Single-cell transcriptomic analysis of honeybee brains identifies vitellogenin as caste differentiation-related factor

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Highlights
scRNA-seq revealed distinct gene expression in the brains of queens and workers

Vitellogenin (vg) may represent a "molecular signature" of the queen caste

Knockdown of vg at early larval stage suppressed development into adult queens

Vg may be involved in regulating caste differentiation

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Single-cell transcriptomic analysis of honeybee brains identifies vitellogenin as caste differentiation-related factor

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SUMMARY
The honeybee (Apis mellifera) is a well-known eusocial insect. In honeybee colonies, thousands of sterile workers, including nurse and forager bees, perform various tasks within or outside the hive, respectively. The queen is the only fertile female and is responsible for reproduction. The queen and workers share similar genomes but occupy different caste statuses. We established single-cell transcriptomic atlases of brains from queens and worker subcastes and identified five major cell groups: Kenyon, optic lobe, olfactory projection, glial, and hemocyte cells. By dividing Kenyon and glial cells into multiple subtypes based on credible markers, we observed that vitellogenin (vg) was highly expressed in specific glial-cell subtypes in brains of queens. Knockdown of vg at the early larval stage significantly suppressed the development into adult queens. We demonstrate vg expression as a "molecular signature" for the queen caste and suggest involvement of vg in regulating caste differentiation.

INTRODUCTION
The honeybee is a well-known eusocial insect. In a colony of honeybees, the queen is primarily responsible for reproduction, whereas the workers are sterile and perform tasks required for the colony’s growth, maintenance, and defense (Johnson, 2010; Page et al., 2012). Workers usually perform tasks based on an age-related (behavioral-developmental) process (Beshers and Fewell, 2001; Calderone, 1998; Robinson and Huang, 1998). In the first two to three weeks following eclosion, an adult worker is responsible for the nursing tasks (e.g., caring for the brood and queen); the worker subsequently switches to other specialized work for a few days, including building and guarding within the hive. Eventually, the role of the worker changes to foraging for foods such as nectar, pollen, and water, for the rest of its life (Ament et al., 2010; Beshers et al., 2001). This behavioral switch is common but not rigid. Instead, honeybees are sensitive to environmental changes and duties are adjusted, reflecting typical phenotypic plasticity (Duncanm et al., 2020; Maleszka, 2018; Sommer, 2020).

Queens and workers develop from diploid fertilized eggs, and therefore share similar genomes. However, they exhibit tremendous differences regarding behaviors and physiologies. The process involved in formation of the two castes is termed caste differentiation, which largely depends on feeding conditions during the early larval stage (within the first 96 h) (Kamakura, 2011; Wang and Li-Byarlay, 2015; Weaver, 1966). However, the molecular basis of the establishment and regulation of caste differentiation remains largely unknown. Previous studies proposed that nutritional conditions during early development profoundly influence the individual’s epigenetic status (e.g., DNA methylation [Foret et al., 2012; Kucharski et al., 2008; Lyko et al., 2010], RNA m6A modification [Wang et al., 2021], and histone acetylation [Spannhoff et al., 2011]) and, thereby, determine the caste fate of the honeybee. In addition, nutrition-related signaling pathways, such as the insulin/insulin-like signaling (Nijhout and McKenna, 2018; Wang et al., 2013; Wheeler et al., 2004) and target of rapamycin nutrient-sensing pathways (Muttì et al., 2011; Patel et al., 2007; Wheeler et al., 2014), have been reported to participate in caste differentiation regulation.

Previous studies investigating neuronal cells in the bee brain suggested that there are remarkable differences in the transcriptomes (Behura and Whitfield, 2010; Liu et al., 2011, 2012; Tholken et al., 2019), proteomes (Hernandez et al., 2012), and methylomes (Forêt et al., 2012; Herb et al., 2012; Lyko et al., 2010) between workers and queens.
RESULTS

Single-cell transcriptomic atlases of honeybee brains

To identify caste-specific cell types among female honeybee brains, we employed 10X Genomics technology to conduct single-cell RNA-seq analysis. For this assay, we collected pools of four to five female honeybee brains from queens, foragers, and nurses, respectively. For each caste or subcaste, we sequenced two independent biological replicates, targeting over 10,000 cells per replicate. The 10X single-cell libraries were constructed, followed by high-throughput sequencing (Figure 1A). Following the recommended standard procedures, FASTQ files were analyzed using the Cell Ranger pipeline to generate feature-barcode matrices. A total of 115,169 cells were obtained after data filtration using the Seurat R package with three parameters: the nCount (number of unique molecular identifiers), nFeature (number of genes), and percent.mt (portion of mitochondrial genes) (Figure S1A). These cells included 40,186 (34.89%), 38,149 (33.12%), and 36,834 (31.98%) cells from the brains of foragers, nurses, and queens, respectively (Table 1).

