeabilization buffer. The sample was centrifuged at 16 000*g* for 20 min at 4 °C to pellet permeabilized cells. Then, the supernatant was carefully removed and the pellet was dissolved using solubilization buffer. After 30 min of incubation at 4 °C with constant shaking, the sample was centrifuged at 16 000*g* for 20 min at 4 °C. Finally, the supernatant containing solubilized membrane proteins and membrane-associated proteins was transferred to a new tube. The protein concentration was quantified using a Bradford assay;[31] then, the samples were stored at −80 °C for future use.

2.3. Filter-Aided Protein Digestion

Protein digestion using filter aided sample preparation (FASP) was performed as previously described with slight modifications.[32] First, ~200 μg of membrane proteins was mixed with 200 μL of strong SDS extraction buffer (100 mM Tris pH 7.4, 4% SDS, and 0.1 M dithiothreitol) and incubated at room

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temperature (RT) for 30 min to break the proteins' disulfide bonds. Then, the sample was concentrated in 10k Microcon centrifugal filter units (Millipore, Billerica, MA) by a centrifugation at 14 000*g* for 20 min. The concentrated sample was mixed with 200 μL of UA solution (8 M urea in 0.1 M Tris/HCl pH 8.5) and centrifuged at 14 000*g* for 20 min. The pellet was diluted in the filtration devices with 200 μL of UA solution and centrifuged again. The enriched pellet was mixed with 200 μL of iodoacetamide (IAA) solution (50 mM IAA in UA solution) and incubated in the dark at RT for 30 min for alkylation. Following a centrifugation at 14 000*g* for 20 min, the pellet was diluted with 0.2 mL of UA solution and centrifuged twice. Afterward, the concentrate was diluted with 0.1 mL of 40 mM NH_4HCO_3 and followed by centrifugation at 14 000*g* for 20 min. The protein samples were digested at 37 °C overnight using trypsin (enzyme to substrate ratio [w/w] of 1:50). The peptides were collected by centrifugation of the filter units. The peptides were dried using a SpeedVac system (RVC 2−18, Marin Christ) for LC−MS/MS analysis.

2.4. Filter-Aided Phosphopeptide Enrichment Using Ti^{4+}-IMAC

Ti^{4+}-IMAC material was prepared according to our previously established protocol.[33] In brief, the immobilized Ti^{4+} polymer beads were obtained with overnight incubation of 10 mg of polymer beads in 100 mM $Ti(SO_4)_2$ solution at RT with gentle stirring. After the removal of the supernatant by centrifugation at 20 000*g* for 2 min, distilled water was used to wash the Ti^{4+}-IMAC beads several times for the removal of the residual titanium ions. The obtained Ti^{4+}-IMAC beads were dispersed in 30% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) before use. The phosphopeptide enrichment was carried out with filter assistant protocol. First, the digested peptide sample was reconstituted in 500 μL of binding buffer (6.0% TFA/80% ACN) and incubated with 5 mg of Ti^{4+}-IMAC material at RT for 60 min with vigorous shaking. Thereafter, the sample was load into 30k Microcon filter units (Millipore) and centrifuged at 14 000*g* for 20 min to remove the supernatant. Second, the beads containing captured phosphopeptides were washed sequentially with 200 μL of binding buffer, wash buffer I (0.6% TFA/50% ACN/200 mM NaCl), and wash buffer II (0.1% TFA/30% ACN). The bound phosphopeptides were then eluted twice with 100 μL of elution buffer (10% ammonia solution). Finally, the two fractions were combined and desalted by Zip-Tip C18 columns (Millipore), followed by vacuum drying and storage at −80 °C for LC−MS/MS analysis.

2.5. LC−MS/MS Analysis

The extracted peptide samples were dissolved in 0.1% formic acid in distilled water, and the peptide concentration was quantified using a Bradford assay.[31] LC−MS/MS analysis was run on Easy-nLC 1000 (Thermo Fisher Scientific, Bremen, Germany) coupled Q-Exactive (Thermo Fisher Scientific) mass spectrometer. Samples were loaded onto a reversed-phase trap column (2 cm long, 100 μm inner diameter fused silica column filing with 5.0 μm Aqua C18 beads, Thermo Fisher Scientific) at a flow rate of 5 μL/min. Buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile) were used as mobile phase buffer. Peptides were separated on an analytical column (15 cm long, 75 μm inner diameter fused silica column filing with 3.0 μm Aqua C18 beads, Thermo Fisher Scientific) at a flow rate of 350 μL/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 remaining at 90% buffer B for 10 min. The peptides were eluted from the analytical column and directly injected into the mass spectrometer via nano-ESI source. Ion signals were collected in a data-dependent mode and run with the following settings: full scan resolution at 70 000, automatic gain control (AGC) target: 3 × 10^6, maximum inject time (MIT): 20 ms, scan range: m/z 300−1,800; MS/MS scans resolution at 17 500, AGC target: 1 × 10^5, MIT: 60 ms, isolation window: 2 m/z, normalized collision energy: 27, loop count 10, and dynamic exclusion: charge exclusion: unassigned, 1, 8, > 8; peptide match: preferred; exclude isotopes: on; dynamic exclusion: 30 s. Raw data were retrieved using Xcalibur (version 2.2, Thermo Fisher Scientific). Each sample was run with technical injections in triplicate.

2.6. Protein Identification and Label-free Abundance Quantitation

The RAW MS/MS data were searched against a composite database for protein identification using in-house PEAKS software (version 8.0, Bioinformatics Solutions, Waterloo, Canada). The database contained 21 765 protein entries of *Apis mellifera ligustica* (downloaded from NCBI on December, 2015) and the common repository of adventitious proteins (cRAP, from The Global Proteome Machine Organization, released on January 1, 2012). The search parameters for membrane proteome were conducted with carbamidomethylation (C, +57.02) as a fixed modification and oxidation (M, +15.99) as a variable modification. For membrane phosphoprotein identifications, carbamidomethylation (C, +57.02) was selected as a fixed modification, and oxidation (M, +15.99) and phosphorylation (S/T/Y, +79.96) were selected as variable modifications. The other parameters used were as follows: parent ion mass tolerance, 15.0 ppm; fragment ion mass tolerance, 0.05 Da; enzyme, trypsin; max missed cleavages, 2; maximum allowed variable post translational modification (PTM) per peptide, 3. A decoy fusion method, an enhanced strategy for conservative estimate false discovery rate (FDR), was used to control FDR at ≤1.0% at both protein and peptide levels. The peptide identification was accepted only if at least two spectra were matched to this peptide sequence. The phosphosites were assigned using Scaffold PTM (version 3.0.0, Proteome Software, Portland, OR) on the basis of the Ascore algorithm.[34] Only a site confidence >90% was considered to be a localized phosphosite.

Relative abundance levels of membrane proteins in the honeybee brain were quantified by the label-free approach in PEAKS Q module. Technical triplicates of each sample were submitted to the software, and one sample was automatically selected as a reference by the PEAKS software to align the retention time shift. 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 using a high-performance retention time alignment algorithm.[35] The average MS signal responses of the three most intensive unique peptides from each protein were plotted against their calculated protein concentrations.[36] When calculating sample or group ratios, normalization factor was used, which was generated by dividing the total ion current (TIC) of the samples by the TIC of the automatically selected reference sample. The level of peptide features and proteins were considered to be significantly changed between different samples when the fold change was ≥3 and the FDR was ≤1% with PEAKS Q as significance method. The identification and quantitative analysis of all samples are included in Supporting Tables S1 and S2. A heat map of differentially expressed proteins was created by

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Gene cluster 3.0 using the unsupervised hierarchical clustering, and the result was visualized using Java Tree view software. The LC−MS/MS data and search results are deposited in ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD006823.

2.7. Bioinformatics Analyses

The membrane associated proteins were identified on the basis of the combination of transmembrane domain (TMD) prediction, signal peptide (SP) prediction, and the annotations of gene ontology (GO). TMD analysis was done as described using a majority-decision based method (MDM).[37] In brief, five topology predictors: MEMSAT3, Phobius,[38] SCAMPI,[39] TOPCONS,[40] and TMHMM,[41] were applied for TMD prediction based on different underlying algorithms, including neural networks (NN), multiple sequence alignment (MSA), and hidden Markov models (HMMs). Proteins were considered transmembrane proteins only when they were predicted by three out of the five methods. For the SP prediction, three methods were used: SignalP4.0,[42] Phobius[38] and TOPCONS.[40] Similar to the MDM for membrane protein prediction, at least two out of three methods needed to predict an SP for a protein to be considered a secreted protein.

The GO enrichment was done by comparing the input dataset with the entire genome of *Apis mellifera* as a background using ClueGO (version 2.2.4),[43] a Cytoscape (version 3.2.1) plugin. ClueGO can automatically recognize protein isoforms and assigned unique gene identifier to a protein with different isoforms. The statistically enriched GO terms were conducted using a two-sided hypergeometric test. The Bonferroni correction was used to control false-positive enrichment by correcting the probability values (pV) of a term, and only a pV ≤ 0.05 was considered a statistically significant enriched GO term.

To enrich the identified membrane protein involved in the canonical pathway in honeybee brain, KEGG Orthology-Based Annotation System (KOBAS, version 2.0, http://kobas.cbi.pku.edu.cn)[44] was used. The provided protein sequences were blasted against the *A. mellifera* database; then, pathway enrichment was conducted by a hypergeometric statistic test. The Benjamini and Hochberg FDR correction was applied to correct the probability values, and only the pathways with corrected $p < 0.05$ were considered statistically significant.

For the exploration of functional connections between membrane proteins involved in brain activity, protein−protein interaction (PPI) networks were constructed by STRING.[45] Medium confidence (0.4) was selected as the required interaction score. PPI networks were visualized using Cytoscape (version 3.2).

