ZOOLOGICAL SCIENCE **21**: 23–28 (2004)

The Changes of Gene Expression in Honeybee (*Apis mellifera*) Brains Associated with Ages

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ABSTRACT—Honeybee (*Apis mellifera)* worker bees (workers) are known to perform wide variety of tasks depending on their ages. The worker's brains also show the activity and behavior-dependent chemical and structural plasticity. To test if there are any changes of gene expression associated with different ages in the worker brains, we compared the gene expression patterns between the brains of newly emerged bees and old foraging workers (foragers) by macroarray analysis. The expression of genes encoding signal transduction pathway components, ion channels, and neurotransmitter transporters is elevated in the old forager brains, suggesting that the neuronal activities would be enhanced. The mRNA levels of cell adhesion protein, transcription related factors, and plasma membrane associated proteins are also increased in the old forager brains. Meanwhile, the mRNA level of one putative cell adhesion protein is decreased in the old forager brains. These results thus suggest that the dramatic changes of gene expression occur in honeybee brains associated with ages.

Key words: honeybee, brain, age, behavior, gene expression

INTRODUCTION

Honeybee, *Apis mellifera,* is one of social insects and has been used as a model system to study complex animal behavior in addition to learning and memory. Honeybees form a colony, which is consisted of single queen, hundreds of male drone, and thousands of female worker bees. In contrast to a queen and male bees, workers perform wide variety of tasks to maintain the colony. Young workers feed and care for larvae and queen, middle-age workers maintain the hive and store food, and old workers forage for nectar and pollen outside the hive (age-related division of labor). In contrast to performing tasks inside the dark hive, foraging involves extensive flight, learning and memorizing food locations, navigation using multiple cues (for example, sun compass), and communication by a dance language (Fahrbach and Robinson, 1995; Menzel and Muller, 1996; Menzel and Giurfa, 2001). These tasks may require better modules for sensory-input processing and enhanced learning and memory capabilities. This behavioral shift is known to accompany changes in endocrine system, brain chemistry, and brain structure. The levels of juvenile hormone and biogenic amines (for example, octopamine) in the brain are higher in old forager compared with young in-hive worker (Fahrbach and Robinson, 1996; Wagener-Hulme *et al.*, 1999). The volumes of certain glomerulus in antennal lobe and the neuropils of mushroom bodies also increase depending on the age and activity (Sigg *et al.*, 1997; Withers *et al.*, 1993). Moreover, it has been recently shown that mushroom body intrinsic neurons of forager brain have longer and more branched dendrites than those of in-hive bee brain (Farris *et al.*, 2001).

It is not known whether the long-term changes in neuronal function, which underlie the age-related division of labor under natural condition are dependent on the changes of gene expression. In fact, there are a few studies reporting the changes of gene expression in the brain that are associated with behavioral plasticity. The expression of *ZENK*

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(one of immediate early genes) and the genes encoding glucocorticoid receptor, serotonin receptor, and nerve growth factor is induced in hippocampus by environmental enrichment in adult rats (Olsson *et al.*, 1994; Rasmuson *et al.*, 1998). In songbirds, the expression of sex steroid receptors and SNAg (song system nuclear antigen) in song behavior control region of brain is modulated by sex, age, and season (Gahr and Metzdorf, 1997; Akutagawa and Konishi, 2001). In honeybees, the levels of *period* and PKG (cGMP dependent protein kinase) mRNAs are higher in the foragers than the in-hive bees (Toma *et al.*, 2000; Ben-Shahar *et al.*, 2002).

To unveil the changes of gene expression in honeybee brains associated with ages, we analyzed the gene expression patterns in the brains of the workers with different ages. The relationship between the changes of gene expression in honeybee brains and the modifications of brain structures and functions associated with ages and behavioral plasticity is discussed.