We then integrated all the high-quality cell data into an unbatched and comparable dataset, which was then projected onto two dimensions via uniform manifold approximation and projection (UMAP) to visualize the cellular heterogeneity. As shown in Figures 1B and 1A total of 49 high-confidence cell clusters were identified. Of these, 37 clusters could be functionally annotated with marker genes predicted by FindAllMarkers (Tables S4–S10) and orthologs of reported marker genes in *Drosophila*. Based on these findings, we divided the cells into five major categories: Kenyon cells (KCs), olfactory projection neurons (OPNs), glial cells, hemocytes, and optic lobe cells (OLCs) (Figures 1C–1E). Most cells were defined as neuron cells because they expressed the pan-neuron marker elav (Robinow and White, 1991), which is annotated as LOC410689 in the honeybee. We recognized three main subgroups of neurons: KCs, OPNs, and optic lobe cells. Notably, KCs (clusters 2, 4, 5, 8–12, 17, 20, 21, 30, 34, 35, 42, and 46) were effectively distinguished by the enrichment of LOC408804 (PLCe) and LOC408372 (mub) (Suenami et al., 2018), and OPNs (clusters 6, 7, 26, and 39) were identified by LOC724282 (otp), LOC724148 (C15), LOC410657 (acj6), and LOC413466 (oaz) (Davie et al., 2018); the highly abundant neurons of the optic lobe, collectively termed OLCs, were identified by a combination of markers. The captured OLCs were further divided into six
In addition to the neurons, we found that cells expressing LOC410151 (rx2), the homolog of repo, were recognized as glial cells (Edwards and Meinertzhagen, 2010; Shah et al., 2018). As a result, 7,268 (~6.3%) glial cells were detected in our datasets, similar to the proportion found in Drosophila (~6.4%) (Davie et al., 2018). Moreover, we also observed a few cells (~0.3%, cluster 41) expressing the hemocyte markers LOC411597 (hml) and LOC551684 (Fer2LCH) that have also been found in the brains of fruit flies and ants (Davie et al., 2018; Sheng et al., 2020). Owing to the lack of marker genes enabling further subdivision, we termed the remaining 12 clusters “undefined neurons”, because elav could be detected in these groups.

Table 1. Numbers of captured cells from different samples in single-cell RNA-seq

| Cell type                        | Forager | Nurse | Queen |
|----------------------------------|---------|-------|-------|
|                                  | rep1    | rep2  | rep1  | rep2  | rep1  | rep2  |
| Glial cells                      | 930     | 1456  | 1354  | 1940  | 768   | 820   |
| Kenyon Cells                     | 7605    | 8892  | 6783  | 6038  | 8862  | 6878  |
| Olfactory Projection Neurons     | 1642    | 1979  | 1399  | 1491  | 2122  | 1770  |
| Optic Lobe Cells                 | 3517    | 3531  | 3340  | 3669  | 3502  | 2670  |
| Hemocyte                         | 14      | 97    | 25    | 13    | 165   | 32    |
| Undefined neurons                | 5490    | 5033  | 5662  | 6435  | 4654  | 4591  |
| Sum                              | 19,198  | 20,988| 18,563| 19,586| 20,073| 16,761|

Neuron classification using neurotransmitters and neuropeptides

During annotation, we observed that various genes associated with neurotransmitters and receptors were differentially expressed in the neuron subtypes. It has been demonstrated that neurotransmitters, including acetylcholine (ACh), glutamate, GABA, and monoamine molecules (e.g., octopamine and dopamine), are important in mediating distinct pathways involved in a variety of cognitive processes (Blenau and Baumann, 2001). On analyzing our datasets, we identified cholinergic (VACHT, CHAT), glutamatergic (Gad1, VGAT), and GABAergic (VGAT) neurons (Figure 2A), based on established markers that participate in the synthesis or vesicle transport of neurotransmitters (Allen et al., 2020; Brunet Avalos et al., 2019), accounting for 40, 8, and 15% of the total neurons, respectively. Vmat has been used to label monoaminergic neurons (Allen et al., 2020), and we found that Vmat-positive cells (~10% of neurons) could be further divided into dopaminergic (ple), octopaminergic (Tdc2), serotonergic (DAT), and histaminergic (TDH) neurons (Figures 2A and 2B). Amongst the monoaminergic neurons, there were remarkably more dopaminergic neurons than the other three subtypes.

Previous studies reported the co-existence of more than one neurotransmitter in the same neuron in Drosophila adult and larval brain cell atlases (Brunet Avalos et al., 2019). To ascertain if this also applied to honeybee brains, we evaluated all possible combinations between the four markers VACHT, Gad1, VGlut, and Vmat. As shown in Figures 2C and 2D, nearly half of the neurons (51.9%) expressed one neurotransmitter, whereas the other neurons expressed two or more neurotransmitters. The statistical analysis revealed no significant differences in the neurotransmitter expression patterns among the three female groups (Figure S3A). Notably, the frequency of dual (17.3%) or multiple (3.7%) neurotransmitter-expressing neurons in the honeybee brains was significantly higher than that in fruit flies (10 and 1.8%, respectively) (Brunet Avalos et al., 2019), revealing the complexity of the neural communication network in honeybee brains.