The residue sequences around the phosphorylated residues are utilized by kinases for site recognition.[46] According to the characteristics of surrounding residues, kinase motifs can be generally grouped into three main classes: pro-directed, acidic, and basic. All phosphosites with confidence >90% were centered on the phosphosite and extended to 13 aa (±6 residues); then, a binary decision tree was used as follows: P at +1 (Pro-directed: P), 5 or more E/D at +1 to +6 (acidic: A), R/K at −3 (basic: B), D/E at +1/+2 or +3 (A), 2 or more R/K at −6 to −1 (B), otherwise (others: O).[47] To extract motifs from our phosphorylation dataset based on the above, all confidence phosphosites were prealigned to ±6 amino acids with a phosphorylated residue in the center, then submitted to the Motif-X algorithm (http://motif-x.med.harvard.edu/motif-x.html) for motif identification.[48] The minimum number of motif occurrences was set to 3% of the entire input sequence number for each residue. The uploaded *Apis mellifera* proteome (a <10 megabytes database that was randomly generated from *Apis mellifera* proteome) database was used as a background, and the significance threshold was set to p-value $<10^{-6}$.

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

To survey the membrane proteins implicated in brain development and behavior regulation at the gene level, total RNA was extracted from the brains of NEBs, 7DBs, NBs, and FBs of the two bee strains in ice using TRIzol reagent (Takara Bio, Kyoto, Japan). Total RNA quantification was performed by NanoDrop ND91000 spectrophotometer (Thermo Fisher Scientific), and the quality of RNA was evaluated by 1.0% denaturing agarose gel electrophoresis according to the bands of 28s and 18s rRNA. Fourteen 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 Supporting Table S3 with glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) 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.[44] 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.

2.9. Measurement of Larval Acceptance Rate, RJ Production, and Pollen Collection

To compare larval acceptance rate, RJ production, and pollen collection between ITB and RJB, the same five colonies of each bee strain with almost identical populations, food storage, and brood composition, as in the above-described brain sampling were used. A frame containing 132 plastic queen cell cups fixed on two strips of wood bars was placed into each colony for RJ production. Once the young worker larvae (∼24 h old) grafted into queen cell cups, the frames were put back into the colonies for nurse bees to take care of the grafted larvae. Approximately 68−70 h after that, the frames were taken out of the colony for RJ gathering. By removing the bees on the frames, the acceptance of queen cells was counted (only queen cells containing larvae and RJ were counted as accepted), followed by removing both the wax caps at the top of the plastic queen cell cups and the larvae in the cells. The RJ was then collected and measured by electronic scale. For the measurement of pollen collection of a colony, a pollen trapper was installed at the entrance of each of the hives (9 am to 11 am), and the collected pollen was weighed by electronic scale. Measurement of RJ yields and pollen collection of each colony were independently repeated three times.

3. RESULTS

3.1. First In-Depth Membrane Proteome of Honeybee Brain

To comprehensively define the membrane proteome of honeybee workers, the brains of both ITB and RJB were sampled in four stages of adult worker bees: NEBs, 7DBs, NBs, and FBs. In total, 2143 protein groups were identified in the

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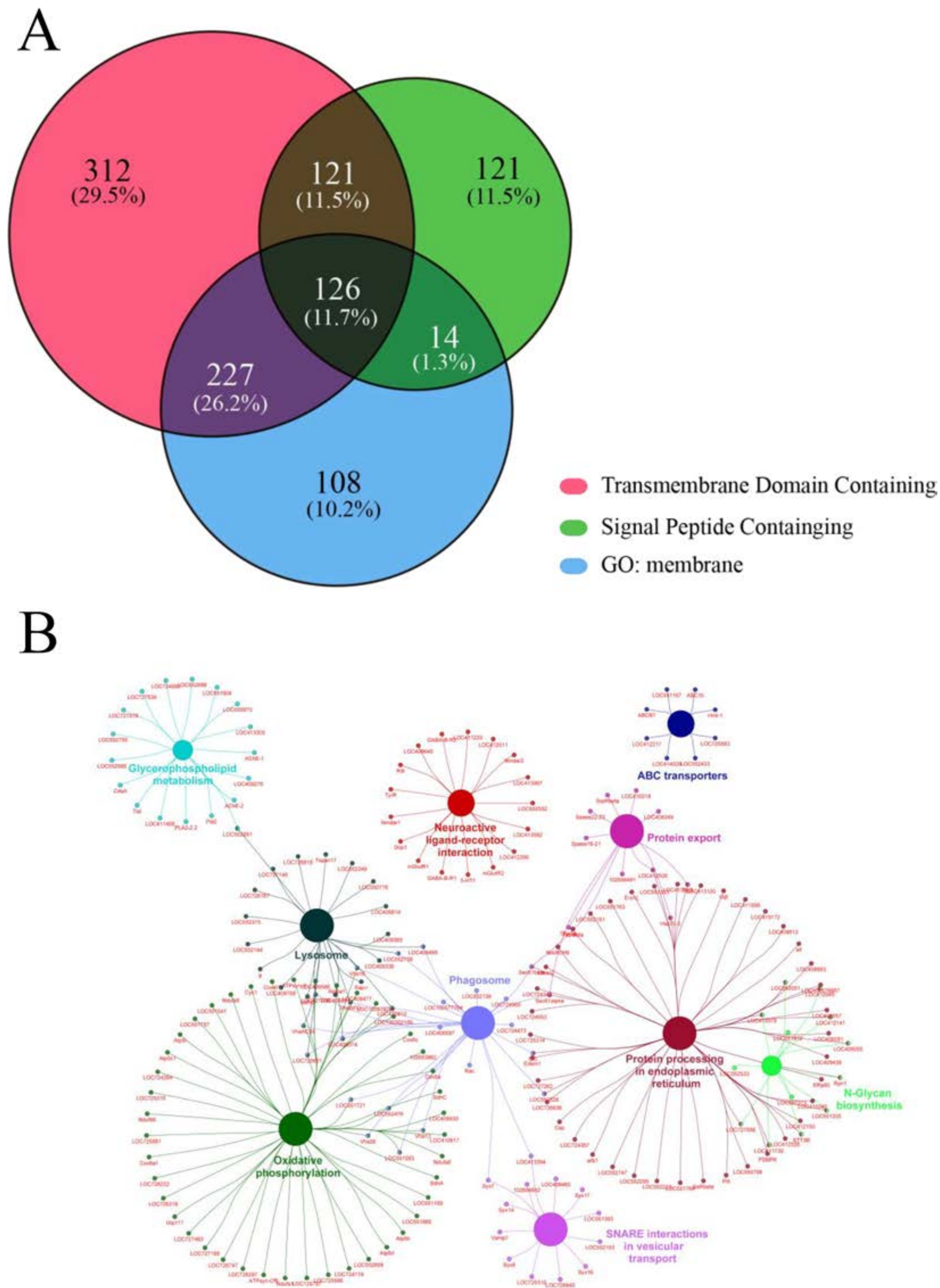

Figure 1. Global view of the totally identified membrane proteins in the brain of honeybee workers (*Apis mellifera ligustica*). (A) Venn diagram shows the constitution of the 2690 nonredundant membrane proteins; see Table S1-3. (B) Significantly enriched KEGG pathways of the identified membrane proteins; see Table S1-6.

brain, of which 1079 (50.35%) were predicted as membrane-associated proteins (Figure S1A). There were 1379 protein groups identified in the ITB workers and 1957 in RJB workers, of which 743 (53.87%) and 1021 (52.17%) protein groups were identified as membrane proteins in ITB (Figure S1B) and RJB (Figure S1C), respectively.

Among the 1079 membrane proteins identified in the brain of both bee stocks, 813 protein groups were found to have at least one transmembrane domain (TMD), 359 protein groups were found to have a signal peptide (SP), and 502 protein groups were assigned to GO term "membrane" (Figure 1A, Table S1-3). The 1079 membrane proteins were strongly enriched in 10 pathways, of which oxidative phosphorylation ($p = 4.93 \times 10^{-22}$) and protein processing in the endoplasmic reticulum (ER, $p = 4.96 \times 10^{-17}$) were the most strongly enriched, followed by lysosome ($p = 4.32 \times 10^{-6}$), phagosome ($p = 6.21 \times 10^{-6}$), and SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment receptor)

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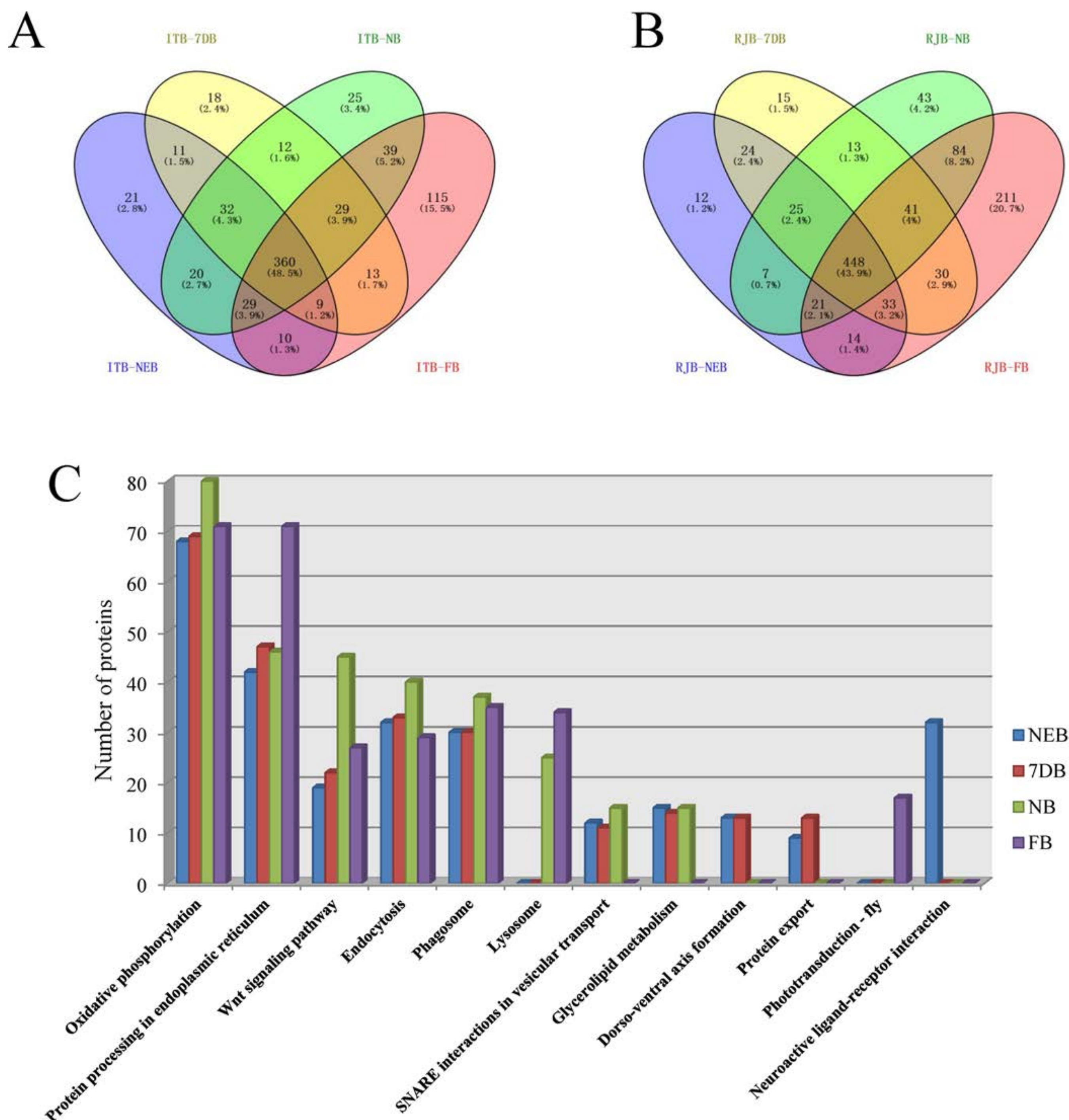