MATERIALS AND METHODS

Construction of honeybee brain specific cDNA library by suppression subtractive hybridization (SSH)

PolyA$^+$RNA was isolated from the brains, hypopharyngeal glands, and abdomens of twenty foragers with a QuickPrep Micro mRNA Purification Kit (Amersham) and used for the preparation of double strand cDNAs. Brain cDNA was used as "tester" and the mixture of hypopharyngeal gland and abdomen cDNAs was used as "driver". The SSH followed by PCR amplification was carried out according to manufacturer's instructions (CLONTECH PCR-Select^{TM} cDNA Subtraction Kit). The PCR amplified DNA was size-fractionated into three pools by agarose gel electrophoresis. The DNA in each pool was cloned in a pGEM-T Easy vector (Promega) by T/A cloning.

Macroarray analysis and cDNA sequencing

The cDNA insert of each recombinant plasmid was amplified by colony PCR with T7 and SP6 promoter primers. The amplification of total 1235 cDNAs was verified by agarose gel electrophoresis. The aliquot (2 μg) of the amplified DNA was denatured by heat and alkaline treatment, and then spotted to nylon membranes by a 96 wells dot blot apparatus. *EF-1* α cDNA was also simultaneously spotted along with other 95 cDNA clones. PolyA$^+$RNA was isolated from the brains of the twenty foragers and the newly emerged workers (within 24 hr after eclosion). First strand ^{32}P-labeled cDNA probes were prepared with each polyA$^+$RNA using ^{32}P-dCTP, oligo dT primer, and reverse transcriptase. The two resulting probes (with equal radioactivity) were used for the hybridization of the nylon membranes prepared as above. The radioactivity of each round DNA spot was measured by a Bioimage analyzer BAS2000 (Fuji). The background correction was made by subtracting the radioactivity of the adjacent area without spotted DNA. We then calculated the ratio of the radioactivity of forager to newly emerged bee (F/NE) for each cDNA. These values were multiplied by the newly emerged to forager bee (NE/F) ratio of the radioactivity of *EF-1* α cDNA for the normalization of hybridization. The level of *EF-1* α mRNA was previously shown to be constant during the adult honeybee development (Toma *et al.*, 2000). These experiments were repeated three times with probes prepared by polyA$^+$RNA isolated from different bee samples. We sequenced the differentially expressed cDNA clones with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The putative protein encoded by each cDNA was determined using a Blast-X program (Altschul *et al.*, 1997).

Northern blot hybridization

PolyA$^+$RNA was isolated from the brains of the twenty newly emerged workers, 7 days old in-hive workers, and 28 days old pollen foragers. 5 μg of polyA$^+$RNA was separated by 1% agarose gel containing formaldehyde and transferred to nylon membranes. They were hybridized with various cDNA probes as indicated. These experiments were repeated three times with polyA$^+$RNA isolated from different bee samples. The radioactivity of each band was measured by the BAS2000 and honeybee *EF-1* α mRNA was used as a control to normalize the levels of loaded RNA.

RESULTS AND DISCUSSION

We compared the gene expression patterns in the brains of newly emerged bees and unknown aged foragers by Macroarray analysis. The level of the expression of each mRNA in brain is varied. Many clones show variable expression levels between the forager and newly emerged bee brains. We sequenced the cDNA clones, which were differentially expressed between forager and newly emerged bee brains. The cDNA sequences were analyzed by a Blast-X program to search the homolog of protein encoded by each cDNA. The putative proteins coded by the cDNAs are classified by their functions and all data are summarized in Table 1. As expected, most of honeybee genes have homologs in a fruit fly, *Drosophila melanogaster*. Meanwhile, when they have homologs in several different species, some show higher homology to, for example, human than fruit fly genes.