Neuropeptides represent another type of essential messenger molecule for communication between neurons. Although numerous putative neuropeptides were previously observed in honeybees, the role of these...
peptides is poorly understood (Nassel and Homberg, 2006). We curated a panel of 27 reported honeybee neuropeptides and examined their expression at the single-cell level, and the heatmap in Figure 2E shows the cell-type-specific expression patterns of these neuropeptides in three female groups. Compared with results analyzed by bulk RNA-seq data, single-cell RNA-seq data showed a detailed differential expression in each cell type. For example, tachykinin and FMRFamide were highly expressed in mushroom bodies and optic lobes (Figure S3C), which is consistent with previously reported findings (Schurmann and Erber, 1990; Takeuchi et al., 2004). Orcokinin, CTLP, and ILP-2 were enriched in Kenyon cells, glia, and hemocytes, respectively. By comparing the datasets from the three female groups, we found that although most peptides exhibited a similar expression pattern, a few displayed caste-specific patterns. For instance, Nus1 and apidaecin 1 were highly expressed in the hemocytes and glial cells of queens, respectively, whereas Crz expression was specifically present in worker subcastes (Figure 2E).

Reclustering of Kenyon cells

Because KCs and glial cells are two major cell types in the honeybee brain, we then focused on elucidating their transcriptomic features. Kenyon cells of two major classes (class-I and class-II) located in the mushroom bodies are vital for sensory information processing, learning, and memory (Farris, 2005; Mobbs, 1982). Previous studies involving imaging analysis led to the class-I KCs being grouped into three subtypes: large (IKCs), middle (mKCs), and small (sKCs) (Suenami et al., 2018). By analyzing our single-cell seq datasets, we confirmed the existence of IKCs and sKCs with the specific markers mblk-1 and E74 (Suenami et al., 2016, 2018), respectively. Moreover, significant clustering of a KC population positively expressing FoxP (FoxP KCs) (Schatton and Scharff, 2017) was confirmed by our findings (Figures 3A, and 3B). In addition, class-II KCs failed to be defined because of a lack of information on suitable marker genes for bees. It is noteworthy that the three female castes shared a repertoire of closely related KC subtypes, although the proportion of all KCs in nurses was less than those in foragers and queens (Figure 3C).

We subsequently employed FindMarkers to analyze the differentially expressed genes (DEGs) in each KC subtype (Table S11) and found that the gene expression profiles of the IKCs, sKCs, and FoxP KCs between queen and worker castes were comparable on the volcano plot (Figures 3D to 3F). The Venn plots in Figure S4A summarize the number of DEGs in the two groups for each cell subtype. It appeared that there were considerably fewer DEGs in each KC subtype between nurses and foragers than between foragers and queens, which was in agreement with the bulk RNA-seq results (Figure S4B). However, the DEGs in these comparisons displayed no significant enrichment of gene ontology items.

Reclustering of glial cells

Given that glial cells are essential for the functioning of the nervous system, we performed glial cell reclustering analysis by employing the marker genes used for classifying different subtypes of glia in Drosophila (Allen et al., 2020; Parker and Auld, 2006). As shown in Figures 4A and 4B, glia reclustering revealed four subtypes: surface glia (Tret1, vkg), astrocytes (ebony, GlnS), ensheathing glia (Idgf4, Tsf1), and cortex glia (wrapper, zy6). By calculating the proportion of each cell type, we identified different compositions among the three female groups (Figure 4C). In particular, the relative ratio of ensheathing glia was higher in queens' brains than in workers' brains. Ensheathing glial cells have been reported to function as phagocytes in the Drosophila brain (Doherty et al., 2009), and are also associated with the aging process (Sheng et al., 2020). We observed that nurse bees had relatively more ensheathing glia than foragers. Recent studies in Harpegnathos reported the expansion of ensheathing glia in the reproductive caste (Sheng et al., 2020). Hence, a high ratio of ensheathing glia could be a biomarker for the queen.
Ensheathing glia expressed vg in a queen-specific manner

Having observed the high number of ensheathing glia in the brains of queens, we sought to analyze the differential gene expression between the ensheathing glia of queens and workers. As shown in Figures 4G and S4C, 111 genes were more highly expressed in the ensheathing cells of queens compared to those of workers (nurse and forager bees). In particular, ensheathing cells in queens displayed a log2-fold-change exceeding three in vg expression compared to the average expression levels of vg in worker ensheathing cells (Figure 5A). To confirm this observation, we performed fluorescence in situ hybridization (FISH) assays, using multiple probes including vg, idgf4, and tsf1. In this assay, idgf4 and tsf1 were used to specifically mark the ensheathing glial cells in adult brains. As shown in Figures 5B–5D, vg transcripts were highly expressed in ensheathing glial cells in queens’ brains compared to that in the ensheathing glial cells in workers’ brains. Although the expression of vg varied in honeybees at different ages, we confirmed that vg was significantly more highly expressed in the queen caste rather than in an age-dependent manner by using qPCR analysis (Figure S5A). As such, our results suggest that vg-positive ensheathing cells represent a specific subtype of glia in the queen brain and are likely to be a biomarker for the queen caste. Moreover, based on our findings, we then investigated whether vg contributes to caste differentiation.