Figure 2. Comparison of the brain membrane proteome across the four stages during age-related polyethism in honeybee workers (*Apis mellifera ligustica*). (A,B) Venn diagrams show the distribution of identified membrane proteins in the Italian bee (ITB) and the royal jelly bee (RJB) at four stages of adult worker bees: newly emerged bee (NEB), 7-day old bee (7DB), nurse bee (NB), and forager bee (FB). (C) Significantly enriched KEGG pathways of the identified membrane proteins in each stage.

interactions in vesicular transport ($p = 2.80 \times 10^{-5}$) (Figure 1B, Table S1-6).

The 1064 (49.65%) identified proteins that were not classified as membrane proteins contained neither TMDs nor SPs. The reasons for this may be that some proteins are connected to the membrane via PPIs or are covalently modified by lipids (lipidation).[49] Besides this, some of the identified proteins are contaminations of cytoplasmic proteins, which cannot be prevented in the process of membrane protein extraction.

3.2. Time-Resolved Comparison of Brain Membrane Proteome during Age Development

To dissect the dynamics of brain membrane proteome driving the neural activity of the adult worker bees during age advancement, the membrane proteome was qualitatively and quantitatively compared at four time points. In the brain of ITB workers, 492, 484, 546, and 604 membrane protein groups were identified in NEBs, 7DBs, NBs and FBs, respectively. An overlapping membrane proteome (core proteome) including

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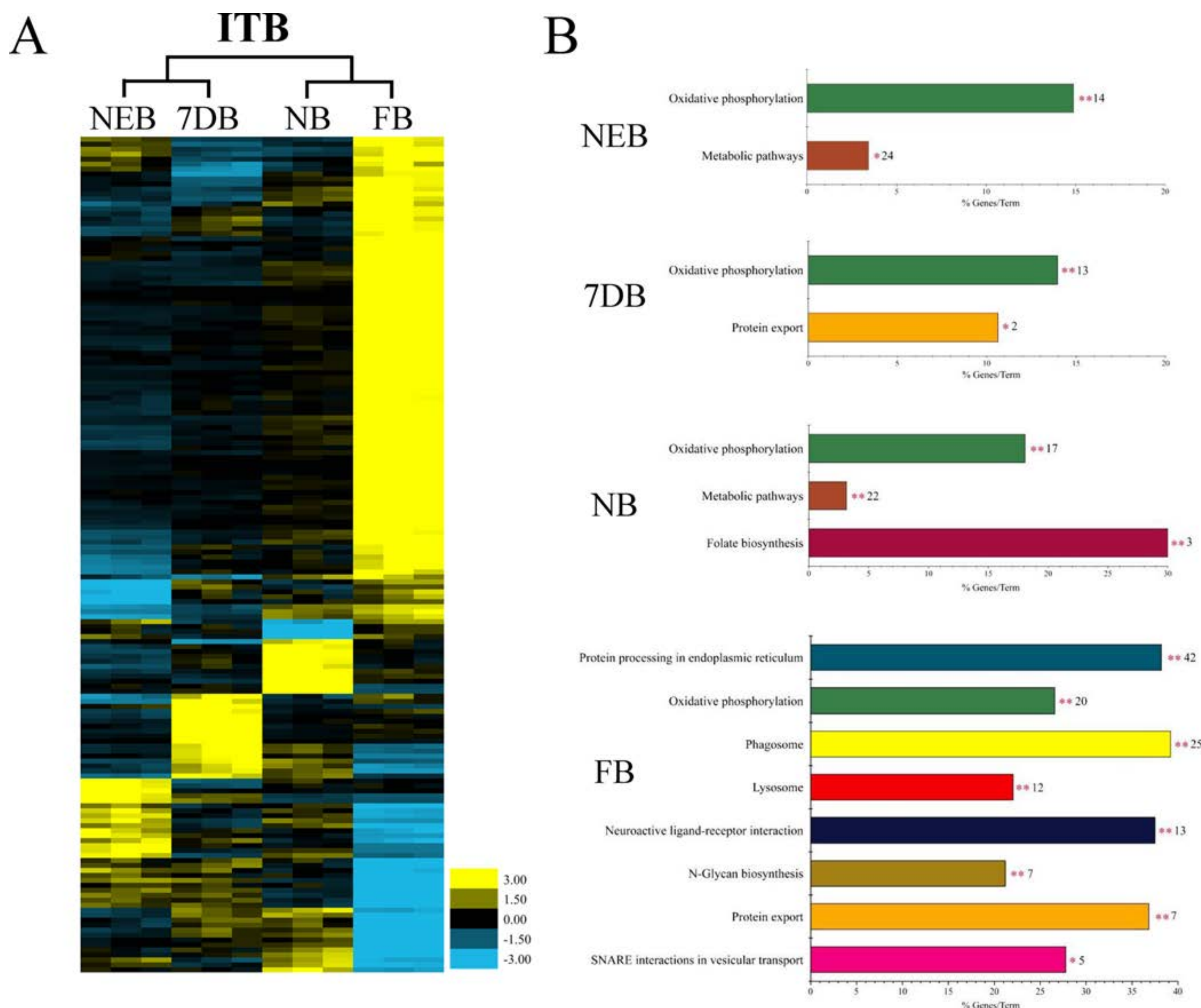

Figure 3. Quantitative comparison of membrane protein expressions during age-related polyethism in honeybee workers of the Italian bee (ITB). (A) Relative abundance of membrane proteins in newly emerged bee (NEB), 7-day old bee (7DB), nurse bee (NB), and forager bee (FB) are represented as a heat map with unsupervised hierarchical clustering. 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. (B) Significantly enriched KEGG pathways of the up-regulated proteins in each stage. For the details of the enrichment analysis results, see Table S1-10.

360 protein groups (representing 48.5% of total identified membrane proteins) was found across the four time points, and the number of uniquely membrane protein groups in the brain was 21, 18, 25, and 115 at each time point, respectively (Figure 2A). For the RJB workers, 585, 630, 683, and 882 membrane protein groups were identified at each time point, of which 448 membrane protein groups (43.9%) overlapped, and 12, 15, 43, and 211 protein groups were exclusively expressed at each time point (Figure 2B).

KEGG pathway enrichment of the identified membrane proteins in each stage of both honeybee strains found similar pathway coverage in both ITB and RJB. Five pathways, oxidative phosphorylation, protein processing in ER, wnt signaling pathway, endocytosis, and phagosome, were significantly enriched and shared by four time points. Dorso-ventral axis formation and protein export pathways were only enriched in NEBs and 7DBs; lysosome pathway was only found in NBs and FBs, and phototransduction was exclusively enriched in FBs (Figure 2C, Tables S1-7 and S1-8).

To evaluate the abundance level alteration of membrane proteins over age development, a total of 168 membrane protein groups were found significantly regulated over four time points in ITB (Figure 3A, Table S1-9). The NEBs and 7DBs were clustered in one branch, whereas NBs and FBs were in another independent branch. Specifically, 26, 17, 24, and 101 protein groups were up-regulated in NEBs, 7DBs, NBs, and FBs, respectively. The up-regulated membrane proteins in the brain of NEBs were mainly enriched in oxidative phosphorylation and metabolic pathway. In 7DBs, the up-regulated membrane proteins were enriched in oxidative phosphorylation, protein export, and glycerolipid metabolism pathways. In NBs, oxidative phosphorylation, metabolic pathway, and folate biosynthesis pathway were enriched. In FBs, the up-regulated proteins were enriched in protein processing in ER, phagosome, oxidative phosphorylation, neuroactive ligand−receptor interaction, lyso-