All genes categorized as neurotransmitter transporters and ion channels are expressed at higher levels in the old forager compared to newly emerged bee brains. These include choline transporter (Okuda *et al.*, 2000), glutamate transporter (Besson, *et al.*, 1999), betaine/GABA transporter (Borden *et al.*, 1995), cationic amino acid transporter (Hoshide *et al.*, 1996), voltage-gated calcium channel subunit (Klugbauer *et al.*, 1999), Na^+/K^+-transporting ATPase α subunit (Sun *et al.*, 1998), and Na^+-driven anion exchanger (with Na^+-dependent Cl^--HCO_3^- exchange activity) (Romero *et al.*, 2000) genes. These results may suggest that the uptake of various neurotransmitters and the exchange of various ions through the plasma membrane in neurons and glia would be more enhanced in the old forager than newly emerged bee brains.

All genes categorized as signal transduction pathway components are expressed at higher levels in the old forager compared to newly emerged bee brains. These include phosphoinositide-specific phospholipase C (PI-PLC) epsilon (Song *et al.*, 2001) and diacylglycerol kinase (DGK) (Harden *et al.*, 1993) genes. These results suggest that the signal transduction mediated by the intracellular Ca^{2+} and phospholipid second messengers would be more intense in the old forager than newly emerged bee brains. The other

Table 1. Summary of genes differentially expressed between newly emerged and forager bee brains.

Clone	Accession number	Putative encoded protein	Identity (%)	Class/Function	Ralative radioactivity to *EF-1* α	F/NE (±SEM)
N12	AB078155	Choline cotransporter	56	Neurotransmitter transporter	0.14(0.02)	2.7(0.4)
3176	AB078156	Glutamate transporter	65		0.20(0.04)	3.3(0.3)
2334	AB078157	Betaine/GABA transporter	40		0.11(0.02)	3.1(0.2)
3324	AB078158	Cationic amino acid transporter	40		0.12(0.02)	1.9(0.2)
3297	AB078159	Voltage-gated calcium channel	26	Ion channel	0.11(0.02)	4.0(0.5)
N23	AB078160	Na^+/K^+-exchanging ATPase	86		0.15(0.03)	2.3(0.3)
N27	AB078161	Na^+-driven anion exchanger	91		0.13(0.02)	3.5(0.3)
1297	AB078163	PI-PLC epsilon	43	Signal transduction	0.32(0.05)	2.5(0.2)
41108	AB078164	Diacyl glycerol kinase	43		0.28(0.03)	4.3(0.3)
2321	AB078165	Orphan receptor tyrosine kinase	42		0.16(0.02)	3.6(0.2)
N182	AB078166	*Drosophila* CG8789	31		0.26(0.04)	3.3(0.3)
22108	AB078167	Protein tyrosine phosphatase sigma	80		0.14(0.03)	2.1(0.2)
1289	AB078168	Guanylyl cyclase	71		0.17(0.03)	2.7(0.3)
N179	AB078169	Still life	74		0.21(0.03)	2.5(0.2)
2182	AB078172	Sidekick	26	Cell adhesion	0.12(0.02)	4.1(0.5)
1227	AB078173	*Drosophila* GH11322	58		0.45(0.07)	0.3(0.1)
4111	AB078176	Trap36	43	Transcription	0.08(0.01)	4.0(0.5)
32101	AB078177	Sin3A	58		0.11(0.02)	3.6(0.4)
N217	AB078179	Dank2	63	Ankyrin	0.42(0.06)	1.4(0.2)
N119	AB078180	Coracle	65	Protein 4.1	0.34(0.06)	1.4(0.1)
22102	AB078181	Pumilio	86	RNA binding	0.18(0.03)	2.3(0.1)
N151	AB078182	Otoferlin	79	Membrane fusion	0.14(0.02)	3.2(0.4)
2330	AB078183	*Drosophila* CG2611	48	Unknown	0.39(0.06)	2.1(0.1)
3298	AB078185	*Drosophila* CG4702	45		0.14(0.02)	3.6(0.5)
N25	AB078186	*Drosophila* CG7781	67		0.31(0.02)	2.4(0.2)
N286	AB078187	*Drosophila* CG7886	33		0.35(0.05)	3.3(0.4)
2149	AB078188	*Drosophila* CG9259	37		0.27(0.03)	3.2(0.3)
3175	AB078189	*Drosophila* CG9487	24		0.12(0.01)	4.2(0.3)
1290	AB078190	*Drosophila* CG9497	33		0.15(0.04)	5.4(0.6)