Indeed, our bulk RNA-seq analysis also showed that vg was one of the most differentially expressed genes in the brains of queens and workers (Table S12). These results indicate that high expression of vg may play more important roles in queens compared to that in workers. Therefore, examining whether vg is involved in caste differentiation is an interesting issue that warrants exploration. Previous studies suggested that knocking down vg expression in adult workers affects the behavioral transition between nurses and foragers (Nelson et al., 2007). Given that caste differentiation is determined during the early larval stage, we knocked down vg throughout the larval stages by conducting RNAi on larvae kept in an in-dish rearing system (Figure 5E). In this assay, we transferred the first instar larvae to a 24-well plate and fed them with adequate high-nutrition food compounds for development. For RNAi treatment, we packaged double-strand (ds)RNAs within a previously described synthetic nanomaterial (Jian et al., 2009; Lynn and Langer, 2000), which was added to the bee food (Figure S5B). The bees were fed with dsRNA-treated food until the pupal stage. Pupae were then transferred to another 24-well plate (Figure S5C), and the emerged adults were used for phenotypic analysis.

There are numerous differences in the body structure of queens and workers (Ament et al., 2010). Queen bees can easily be recognized by specific morphological features, particularly birth weight and body size. In our in-dish-rearing system, three groups of adults with different body phenotypes developed (Figure 5F): (1) over 22% of adults appeared similar to natural queens (birth weight > 190 mg), and we termed this group the “queen-like” bees; (2) ~75% of adults were lighter than queens but heavier than workers (birth weight 120–190 mg), and we termed them “intermediate” bees; and (3) ~3% of adults had body weights similar to those of workers (birth weight < 120 mg), and we called this the “worker” phenotype. The worker phenotype was also confirmed at the early larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966). We did not observe any effects in this assay (Figure S5T), indicating that vg was knocked down at the late larval stage (after 96 h) when the queen fate had already been established (Weaver, 1966).
DISCUSSION

Caste differentiation is a hallmark of a social insect community. The honeybee is an excellent model organism for studying the mechanisms underlying determination and regulation of the caste fate of queens. Previous studies proposed that nutritional conditions in coordination with epigenetic regulation and nutrition-related signaling pathways control caste differentiation (Kucharski et al., 2008; Mutti et al., 2011; Spannhoff et al., 2011; Wang et al., 2021). Given that the behaviors of animals in a colony are relatively inflexible, and the relatively complex behaviors are controlled by the brain (Leadbeater and Chittka, 2007), we speculated that caste determination might be accompanied by differentiation of specific cell types. To identify the difference between queen and worker castes, we performed transcriptomic analysis of different caste brains at the single-cell level, and we identified a specific subtype of ensheathing cell with high levels of *vg* expression in queen brains. In particular, *vg* was more highly expressed compared to the average *vg* expression level in the ensheathing cells of workers. Importantly, we found that the knockdown of *vg* at the early larval stage, but not the late stage, significantly suppressed the developmental trajectory to queens. Therefore, our study not only identifies the high expression of *vg* in a subset of glial cells as a “molecular signature,” but it also suggests that *vg* is involved in caste differentiation.

By analyzing transcriptomic features in this study, we obtained an important finding that glial cell populations vary significantly between queen and worker castes. Specifically, we found that the ratio of ensheathing glia was most variable among the castes and subcastes. The ensheathing glia population has been reported to be associated with caste transition, injury, and aging in ants (Sheng et al., 2020). In addition, the function of the ensheathing glia has been extensively studied in other model organisms, including *Drosophila* and mammals (Freeman, 2015; Ramon-Cueto and Avila, 1998). Our findings further emphasize the importance of this type of cell in regulating development and behavior.

Vitellogenin is a well-studied factor that is involved in a variety of biological processes, including reproduction, immunity, aging, and behavioral transition (Amdam et al., 2003; Havukainen et al., 2011; Ihle et al., 2015; Nelson et al., 2007; Seehuus et al., 2007). In particular, *vg* is synthesized by the fat body and then released to the hemolymph. Its expression in the hemolymph of queens has been reported to be much higher than that in workers (Barchuk et al., 2002; Guidugli et al., 2005). In this study, we identified a specific subtype of ensheathing glial cells that expresses *vg* at extremely high levels, suggesting that *vg* expression might be associated with the differentiation of this cell subtype. After knocking down *vg* with RNAi at an early larval stage, we found that the fate of queens was inhibited. In conclusion, our work identifies a subset of glial cells with *vg* high expression as a “biomarker” for the queen caste. It also suggests that *vg* plays a role in regulating caste differentiation in honeybees.

Limitations of the study

There are some limitations to this study. First, from a technical standpoint, the single-cell atlas cannot completely cover all the cell types, and rare cell populations might be omitted. Second, available genomic information of honeybees is insufficient and has not been fully elucidated. As a result, ~30% of captured cells in our atlases are functionally undefined. Third, the variation between replicates increased during reclustering analysis, particularly for glia subtypes, which was also an inherent issue of the approach of single-cell RNA-seq. It is easy to produce the variation when we capture those rare cell types. Nevertheless, the findings from our “FISH” assays provided experimental evidence to support our conclusions.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
**A**

Bar chart showing average expression levels in different cell types.

- Red: Forager Rep1
- Pink: Forager Rep2
- Green: Nurse Rep1
- Orange: Nurse Rep2
- Blue: Queen Rep1
- Light blue: Queen Rep2

**B**

Images showing cell expression levels for Queen, tsf1, idgf4, vg, DAPI, and merge.

**C**

Images showing cell expression levels for Nurse, tsf1, idgf4, vg, DAPI, and merge.