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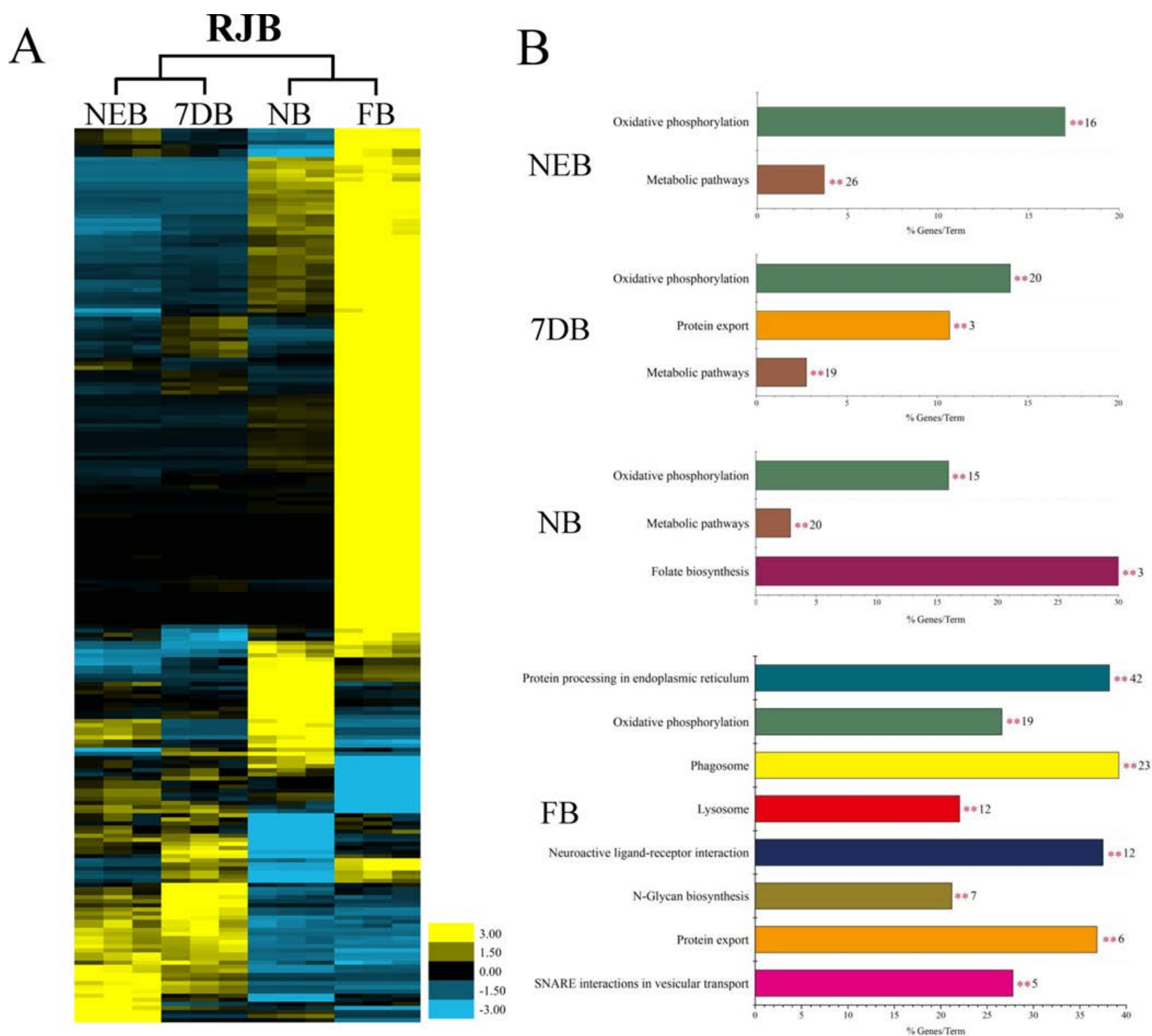

Figure 4. Quantitative comparison of membrane protein expressions during age-related polyethism in honeybee workers of the royal jelly bee (RJB). (A) Relative abundance of membrane proteins in newly emerged bee (NEB), 7-day old bee (7DB), nurse bee (NB), and forager bee (FB) are represented as a heat map with unsupervised hierarchical clustering. 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. (B) Significantly enriched KEGG pathways of the up-regulated proteins at each stage. For the details of the enrichment analysis results, see Table S1-12.

some, protein export, *N*-glycan biosynthesis, and SNARE interactions in vesicular transport (Figure 3B, Table S1-10).

Regarding RJB, 217 membrane protein groups significantly changed in abundance levels, of which 28, 33, 29, and 127 protein groups were up-regulated in NEBs, 7DBs, NBs, and FBs, respectively (Figure 4A, Table S1-11). A similar protein expression pattern and comparable enriched pathways to those of ITB were found (Figure 4A, Table S1-12).

To reveal specific interaction patterns of the membrane proteins in the regulation of brain functions, especially in modulating age-related behavior variations, the differentially expressed membrane proteins were used to construct a PPI network. As a result, 153 proteins were linked into a PPI network, and significantly enriched into six functional groups: metabolic pathway, oxidative phosphorylation, protein processing in ER, phagosome, protein export, and neuroactive ligand–receptor interaction (Figure 5).

3.3. Comparison of Brain Membrane Proteome between ITB and RJB

To follow the membrane proteome variation between ITB and RJB, the proteomes were compared between ITB and RJB at each of the four time points (Tables S1-13 to S1-16).

Of all 1079 identified membrane protein groups, 697 (64.6%) were shared in both honeybee strains, while 46 and 324 protein groups were uniquely identified in ITB and RJB, respectively. In ITB worker brains 360 membrane proteins were shared at all four time points; in RJB this number was 448. Further GO analysis of these two core membrane proteomes shows that both honeybee strains are significantly enriched in the same nine biological processes, with transport, intracellular signal transduction, and

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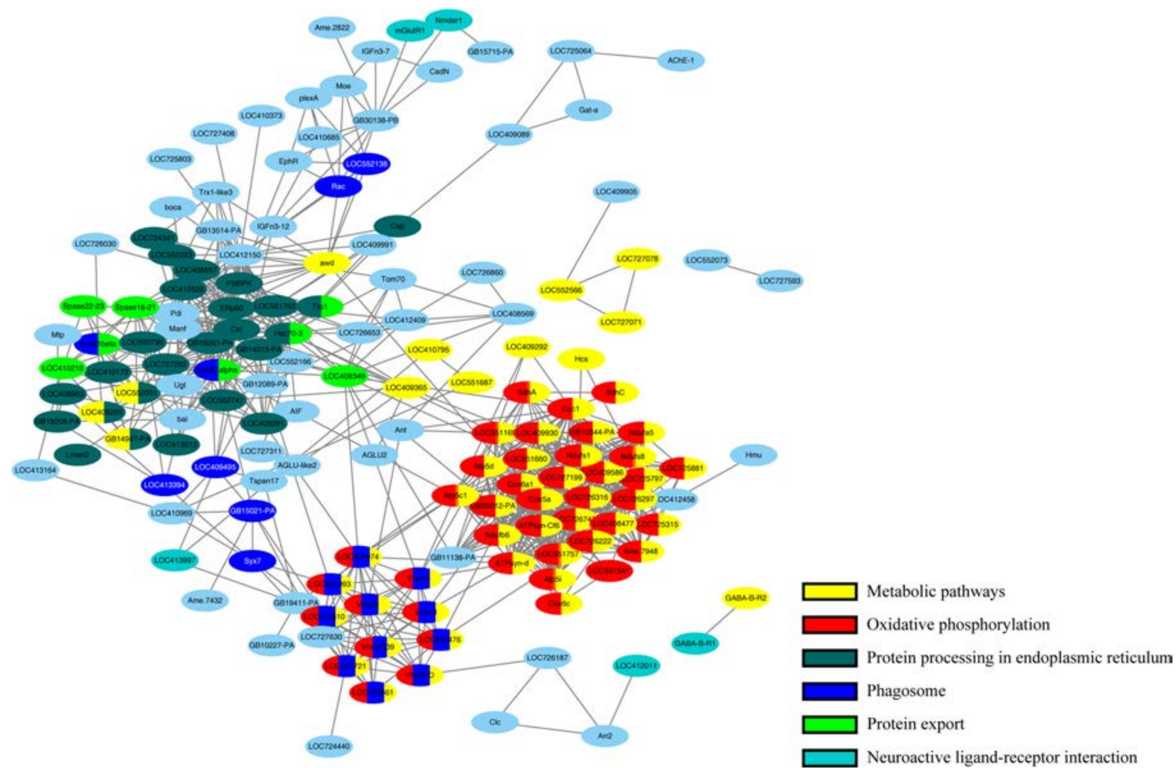

Figure 5. Protein−protein interaction (PPI) network of the differentially expressed membrane proteins during age-related polyethism in honeybee workers. 153 proteins were linked into the network, and 101 proteins were significantly enriched into six functional groups: metabolic pathway, oxidative phosphorylation, protein processing in ER, phagosome, protein export, and neuroactive ligand−receptor interaction.

ribose phosphate metabolic process as the top three terms (Figure S2, Tables S1-17 and S1-18).

In NEBs, 645 membrane protein groups were identified in both honeybee stocks, with 61 and 153 protein groups uniquely identified in ITB and RJB, respectively. Among the 157 differentially expressed proteins (fold change ≥3, $p \leq 0.01$), 134 were up-regulated in RJB and 23 in ITB. The up-regulated membrane proteins in RJB were enriched in pathways involved in oxidative phosphorylation, protein processing in ER, and phagosome. In contrast, the ITB up-regulated membrane proteins were not significantly enriched in any pathways. In 7DBs, 668 membrane protein groups were identified in both stocks, with 39 and 184 protein groups uniquely identified in ITB and RJB, respectively. Among the 320 differentially expressed membrane proteins, 295 were highly abundant in RJB and 25 in ITB. The proteins with high level of abundance in RJB, were enriched in pathways including oxidative phosphorylation, protein processing in ER, metabolic pathway, and phagosome. In NBs, 762 membrane protein groups were identified in both honeybee strains. Among the 225 differentially expressed proteins, 212 were up-regulated in RJB and mainly significantly enriched in the following pathways: wnt signaling pathway, SNARE interactions in vesicular transport, phagosome, and oxidative phosphorylation. Similarly, the proteins highly abundant in ITB were significantly enriched in pathways associated with ABC transporters and glycerolipid metabolism. In FBs, 942 membrane proteins were identified by both strains. Among the 269 differentially expressed proteins between the two bee stocks, 255 had a higher level of expression in RJB and 14 were up-regulated in ITB. The proteins up-regulated in RJB were mainly enriched in oxidative phosphorylation, SNARE interactions in vesicular transport, and endocytosis pathways. On the contrary, the membrane proteins up-regulated in the ITB were significantly enriched only in the lysosome pathway. Furthermore, there were 28 proteins consistently up-regulated in RJB at all four time points, of which 15 were linked into PPI network and mainly involved in oxidative phosphorylation, SNARE interactions in vesicular transport, and phagosome (Figure S3).