The cDNA clones differentially expressed between the newly emerged (NE) and forager (F) bee brains are classified by the functions of the putative encoded proteins. The cDNA sequences were deposited in a DDBJ DataBank with the specific accession number. The radioactivity of each round DNA spot was determined by subtracting the radioactivity of the adjacent non-spotted portion with the same area (background correction). The changes of gene expression are shown as the ratios of the radioactivity of F to NE (F/NE), which are corrected by the NE/F ratio of *EF-1* α mRNA. These values are means±SEM of three independent experiments. The cDNA clones consistently showing more than 1.2 fold difference between NE and F are listed. The cDNA clones with large SEM are eliminated from this list. The ratios (means±SEM of three independent experiments) of the radioactivity of each cDNA clone to *EF-1* α in the membrane hybridized with ^{32}P-labeled cDNA probes prepared with newly emerged bee brains poly A^+ RNA are also listed.

genes expressed at higher levels in the old forager brains are orphan receptor tyrosine kinase (RTK) homologous to mRor1 (Oishi *et al.*, 1999), protein kinase homologous to *Drosophila* CG8789, and protein tyrosine phosphatase (PTP) homologous to human PTP sigma (Krueger *et al.*, 1990). Their functions in the adult brains are not yet identified in any species. Nevertheless, the cell signaling activities mediated by these protein kinases and phosphatase may be enhanced in the old forager compared to newly emerged bee brains. We identify the honeybee gene encoding the homolog of *Drosophila* soluble guanylyl cyclase β subunit, that catalyzes the synthesis of intracellular cGMP (Shah and Hyde, 1995). The resulting cGMP directly or indirectly activates PKG, cGMP-regulated phosphodiesterase, PKA, and cyclic nucleotide-gated channels. These cGMP dependent intracellular events would be more facilitated in the old forager than newly emerged bee brains. We also find the honeybee gene homologous to *Drosophila still life (sif).* The *sif* encodes a guanine nucleotide exchange factor for RAC, a member of RHO family small G proteins. SIF was shown to

be necessary for the formation or maintenance of synapses (Sone *et al.*, 2000). The higher expression of *sif* mRNA in the old forager brains may demonstrate that new synaptic formation or the reorganization of preexisting synapses is activated.

We identify two honeybee genes encoding the homologs of cell adhesion proteins, *Drosophila* Sidekick (SDK) and GH11322 in this screen. Expression of the former gene but not the latter is enhanced in the old forager brains. It is intriguing that the expression of the *GH11322* homologous gene is in fact reduced in the old forager brains (see Fig. 1). *Drosophila* SDK appears to be necessary for the correct pattern formation of photoreceptor cells (Nguyen *et al.*, 1997). Thus, new synaptic formation or the modifications of synaptic terminals in visual system may occur more frequently in the old foragers. *Drosophila GH11322* cDNA encodes a protein with four immunoglobulin domains, two fibronectin type III domains, and one transmembrane domain. In *Drosophila,* this gene lies next to *turtle (tutl),* which has an essential role for coordinated motor output. The phenotypes of the disruption of both *tutl* and *GH11322* genes are identical to those of the single disruption of *tutl,* suggesting that their functions are not redundant (Bodily *et al.*, 2001). Because these two proteins have similar extracellular but different intracellular domains, they can bind the same targets but result in the different outcomes. The functions of GH11322 in the brain remain to be established.

We find that two honeybee genes encoding transcription related factors are expressed at higher levels in the old forager brains. These are one of transcription mediator complex subunits (Trap36) (Boube *et al.*, 2000) and transcription repressor for particular genes (Sin3A) (Neufeld *et al.*, 1998). These results may suggest that the stimulation and repression of the transcription of specific genes are more tightly regulated in the old forager compared to newly emerged bee brains.