**D**

Images showing cell expression levels for Forager, tsf1, idgf4, vg, DAPI, and merge.

**E**

Diagram illustrating the process:
- Step 1: Egg collection, Honeycomb
- Queen lays eggs
- Step 2: Larvae transfer and dsRNA treatment
- 24 Cell plate to 24 Cell plate
- dsRNA
- Step 3: Different phenotype

**F**

- **Queen-like Phenotype**: Birth Weight > 190 mg
- **Intermediate Phenotype**: Birth Weight 120-190 mg
- **Worker Phenotype**: Birth Weight < 120 mg

**G**

Bar graph showing birth weight distribution.

- Blue: <120
- Orange: 120-190
- Green: >190

| Birth Weight (mg) | N=106 | 65 | 127 | 51 |
|-------------------|-------|---|-----|----|
| <120              | 2.8%  | 4.6% | 16.5% | 19.6%
| 120-190           | 68.9% | 70.8% | 73.2% | 68.6%
| >190              | 28.3% | 24.6% | 10.2% | 11.8%
Figure 5. Vg is expressed in ensheathing glia in a queen-specific manner and vg RNAi affects caste differentiation

(A) Bar plot showing the average expression level of vg in four glia subtypes among replicates of nurses, foragers, and queens.
(B–D) Simultaneous detection of tsf1 mRNA (magenta), idgf4 mRNA (green), vg mRNA (red), and (4′, 6-diamidino-2-phenylindole) DAPI-stained nuclei (blue), using confocal microscopy. The co-expression of tsf1 and idgf4 was used to mark ensheathing glia. Brain sections from the queen (B), nurse (C), and forager (D), respectively. Left: whole brain; Right: successively amplified regions with positive probe signals. Scale bars were indicated on the corresponding images.
(E) Scheme of RNAi treatment assay based on the in-dish queen rearing system. First instar larvae were transferred into a 24-well plate and fed high nutritional foods. The double-strand RNAs were added or not added to the food in the treatment or control group, respectively. The pupae were transferred to another 24-well plate and kept still for eclosion.

(F) The adults reared in the in-dish-rearing system are divided into three typical phenotypes according to birth weight.

(G) Statistics for three phenotypes of four experimental groups (blank, ds GFP, ds vg and ds Tor). The phenotypes are colored according to different birth weight standards. The number of individuals and percentage of each phenotype are shown. See also Figure S5 and Table S12.

**RESOURCE AVAILABILITY**
- Lead contact
- Materials availability
- Data and code availability

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**
- Honeybees and brain sample collection

**METHOD DETAILS**
- Preparation for single-cell suspension
- 10x Genomics and sequencing
- Bulk RNA-seq
- In-dish rearing of honey bee queens
- Fluorescent in situ hybridization
- Confocal imaging
- dsRNA synthesis and RNAi treatment
- Synthesis of transfection polymer
- Quantitative real-time PCR
- 10x data preprocessing
- Data processing by seurat
- Analysis of bulk RNA-seq
- Analysis of neuropeptides
- Differentially expressed genes
- Comparisons to bulk RNA-seq and single-cell RNA-seq
- Gene ontology enrichment analysis

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at [https://doi.org/10.1016/j.isci.2022.104643](https://doi.org/10.1016/j.isci.2022.104643).

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**AUTHOR CONTRIBUTIONS**
D.C. and W.Z. conceived the project and designed the experiments. Y.Z., Y.W., C.C., Y.H., Y.Z., and H.S. conducted the experiments. W.Z., L.W., and Y.C. analyzed the results. D.C., Q.S., J.Z., and W.Z. wrote the manuscript. All the authors read and approved the final manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Biological samples** | | |
| Brains from queens for single-cell RNA-seq, 2 replicates | This paper | N/A |
| Brains from nurses for single-cell RNA-seq, 2 replicates | This paper | N/A |
| Brains from foragers for single-cell RNA-seq, 2 replicates | This paper | N/A |
| Brains from queens for bulk RNA-seq, 2 replicates | This paper | N/A |
| Brains from nurses for bulk RNA-seq, 2 replicates | This paper | N/A |
| Brains from foragers for bulk RNA-seq, 2 replicates | This paper | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| D (-) Fructose | Sigma-Aldrich | Cat# F3510 |
| D (+) Glucose | Sigma-Aldrich | Cat# G7021 |
| Bacto yeast extract | Thermo Fisher | Cat# 288620 |
| fresh royal jelly | This paper | N/A |
| Papain, Lyophilized | Worthington | Cat# LS003119 |
| SF-900™ III SFM | Thermo Fisher | Cat# 12658019 |
| DPBS | Thermo Fisher | Cat# 14190144 |
| Bovine Serum Albumin | Sigma | Cat# A1933 |
| 10% Tween 20 solution | Bio-Rad | Cat# 1610781 |
| Elution buffer | QIAGEN | Cat# 19086 |
| TE buffer | Thermo Fisher | Cat# 12090015 |
| Nuclease-Free water | Thermo Fisher | Cat# AM9937 |
| Sodium acetate | Sigma | Cat# S5636 |
| 1,4-butanediol diacrylate | Alfa Aesar | Cat# 32780 |
| Polyoxymethylene | Aladdin | Cat# C104190 |
| Formamide | Macklin | Cat# F6287 |
| N, N-Dimethylethylenediamine | TCI | Cat# D0719 |
| Tetrahydrofuran | Energy Chemical | Cat# W310075 |
| DMSO | Sigma | Cat# 472301 |
| Trizol | Thermo Fisher | Cat# 15596018 |
| AceQ Universal SYBR mixture | Vazyme | Cat# Q511 |
| DAPI | Thermo | Cat# 62248 |
| Tween®20 | Sigma | Cat# P1379-1L |
| Antifade Mounting Medium | VECTASHIELD | Cat# H-1000 |
| Stellaris® RNA FISH Wash buffer A | Biosearch | Cat# SFM-WA1-60 |
| Embedding medium for frozen | Sakura | Cat# 4583 |
| Hiscrcript III RT SuperMix for qPCR(+gDNA wiper) | Vazyme | Cat# R323-01 |
| **Critical commercial assays** | | |
| Chromium Next GEM Single Cell 3’ Kit v3.1 | 10X Genomics | Cat# 1000121 |
| Chromium Chip G Single Cell Kit | 10X Genomics | Cat# 1000120 |
| Single Index Kit Set A | 10X Genomics | Cat# 1000213 |
| AHTS® Universal V8 RNA-seq Library Prep Kit for Illumina | Vazyme | Cat# NR605-02 |
| Qubit dsDNA HS Assay Kit | Thermo Fisher | Cat# Q32854 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dahua Chen (chendh@ynu.edu.cn or chendh@ioz.ac.cn).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- Raw sequencing data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Raw micrograph data were deposited at online repository in Mendeley (https://doi.org/10.17632/xrd623n43s.1).