3.4. Verification of Differentially Expressed Membrane Proteins

To test the altered abundance of membrane proteins at their gene level, key proteins implicated in important pathways during brain development and behavior regulation were selected. During different brain development stages, eight membrane proteins were tested, including three proteins (trehalase, acylglycerol kinase, and nucleoside diphosphate kinase) involved in the metabolic pathway, three proteins (NMDA receptor 1, metabotropic glutamate receptor 1, and gamma-aminobutyric acid type B receptor subunit 1) in the neuroactive ligand−receptor interaction pathway, and two proteins (syntaxin-12 and vesicle-trafficking protein SEC22b-B) in SNARE interactions in the vesicular transport pathway. As a result, genes of trehalase, acylglycerol kinase, and nucleoside diphosphate kinase were up-regulated in NEBs, and the expressions of five other genes were significantly up-regulated in FBs, which is in accordance with their protein expression (Figure S4A).

In the comparison between RJB and ITB, genes encoding six proteins were tested, of which NADH-ubiquinone oxidoreductase 75 kDa subunit and NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 are implicated in oxidative phosphorylation pathway; calreticulin and syntaxin-12 in SNARE interactions in vesicular transport pathway; and V-type proton ATPase subunit E and V-type proton ATPase subunit G in both pathways. All of them were observed as up-regulated in

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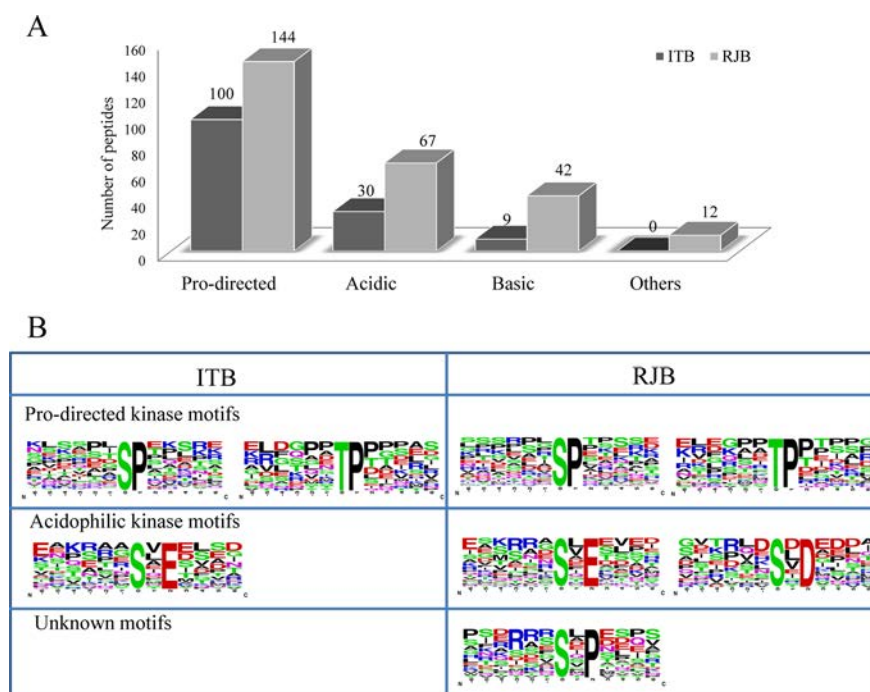

Figure 6. Comparison of kinase motifs extracted from the Italian bee (ITB) and the royal jelly bee (RJB). (A) All localized phosphosites were classified into one of three general kinase motif categories (pro-directed, acidic, basic) or "others". (B) Frequency-corrected phosphorylation motifs were extracted using the Motif-X algorithm. The minimum number of motif occurrences was set to 3% of the entire input sequence number for each residue, and the significance threshold was set to p-value $<10^{-6}$.

RJB in each stage, which is consistent with their protein expression (Figure S4B).

3.5. Phosphoproteomic Profiling of Brain Membrane Proteins

To acquire the comprehensive phosphorylation profiling of brain membrane proteins of the adult worker bees, Ti^{4+}-IMAC based phosphopeptides enrichment was applied. At four time points of ITB and RJB, 425 phosphopeptides carrying 417 unique phosphosites from 165 membrane protein groups were identified. Specifically, in ITB, 267 phosphopeptides harboring 241 phosphosites corresponding to 125 membrane protein groups were identified, and in RJB, 405 phosphopeptides containing 366 phosphosites from 172 membrane protein groups were identified (Tables S2-1 and S2-2). The phosphorylated membrane proteins were significantly enriched in oxidative phosphorylation, neuroactive ligand−receptor interaction, SNARE interactions in vesicular transport, and mTOR signaling pathways (Table S2-3).

In the brain of ITB, 163, 119, 203, and 184 unique phosphopeptides from 102, 84, 116, and 110 membrane protein groups carrying 149, 109, 182, and 172 phosphosites were found at each of the four time points, respectively (Figure S5A). Of the 125 phosphorylated membrane proteins, 72 unique phosphosites on 62 protein groups were observed at all four time points. The numbers of specifically phosphorylated membrane protein groups were 10, 3, 17, and 11 in NEBs, 7DBs, NBs, and FBs, respectively. At each of the four time points in RJB, 181, 120, 274, and 315 unique phosphopeptides from 107, 83, 140, and 157 membrane protein groups carrying 160, 108, 248, and 298 phosphosites were identified (Figure S5B), of which 97 phosphopeptides containing 85 unique phosphosites from 73 membrane protein groups were shared in all samples. In view of each time point, 8, 25, and 49 unique phosphoprotein groups were identified in NEBs, NBs, and FBs, respectively.

To better understand the peptide substrates and their corresponding kinases implicated in the phosphorylation of brain membrane proteins, all of the localized phosphosites were classified into one of three general kinase motif categories (pro-directed, acidic, basic) or "others". In both brain samples, phosphosites that belong to the pro-directed motif category were most abundant (>50%), followed by phosphosites belonging to the acidic motif category (accounting for ∼20%) and the basic motif. However, in RJB, apart from those three classes, 5% of phosphosites were categorized as "Others" which was a unique motif class (Figure 6A, Table S2-4).

To further extract frequency-corrected phosphorylation motifs from our data, the Motif-X algorithm was applied. In ITB, three kinase motifs were identified: two pro-directed motifs (pS-P and pT-P) and one acidophilic kinase motif (pS-x-E). In contrast, more motifs were found in RJB: two pro-directed kinase motifs (pS-P and pT-P), two acidophilic kinase motifs (pS-x-E and pS-x-D), and one motif (pS-x-P) that did not relate to any particular type of kinases (Figure 6B, Table S2-5).

3.6. Comparison of Brain Membrane Phosphoproteome across Ages

To reveal phosphorylation networks in the regulating function of brain membrane proteins during age development of adult worker bees, phosphopeptides were quantitatively compared. In ITB workers, 101 phosphopeptides from 81 membrane proteins significantly changed in their abundance and were mainly located in the membrane protein complex and plasma membrane part (Figure 7a, Table S2-6). The 81 membrane proteins were classified into nine GO categories. The top three categories were proteins related to transport (26, 32%), development and differentiation (15, 19%), and signal transduction (8, 10%) (Figure 7B, Table S2-7). Regarding each age development stage, 35, 47, and 19 phosphopeptides were highly abundant in NEBs, NBs, and FBs, respectively (Table S2-8). Notably, no

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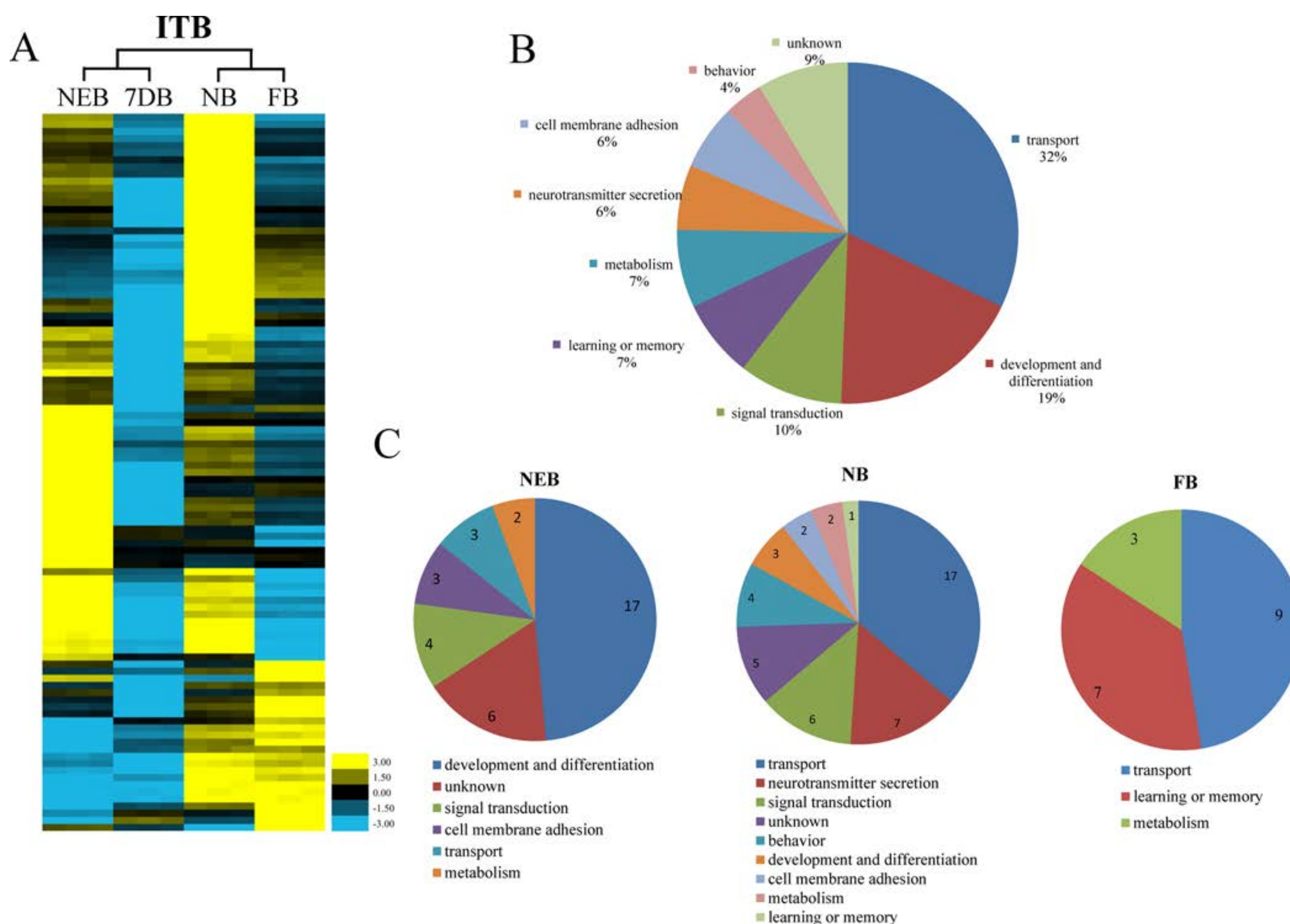

Figure 7. Quantitative comparison of phosphopeptides during age-related polyethism in honeybee workers of the Italian bee (ITB). (A) Relative abundance of membrane proteins in newly emerged bee (NEB), 7-day old bee (7DB), nurse bee (NB), and forager bee (FB) is represented as a heat map with unsupervised hierarchical clustering. 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. (B) Functional classification of differentially expressed membrane phosphoproteins in all stages. (C) Functional classification of the up-regulated phosphoproteins in each stage.

phosphopeptide was highly abundant in 7DBs. In NEBs, 35 up-regulated phosphopeptides were assigned to six GO categories, of which development and differentiation (17) was the most representative term. The highly abundant phosphopeptides in NBs were classified into nine GO classes, of which transport (17), neurotransmitter secretion (7), and signal transduction (6) were major functional groups. In FBs, the most abundant phosphopeptides were related to transport (9), learning or memory (7), and metabolism (3) (Figure 7C).

In RJB workers, 153 phosphopeptides from 93 proteins significantly changed in their abundance level across ages (Figure 8A). The 93 proteins were grouped into 9 GO categories, of which transport (28, 30%), development and differentiation (16, 17%), and signal transduction (11, 12%) were the top three (Figure 8B), similar to the case in ITB. Regarding the different stages of RJB, 50, 72, and 31 phosphopeptides had a high level of abundance in NEBs, NBs, and FBs, respectively. In NEBs of RJB, development and differentiation (22), transport (9), and cell membrane adhesion (7) were the abundant groups. In NBs, the highly abundant phosphopeptides were mostly related to transport (25), neurotransmitter secretion (13), and signal transduction (13). In FBs, the phosphopeptides with increased level of abundance were assigned to four categories: learning or memory (18), transport (7), behavior (4), and metabolism (2). Of the differentially expressed phosphoproteins, 53 were connected in PPI network (Figure 8C).

3.7. Comparison of Brain Membrane Phosphoproteome between ITB and RJB

To deeper understand the functional differences of phosphorylation in both bee strains, the brain membrane phosphoproteomes of ITB and RJB workers were quantitatively compared at each of the four stages. Only the phosphopeptides differing in changing patterns different from their whole proteins or showing abundance change exclusively at the phosphopeptide level were kept (Tables S2-9 and S2-10).

In NEBs, 42 phosphopeptides from 35 membrane proteins were up-regulated in RJB, and they were grouped into nine terms with development and differentiation as the top category containing 12 phosphopeptides. In contrast, the 19 phosphopeptides from 18 membrane proteins with increased abundance level in ITB were mostly associated with transport. In 7DBs, 61 phosphopeptides from 45 proteins had a high level of abundance in RJB and were grouped into 9 GO terms with transport as the top one containing 12 phosphopeptides. By comparison, only three phosphopeptides from three proteins were up-regulated in ITB, and two of them were implicated in development and differentiation. In NBs, 20 phosphopeptides from 17 membrane proteins were increased in the abundance level in RJB and mainly

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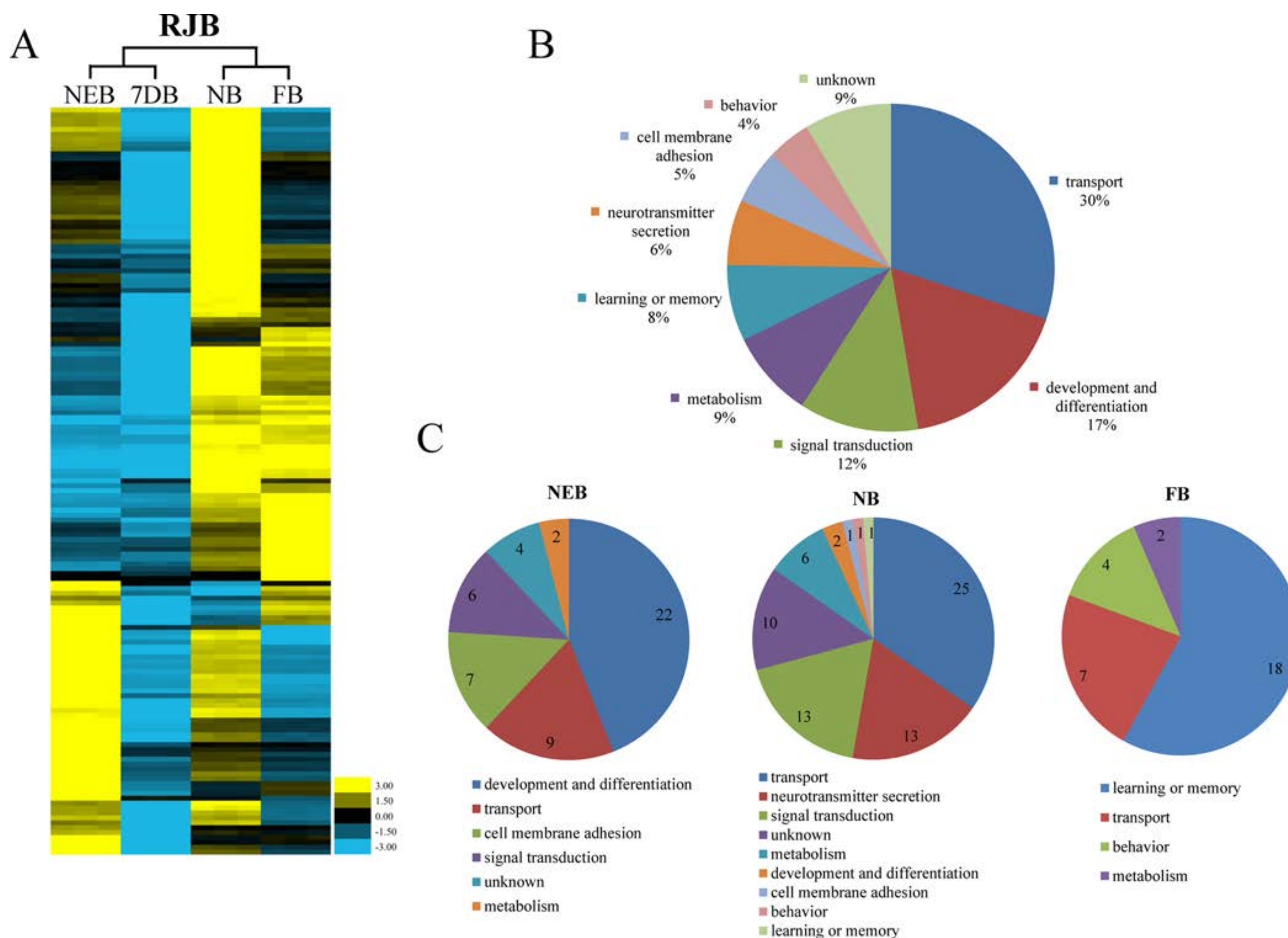

Figure 8. Quantitative comparison of the phosphopeptides during age-related polyethism in honeybee workers of the royal jelly bee (RJB). (A) Relative abundance of membrane proteins in newly emerged bee (NEB), 7-day old bee (7DB), nurse bee (NB), and forager bee (FB) are represented as a heat map with unsupervised hierarchical clustering. 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. (B) Functional classification of differentially expressed membrane phosphoproteins in all stages. (C) Functional classification of the up-regulated phosphoproteins in each stage.

associated with transport, development, and differentiation. In ITB, however, four phosphopeptides from four up-regulated proteins were involved in different functional categories. In FBs, 21 phosphopeptides from different proteins were highly abundant in RJB, and 8 of them were implicated in transport. In FBs of ITB, eight phosphopeptides from seven membrane proteins were highly abundant and mainly associated with development and differentiation and learning or memory.

3.8. Determination of Acceptance of Queen Cell Cups, RJ Production, and Pollen Collection

To compare the differences in acceptance of queen cell cups, RJ production, and pollen collection between ITB and RJB, five colonies of each bee strain were used, and all data are shown in Supporting Table S2-11. The acceptance rate of queen cell cups of RJB was 90.60 ± 2.93%, which was significantly higher than that of ITB with 60.80 ± 1.73% (Figure 9A). The RJ production of the RJB colony was 97.45 ± 3.29 g and of the ITB colony was 9.81 ± 0.27 g, an almost 10-fold difference (Figure 9B). The pollen collection of RJB was 34.95 ± 1.86 g, which was also significantly higher than the 22.17 ± 1.47 g of ITB (Figure 9C).

4. DISCUSSION

The honeybee brain is a plastic organ changing its physiological properties in response to internal and external stimuli to accomplish defined tasks according to age.[11] The membrane proteins in the brain cells play key roles in cell signaling, cell adhesion, protein trafficking, transport of ions, and so forth.[50] To understand membrane proteome and phosphoproteome driven for honeybee brain functionality, we identified an unprecedented depth of coverage of membrane proteome and phosphoproteome in adult worker bees at age-resolution. In younger worker bees, the neuron construction and neural circuit refinement are the basic requirements of the neonatal nervous system. The enriched protein export and metabolism pathways and the up-regulated phosphoproteins are thought to stimulate the development and differentiation, and cell membrane adhesion is supposed to support the brain cell maturation. In NBs and FBs, to satisfy the demand of intensive information processing in hive and field works, the enriched oxidative phosphorylation, pathways of SNARE interactions in vesicular transport, and neuroactive ligand−receptor interaction are supposed to underpin signal transduction. The strongly activated phosphoproteins related to ion transport and signal transduction suggest that phosphorylation plays vital roles in driving neural activities.