### Deposited data

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| NEBNext® Poly(A) mRNA Magnetic Isolation Module | New England BioLabs | Cat# E7490 |
| SPRselect beads | Beckman Coulter | Cat# B23318 |
| TransScript® First-Strand cDNA Synthesis SuperMix | TransGen | Cat# AT301-03 |
| T7 RiboMAX™ Express Large Scale RNA Production System | Promega | Cat# P1320 |

**Raw micrograph data**
This paper, Mendeley Data
https://doi.org/10.17632/xrd623n43s.1

**Experimental models: Organisms/strains**
Honey bees (Apis mellifera) Institute of Biomedical Research, Yunnan University (China) N/A

**Oligonucleotides**
- Primers for dsRNA DNA templates synthesis, see Table S1 This paper N/A
- Primers for qRT-PCR, see Table S2 This paper N/A

**Recombinant DNA**
- pAc5.1-Flag-EGFP This paper N/A

**Software and algorithms**
- Cell Ranger (v3.1.0) 10X Genomics https://support.10xgenomics.com
- Seurat (v3.1.5) (Butler et al., 2018) https://satijalab.org/seurat
- FastQC (v0.11.9) Babraham Bioinformatics https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
- Trimmomatic (v0.39) (Bolger et al., 2014) http://www.usadellab.org/cms/?page=trimmomatic
- STAR (v2.7.9a) (Dobin et al., 2013) https://github.com/alexdobin/STAR
- RSEM (v1.3.1) (Li and Dewey, 2011) http://deweylab.github.io/RSEM/
- DESeq2 (v1.32.0) (Love et al., 2014) http://www.bioconductor.org/packages/release/bioc/html/DESeq2.html
- DAVID (v6.8) (Huang da et al., 2009a, b) https://david.ncifcrf.gov/
- TBtools (v1.082) (Chen et al., 2020) https://github.com/CJ-Chen/TBtools
- GraphPad Prism (v8.2.1) GraphPad Software, La Jolla, CA N/A
- ZEN lite blue (v3.1) Zeiss https://www.zeiss.com.cn/microscopy/products/microscope-software/zen.html
- Imaris (v9.0.1) Oxford Instruments Group https://imaris.oxinst.com/support/imaris-release-notes/9-0-0#f-0-1
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Honeybees and brain sample collection
All the *Apis mellifera* colonies used in this study were kept in apiary supported by the institute of biomedical research, Yunnan University. We chose morphologically distinguishable nurses and foragers from the same colony. The workers those entered cells and nursed larvae were recognized as nurses, while the foragers could be easily identified by the yellow pollen loaded on their hind legs. The mated queens were obtained by artificial rearing and inseminating methods.

METHOD DETAILS

Preparation for single-cell suspension
Before dissection, all bees were anesthetized by carbon dioxide. The whole brains of alive bees were dissected in ice-cold SF9-III medium for each biological replicate and immediately transferred to a precooling tube containing SF9-III medium. Four to five brains were pooled. The dissociated brains were incubated with papain solution (Worthington, 1 μg/mL) at 35°C for 30 min, and briefly centrifuged (50 g, 10 s) to collect cell suspension and remove undigested tissue fragments. The cell suspension was centrifuged again (300 g, 5 min), and the pellet was washed twice with DPBS. Subsequently, the cell pellet was resuspended into 3 mL PBSB (DPBS+0.04% BSA) and filtered through a 20 μm pluriStrainer. An AO/PI Dual-fluoresces counting assay performed on the Countstar FL system detected cell counting and viability. The cell suspension (cell viability >95%) was adjusted to a final concentration of 700~800 cells/μL.