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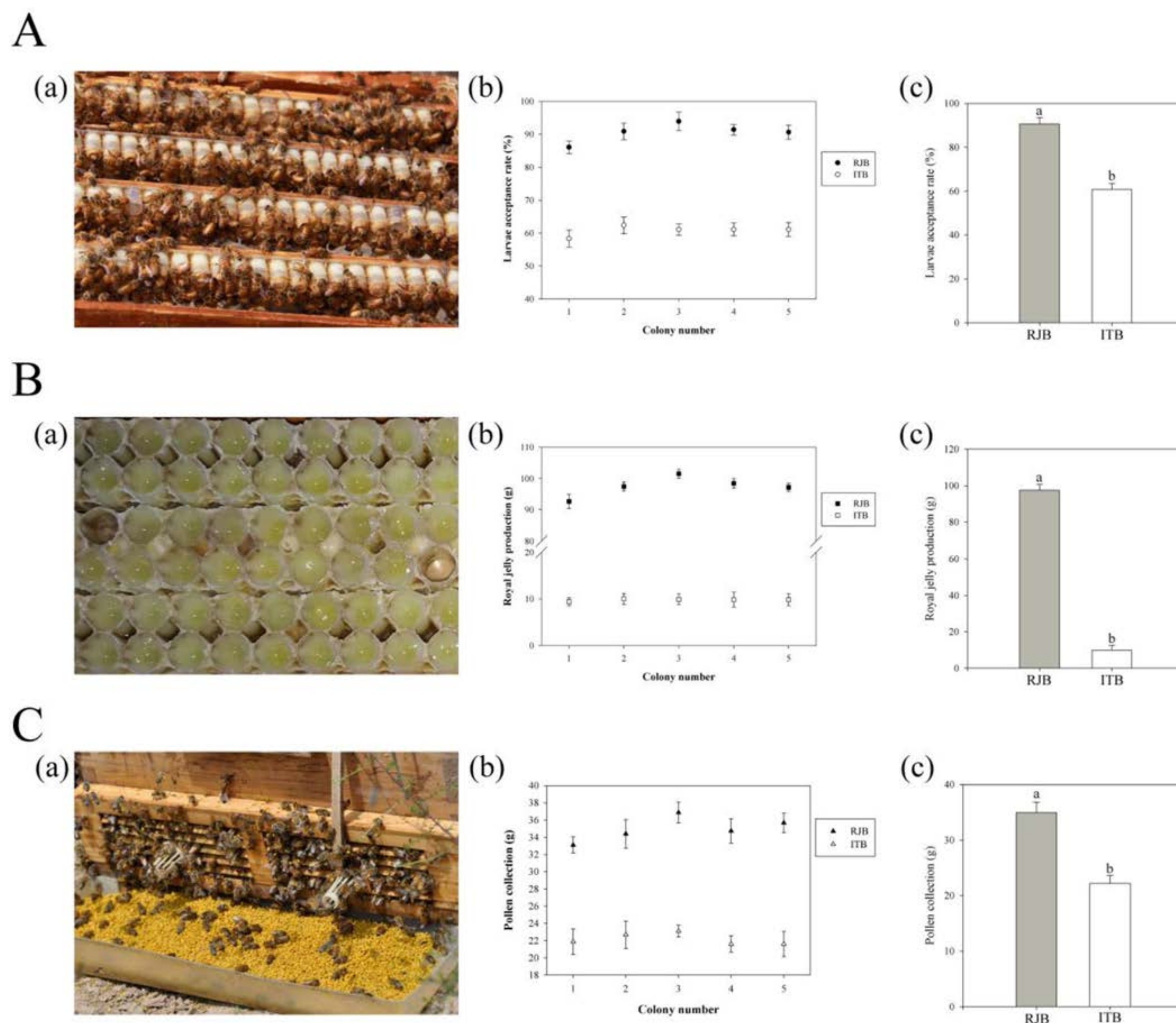

Figure 9. Measurement of larval acceptance rate, royal jelly (RJ) production and pollen collection in both Italian bee (ITB) and royal jelly bee (RJB). (A-a) RJ frame with artificial plastic queen cell cups containing grafted larvae for nurse bees to feed them. (B-a) Wax caps and larvae were removed and RJ was left in the queen cell cups for harvesting. (C-a) The pollen collection by installing a pollen trapper at the entrance of the colony. (A-b), (B-b), and (C-b) are the measurement larval acceptance rate, the weight of RJ yield, and the weight of pollen collection, respectively. Five colonies of each bee strain (the ITB and the RJB) were measured with three replicates. Error bar is standard deviation. (A-c), (B-c), and (C-c) are the comparison of larvae acceptance rate, RJ yield, and pollen collection between ITB and RJB in the overall level. Error bar is standard deviation. "a" is significantly higher ($p < 0.05$) than "b".

Furthermore, RJB has adapted distinct membrane proteome and phosphoproteome signatures in regulating the behaviors to cement the performance of RJ secretion. The up-regulated proteins related to SNARE interactions in vesicular transport, endocytosis, and the wnt signaling pathway in the brain of RJB NBs and FBs are important for the release and absorption of signal molecules to increase nerve sensitivity for facilitating larvae pheromone and flower odor recognition. Moreover, the up-regulated phosphoproteins associated with efficient neuro-transmitter transmission and recycling in RJB indicate their key roles in enhancing the performance of nursing and foraging behavior, which are vital to boosting larval feeding of NBs for increased RJ yields and to food collecting of FBs for nutrient support of the colony.

4.1. Membrane Proteins and Phosphoproteins Are Vital for Honeybee Brain Energy Supply and Signal Transduction

The brain cells are highly specialized to process and transmit information using electrical and chemical signals.[51] In this context, the larger number of identified receptor proteins, transporter proteins, and ion channel proteins is believed to serve this purpose. Moreover, the membrane proteins-involved pathways are likely to establish a solid foundation for the unique biological requirements of the brain. For instance, oxidative phosphorylation is critical for cells to convert energy from the environment into biological fuel, the ATP, and the enzymes that catalyze this chain reaction are located in the inner membrane of mitochondria.[52] Having the highest pathway coverage here indicates the key role of oxidative phosphorylation in supplying metabolic energy required for neural activity, which is in

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accordance with the fact that neurons and astrocytes are greatly dependent on mitochondria for their energetics and viability.[53] Another pathway with high coverage is protein processing in the ER. The ER is widely present in synaptic terminals and postsynaptic densities in nerve cells as an organelle for synthetization and modification of neuro-secretory peptides and neuronal proteins.[54,55] By producing local or global Ca^{2+} signals, the ER is critically involved in fast neuronal signaling.[56] Thus a large number of proteins identified as ER membrane proteins signify the vital role of the ER in neural activity for the honeybee brain. Although the biological mission of adult worker bees shifts according to age,[57] the shared membrane proteome over ages (~400 protein groups) represent its centrality to sustain the basic neural activity of the brain. Examples of pathways enriched by the shared proteome are oxidative phosphorylation, protein processing in the ER, phagosome, endocytosis, and wnt signaling pathway, which are vital to the provision of efficient energy production, protein synthesis and degradation, and signal transduction for brain cells to function.

Reversible phosphorylation−dephosphorylation reactions are crucial to living cells for regulating the function of a protein transiently or permanently.[58] Given the high efficiency in signal transduction and regulation, protein phosphorylation has widespread effects on modulating membrane proteins in neurons.[59−61] In the worker bee brain, the phosphorylated membrane proteins associated with oxidative phosphorylation, SNARE interactions in vesicular transport, neuroactive ligand−receptor interaction, and mTOR signaling pathways suggest that phosphorylation events play key roles in modulating energy production, vesicular transport, and signal transduction to support the neural activity in the brain. This is different from our previous work on the brain phosphoproteome, in which glycerolipid metabolic process, phosphate-containing compound metabolic process, and transport were the predominant biological pathways.[28] These observations indicate that phosphorylated membrane proteins may have distinct functions in cerebral activities. Furthermore, the extracted pro-directed kinase motifs from our phosphoproteome data are known substrates of mitogen-activated protein kinases (MAPKs) and cyclin-dependent protein kinases (CDKs).[62] The MAPK signaling can control the learning and memory in different invertebrate and vertebrate models.[63] It is also associated with neural plasticity by regulating gene transcription in the honeybee mushroom body and optic lobes.[64,65] The CDKs are involved in regulating proliferation, differentiation, and migration of neurons, which are essential for the brain morphology development and thus influence the neural functions.[66] Moreover, the two acidophilic kinase motifs extracted here are reported to be recognized by casein kinase 2 (CK2),[67] a ubiquitous protein serine/threonine kinase, which is a key regulator in variety of biological pathways.[68] In honeybees, CK2 is reported in regulating circadian rhythm and mushroom body development.[2] Hence, MAPKs, CDKs, and CK2 are potentially the major kinase families in honeybee brains that modulate the phosphorylation network of membrane proteins in their respective signaling pathways.

4.2. Brain Membrane Proteome and Phosphoproteome Dynamics Coordinate with Physiology of Age-Related Polyethism

In honeybee society, task specialization of worker bees is strongly related to their age; that is, the younger bees work inside the hive, and older bees forage the nectar and pollen outside of the hive.[57,69] To this end, the physiology of honeybee brains is tuned accordingly,[15,16] and this is reflected in the variation of membrane proteomes here. First, the enriched oxidative phosphorylation pathway across all stages emphasizes that the brain as a command center requires a sufficient energy provision. However, in this pathway a higher number of up-regulated proteins was found in FBs relative to the other three ages, such as components of cytochrome *bc*1 complex and cytochrome *c* oxidase. This finding implies a stronger ATP requirement in forager brains for information processing to prime the complex environment during foraging in the field. Second, the brains undergo remarkable structural and organizational changes during postnatal development, including synaptic density, cell proliferation, and migration, especially for the early phase.[70] Toward this goal, in NEBs, the up-regulated proteins implicated in the metabolism of carbohydrates, nucleosides, and lipids (such as trehalase, acylglycerol kinase, and nucleoside diphosphate kinase) are thought to satisfy the high metabolic rate and to stimulate brain cell development. This is also supported by the up-regulation of their encoding genes here. Third, as age develops, the adult worker bees participate in more complicated tasks than at young age. In particular, FBs have to process tremendous olfactory, visual, and tactile information during forage activity outside the hive. The higher number of membrane proteins and phosphoproteins identified in the older bees may support neural signal transduction and modulation in NBs and FBs to consolidate the large-scale information processing in the brain. To enhance signal transduction, the up-regulated membrane proteins enriched in SNARE interactions in vesicular transport pathway in FBs are thought to function as fusion proteins that universally partake in membrane fusion.[71] In neurons, SNAREs regulate neurotransmitter release by mediating synaptic vesicle trafficking and catalyzing fusion of vesicles with plasma membrane.[72] Furthermore, the enriched ligand−receptor interaction pathway and up-regulated receptors in FBs are likely to underpin neuronal signal modulation. By recognizing specific small signaling molecules, neuroreceptors coordinate diverse intracellular regulatory proteins to initiate appropriate responses by the cell.[73] Given that chemical signaling between neurons is mainly dependent on chemical release in the presynaptic terminal and receptor binding at the postsynaptic membrane of the target cells,[74] the functionally enhanced pathways of SNARE interactions in vesicular transport and ligand−receptor interaction emphasize that elevated neural signal transduction and modulation are required in older bees to respond to complicated stimuli in the hive and field. Notably, the uniquely enriched phototransduction pathway in FBs, which converts light signals (photons) into a change of membrane potential in photoreceptor cells,[75] indicates its key role in processing visual stimulations to fit their physiology when working in the field. Moreover, the specifically enriched lysosome pathway in NBs and FBs is thought to guide the aged neurons to lysosomal cell death, which is in line with the fact that lysosome number and size increase with aging in most cells.[76]

Phosphorylation has regulatory roles in organs to match with their distinct physiology in different stages.[77] The phosphorylated membrane proteins are also pivotal in tuning age-specific performance of the brain.[28] In NEBs, the up-regulated phosphoproteins, mainly implicated in development and cell membrane adhesion, suggest that phosphorylation plays a key role in the regulation of brain development. For instance, the membrane protein reticulon-4, acting as a negative regulator of

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neurite fasciculation, branching, and extension in the developing nervous system, prevents unwanted side branch formation, thus stabilizing in the adult CNS maturation of bees.[78] Neuroligin 5 is a postsynaptic adhesion molecule with a role in the maturation of the postsynaptic site.[79] Neuroglian functions in synapse formation by organizing microtubules in the synaptic terminal.[80] Hence, the high levels of their phosphorylation are supposed to enhance synapse formation and stabilization for promoting the maturation of neuronal connections. Likewise, the phosphoproteins in high abundance levels related to ion transport and signal transduction are suggestive of the fact that phosphorylation events may prime brain functionality in nursing and foraging behavior in NBs and FBs. Effective desensitization of olfactory signaling is essential for animals to detect and respond to odorants. The identified adenylate cyclase 3 can be inhibited by Ca^{2+} through Ca^{2+}/calmodulin kinase II (CaMKII) phosphorylation to terminate olfactory signaling in neurons.[81] Thus the high level of phosphorylated adenylate cyclase 3 is likely to promote rapid and reversible olfactory signal transduction in NBs and FBs. Activity-dependent synaptic plasticity is a critical neural underpinning of learning and memory, in which voltage-dependent Ca^{2+} channel type A subunit alpha-1 (CAC1A) modulates presynaptic Ca^{2+} currents and plays crucial signaling roles.[82,83] Phosphorylation of CAC1A by cAMP-dependent protein kinase is sufficient to modulate of Ca^{2+} channels.[84] The up-regulated phosphorylated CAC1A is supposed to enhance learning and memory performance in older bees.

4.3. RJB Reshapes Brain Membrane Proteome and Phosphoproteome To Prime the Enhanced Performance of RJ Secretion

One of the most important duties of NBs is to feed the queen and the larvae aged <3 days with RJ. RJ is secreted by hypopharyngeal glands and mandibular glands, and these two glands have reshaped their proteome to drive the gland development and functionality for enhanced RJ production.[30,85] Because the secretion of RJ is a behavior performed by NBs, the RJB has developed a distinct brain neuropeptidome to regulate behavior in response to the enhanced RJ yields.[19] As expected, membrane proteome and phosphoproteome have also developed unique architectures to adapt to the elevated RJ secretion. This is manifested by the fact that more membrane proteins were expressed in the brain of RJB than of ITB across all adult phases. Furthermore, the higher number of phosphoproteins and phosphosites identified in RJB than in ITB in each stage suggests that generally more active phosphorylation events occur to match the physiological requirements of producing voluminous RJ in response to decades of breeding selection.

All behaviors are the consequences of the CNS responding to stimuli. Nursing behavior of secreting RJ to feed young larvae is induced after pheromone signals transmit to the brain of NBs.[86] Similarly, foraging and forager recruitment behaviors are performed by FBs according to the signals of hive requirements and food resources.[86] The fact that RJB has evolved a much higher level of larval acceptance in queen cells relative to ITB (Figure 9A) indicates that RJB NBs have developed a stronger olfaction sensation to respond to pheromone stimulation. The higher acceptance of queen cells definitely demands more protein as food to ensure brood growth, thereby requiring RJB FBs to have a stronger tenacity in foraging pollen (Figure 9C). All of these facts are reflected in the brain membrane proteome of honeybees to match their behaviors. First, in all stages of RJB, functions of energy supply and protein synthesis are enforced for the neural activity by the up-regulated membrane proteins implicated in oxidative phosphorylation and protein processing in the ER. Second, in RJB NBs and FBs, the release and absorption of signal molecules between neurons are strengthened by the up-regulated membrane proteins enriched in SNARE interactions in vesicular transport, endocytosis, and wnt signaling pathways. Hence, the elevated signal transduction in the RJB brains can possibly increase nerve sensitivity that facilitates larvae feeding and food collecting. In RJB NBs, the consolidated intercellular communication in the CNS is further supported by specifically enriched phosphatidylinositol signaling and arachidonic acid metabolism pathways. The phosphatidylinositol signaling is critical for synaptic development and plasticity.[87,88] To digest superfluous arachidonic acid created by phosphatidylinositol degradation, the activity of the arachidonic acid metabolism pathway is also enhanced.[89] Therefore, the elevated phosphatidylinositol signaling is supposed to boost synaptic plasticity of feeding behavior. Moreover, in RJB FBs, the uniquely enriched pathways of TGF-beta signaling and taurine and hypotaurine metabolism are important for the neuronal remodeling and learning protection in the brain,[90−92] suggesting their key roles in neuronal plasticity and learning abilities of RJB FBs during food foraging outside of the hive.

Protein phosphorylation provides a wide variety of neuronal process regulations to the specific biological responses.[93,94] Compared with ITB, a larger number of up-regulated phosphoproteins implicated in a wide spectrum of functional terms in RJB suggests that neural function is intensively regulated by phosphorylation in RJB. This is reflected in newly emerged RJB bees by the up-regulated phosphoproteins closely related to development and differentiation. For instance, ALK tyrosine kinase receptor (ALK) is a key transmembrane receptor in cellular proliferation and differentiation.[95] ALK mediates neuronal circuit assembly in the visual system and synapse stabilization in different species.[96,97] As a kinase, the activated ALK also triggers autophosphorylation, which initiates a series of activation of downstream signaling molecules.[98] Likewise, functions of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and its autophosphorylation are important to synaptic plasticity by modulating synaptic transmission and cell morphology.[99] These observations suggest that neuronal development and plasticity in RJB are enhanced by phosphorylation at the very beginning of adult brain development. Robust neural activity relies on efficient synaptic transmission, in which the release and absorption of enormous neurotransmitters and ions should be well-coordinated. In RJB NBs and FBs, membrane transporters were activated by phosphorylation, such as excitatory amino acid transporter 2, glucose transporter type 1, and vesicular glutamate transporter 3. These findings suggest that signal transduction is reinforced in RJB to boost their performance in nursing and foraging behavior to enhance RJ secretion. This is also supported by the higher acceptance of queen cells and pollen collection observed in RJB. At the same time, synaptic transmission places a great burden upon neurons for recycling the neurotransmitter molecules. The elevated phosphorylation level of sodium-dependent neutral amino acid transporter SLC6A17 is thought to remove neurotransmitters from the synaptic cleft.[100]

5. CONCLUSIONS

We report the first and most comprehensive depth of membrane proteome and membrane phosphoproteome of the honeybee

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brain at age-resolution, which reveals age-specific molecular bases closely correlated with behavior regulation driven by cerebral functionality. To accomplish the modulation of brain performance, membrane protein phosphorylation is closely regulated by major kinase families of MAPKs, CDKs, and CK2. Age-resolved dynamic of brain membrane proteome and phosphoproteome implicated in neurons' metabolic activity and construction are fundamentally important in driving physiological maturity of younger bee brains, and pathways related to neuronal signal transduction consolidate learning and memory performance in older bees. It is noted that RJB has tailored distinct membrane proteome and phosphoproteome arsenals to fit with the increased RJ yields during decades of selection. In RJB, the enriched phosphatidylinositol signaling system, arachidonic acid metabolism pathway, and SNARE interactions in vesicular transport pathway are strengthened at proteome level, and brain signaling and nerve sensitivity are enhanced by phosphorylation modifications. Both are potentially important to facilitate larvae feeding and food collection to prime the elevated RJ yields. Our data provide novel mechanistic understanding of brain development and functional underpinnings by membrane proteome and phosphoproteme and add a valuable resource for further functional investigation of honeybee neurobiology as well as of the neurobiology of other social insects.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.7b00371.

Figure S1. Cellular localization of the identified proteins in the brain of honeybee workers. Figure S2. Gene ontology (GO) analysis of the brain membrane proteins shared in all four time points. Figure S3. Protein–protein interaction network of the membrane proteins consistently up-regulated in the royal jelly bee (RJB). Figure S4. Validation of the mRNA expression level of the differentially expressed membrane proteins by quantitative PCR analysis. Figure S5. Overview of the brain membrane phosphoproteome of both Italian bee (ITB) and royal jelly bee (RJB) at four time points. (PDF)

Table S1. Identification, quantification, and bioinformatics analyses of membrane proteins. (XLSX)

Table S2. Identification, quantification, and bioinformatics analyses of phosphoproteins. (XLSX)

Table S3. Primer sequences used for qRT-PCR analysis. (XLSX)

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Notes

The authors declare no competing financial interest.

The LC–MS/MS data and search results are deposited in ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD006823.

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