10x Genomics and sequencing
The single-cell 3’ gene expression libraries were generated following the manufacturer’s instructions (Chromium Next GEM Single Cell 3’ Reagent Kits v3.1 User Guide). Each sample was processed on an independent Chromium Next GEM Chip G with a target capture of 10,000 cells. The quality of libraries was analyzed by Agilent 2100 Bioanalyzer. The libraries were sequenced by Illumina Novaseq 6000 platform.

Bulk RNA-seq
Five adult brains were pooled and homogenized in the Trizol reagent to extract total RNA. mRNA was isolated using the Oligo d(T)25 magnetic beads (NEB). The purified mRNA samples went through the steps of fragmentation, random primers annealing, double-stranded cDNA synthesis, adaptor ligation, ligation product purification, and library amplification according to the manufacturer’s instructions (VAHTS® Universal V8 RNA-seq Library Prep Kit for Illumina, Vazyme). Finally, a sequencing library suitable for the Illumina® platform was obtained. The quality of libraries was analyzed by Agilent 2100 Bioanalyzer. The libraries were then sequenced by Illumina Novaseq 6000 platform.

In-dish rearing of honey bee queens
Healthy mated queens were caged in a clean frame to lay eggs for 3-6 h (depending on the number of eggs). Then released the queen and waited for eggs to hatch. Before larvae transfer, prepared larval diet: 55% fresh royal jelly, 5% glucose, 10% fructose, 1% yeast extraction, and 30% distilled water; mix thoroughly. The 1st instar larvae were transferred from the comb to sterile 24-well plates. Each larva was fed with a 250 μL pre-heated (31°C) larval diet once every two days. The plates filled with larvae were placed horizontally in the constant temperature and humidity incubator (at 35°C, 95% R.H.). The mature larvae were moved to new sterile 24-well plates for pupation. The plates filled with pupae were placed horizontally in another incubator (at 35°C, 75% R.H.). When the color of pupae turned dark utterly, adult honeybees would begin to emerge.

Fluorescent in situ hybridization
Day1: Bee brains were dissected to remove Compound eye and ocellus, embedded in O.C.T and frozen in −80°C ready for section. 30μm is an appropriate thickness for the bee brain section to maintain tissue shape. The sections were washed with PBST for 2 min twice and then fixed with 4% paraformaldehyde.
for 30 min. Washed for 5 min twice in PBST, and sections were dehydrated with gradient ascending methanol (25%, 50%, 75%, 100%) (5 min for each concentration on ice) when sections were treated in 100% methanol, -20°C overnight. Day2: Dehydrated sections were treated with gradient descending methanol (100%, 75%, 50%, 25%) to rehydrated, PBST washed for 5 min twice on ice. And proteinase K (10 μg/mL) for 3 min, washed with PBST 5 min twice, then fixed again with 4% paraformaldehyde for 1 h. Washed with PBST for 5 min thrice. And they were treated with wash buffer (wash buffer A: PBST = 1:1) for 10 min, wash buffer A (6 mL Stellaris® RNA FISH Wash buffer A, 21 mL DEPC H2O, 3mL Formamide) for 5 min twice, HYB-(10 mL 20x saline sodium citrate, 20 mL formamide, 10 mL DEPC H2O, 40 μL Tween®20) for 5 min at room temperature then 1h at 37°C. Add probes (1: 600) diluted with HYB-(Probes for FISH were shown in Table S3). Placed sections at 37°C in an incubator for 18 h. Day3: Probes were removed thoroughly, HYB- 30 min at 37°C, PBST 2 min twice, DAPI (0.5 μg/mL) 30 min at room temperature, PBST 10 min thrice. The sections were mounted with Antifade Mounting Medium. Image reconstruction was processed using confocal microscopy.

Confocal imaging
The whole-brain imaging was performed using Zeiss LSM 980 confocal microscopes with Plan-Apochromat 40x/1.3 oil objective. Z-stack and tile scan features were used to image the large, wavy surfaces of the brain slices. The resulting tiles were then stitched into a single large image (ZEN 3.1 Blue software, Zeiss), which enabled visualization of the whole brain at high resolution. Imaris software (Imaris 9.0.1, Bitplane) was used to visualize images in 3D.

dsRNA synthesis and RNAi treatment
dsRNAs were synthesized in an in vitro transcription reaction using T7 RiboMAX™ Express Large-Scale RNA Production System (Promega). The DNA templates (~500bp) of dsRNAs were amplified from the queens’ ovaries cDNA. The T7 promoter sequence was added to the 5’end of all the primers for DNA template amplification. The primers used in this study were listed in Table S1.

To promote the efficiency and specificity of gene silencing, three dsRNAs targeted to different gene regions were mixed in equal proportions and diluted to a final concentration of 1 μg/μL of total dsRNA. For one treatment on one 24-well plate filled with larvae, 2.4 μg dsRNA mixture and 8μL transfection polymer material ([Poly]oxy-1,4-butanedioloxyl]-1-oxo-1,3-propanediyl)[2-(dimethylamino)ethyl]limino][3-oxo-1,3-propanediy]] were diluted in 80 μL 25 mM NaOAc buffer (pH5.0), vortexed vigorously for 30 s and then incubated at room temperature for 20 min. Dropwise added 3 μL mixture to the region near the larva’s head. The tips should avoid touching larva. This treatment was begun on the day after 1st instar larvae transfer and repeated once a day until the larval stage ended.

Synthesis of transfection polymer
To a solution of 1,4-butanediol diacylate (10 mmol, 1982.2 mg, 1.982 mL) in THF (20 mL), diamine (10 mmol, 881.5 mg, 1.092mL) was added. The reaction mixture was stirred at 50°C for 48 h. After that, the reaction was cooled to room temperature and concentrated under reduced pressure. The polymer was prepared with DMSO into a solution of 500 mg/mL.

Quantitative real-time PCR
Total RNA was isolated with Trizol Reagent (Invitrogen). cDNA was synthesized using TransScript® First-Strand cDNA Synthesis SuperMix (TransGen). Quantitative RT-PCR was performed using AceQ Universal SYBR mixture (Vazyme) in duplicate on LightCycler 480 II (Roche). Template concentrations were normalized to endogenous reference RpL32. Primers for quantitative RT-PCR were designed by PerlPrimer and shown in Table S2.

10x data preprocessing
The 10x honeybee brain samples were each processed by the Cell Ranger (version 3.1.0) count pipelines provided by 10X Genomics to generate single feature-barcode matrices. The reference genome was built based on A. mellifera (assembly Amel_HAv3.1) from NCBI.
Data processing by seurat

The feature-barcode matrices of each sample (two biological replicates were performed for each queen, forager, and nurse experiment) were processed by Seurat (version 3.1.5) (Butler et al., 2018). Genes expressed in at least one cell were reserved for further processing. The duplicates of three types of honeybee brain samples were merged by the Seurat merge function. In addition, cells with unique feature counts between 200 and 5000, the number of UMIs between 500 and upper limit (upper limit = Q3 + 1.5 * (Q3 – Q1), Q1 and Q3 are the first and third quartiles) and less than 15% of mitochondrial genes were screened for further analysis.

After the filtered datasets were normalized using the default parameters, variable genes were found using the FindVariableFeatures function in Seurat with the parameters including selection.method = "vst", nfeatures = 3000. Then, three types of honeybee brain datasets were integrated using the IntegrateData function in Seurat with dims = 1:30 parameter.

Then, the data were reduced and clustered using principal component analysis (PCA) and uniform manifold approximation and projection (UMAP) in Seurat. And Clustering of the data was processed using the FindClusters function with resolution = 1 parameter. Marks genes for each cluster were determined by Seurat FindAllMarkers function with parameters including only.pos = TRUE, min.pct = 0.1, logfc.threshold = 0.5, return.thresh = 0.01, test.use = "bimod".

For reclustering of glia and Kenyon cells, cells set were selected by their respective marker genes, and analysis was carried out by the above method. The only difference was that the datasets of glia and Kenyon cells adopt 0.2 and 0.5 resolutions respectively. The information of all described genes in this study is summarized in Table S13.

Analysis of bulk RNA-seq

Bulk RNA-seq libraries were paired-end-sequenced. FastQC software (version 0.11.9) was used for quality control. Adapters were trimmed off with the Trimmomatic program (version 0.39) (Bolger et al., 2014). After filtering, the paired reads were aligned to the honeybee reference genome (assembly Amel_HAv3.1) using STAR software (version 2.7.9a) (Dobin et al., 2013). The STAR mapping produced reads were accurately quantified by the RSEM program (version 1.3.1) (Li and Dewey, 2011) to obtain count matrices about gene expression. The count matrices were processed by DEseq2 (version 1.32.0) (Love et al., 2014) R package.

Analysis of neuropeptides

The expression profiles of 27 reported bee neuropeptides were obtained from single cell RNA-seq and bulk RNA-seq. TBtools software (version 1.082) (Chen et al., 2020) was used to generate a heatmap.

Differentially expressed genes

For single cell RNA-seq data, differentially expressed genes were calculated by Seurat FindMarkers function with parameters including logfc.threshold = 0, min.pct = 0, test.use = “bimod”. The significance threshold was adjusted p value < 0.01, log2 foldchange of the average expression between the two groups >0.5 or < -0.5. For bulk RNA-seq data, adjusted p value < 0.01 and log2 foldchange >1 or < -1 in DEseq2 results were considered as differentially expressed genes.

Comparisons to bulk RNA-seq and single-cell RNA-seq

Pseudo-bulk normalized expressions of single-cell RNA-seq were obtained from three Seurat objects of forager, nurse, and queen. Compared with the normalized counts of bulk RNA-seq, the genes that were simultaneously detected were retained. The normalized expressions of single-cell RNA-seq and bulk RNA-seq were converted by log2 to calculate Spearman’s correlation coefficients using the stat.cor function from the ggpubr package.

Gene ontology enrichment analysis

The FindMarkers function of the Seurat R package was used to conduct data analysis. Genes with average log2 foldchange >0.5 and adjusted p value < 0.01 were considered as DE-Gs. GO term enrichment was performed using DAVID (Huang da et al., 2009a, b).
QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed using GraphPad Prism 8 software (version 8.2.1). Unpaired two side t-test was applied to determine whether the means of two populations were different, and differences were considered to be statistically significant at a value of p < 0.05.