All genes categorized as other functional protein genes are expressed at higher levels in the old forager compared

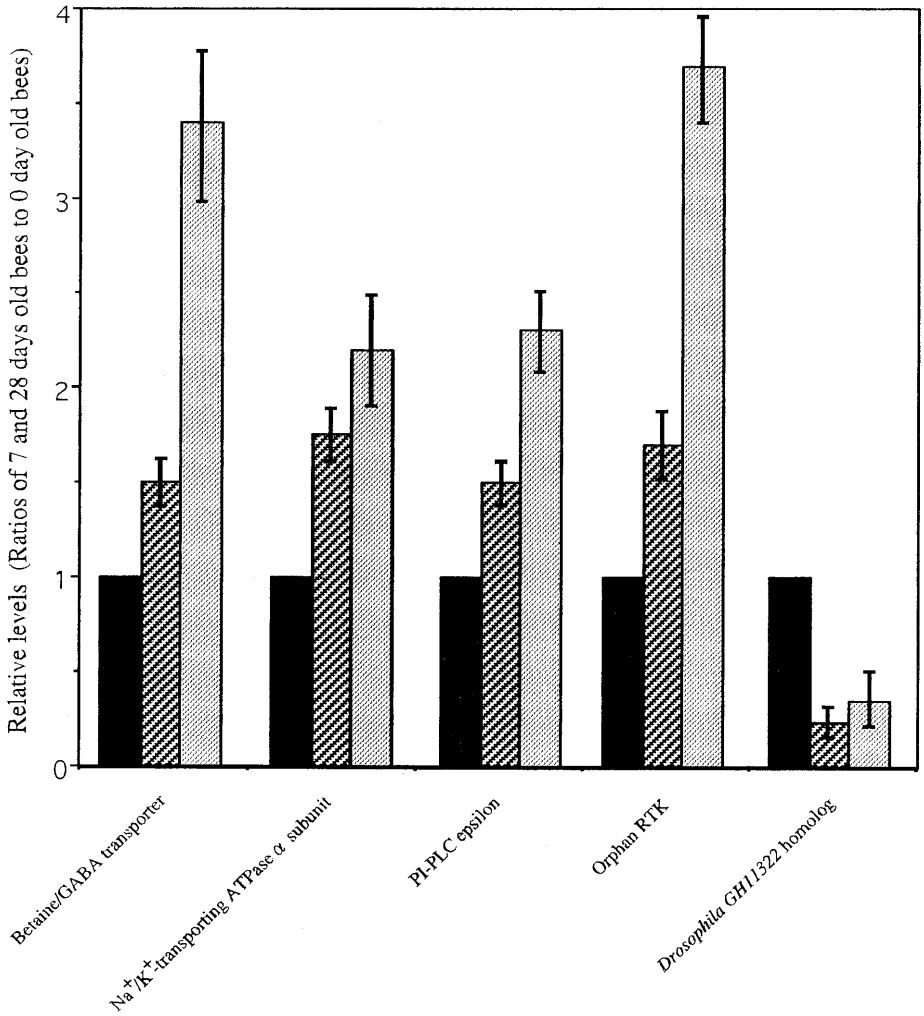

Fig. 1. The relative levels of betaine/GABA transporter, Na^+/K^+-transporting ATPase α subunit, PI-PLC epsilon, orphan RTK, and *Drosophila GH11322* homolog mRNAs in the brains of bees at 0 (solid bars; normalized as 1), 7 (striped bars), and 28 (hatched bars) days after eclosion. The 7 and 28 days old bees are the in-hive and pollen forager bees, respectively. These values are means±SEM of three independent experiments.

to newly emerged bee brains. These include the honeybee genes homologous to *Dank2, coracle, pumilio,* and human otoferlin. *Dank2* is one of two *Drosophila* ankyrin genes (Bouley *et al.*, 2000). The ankyrins are linker proteins, which connect various membrane proteins with submembranous actin-spectrin skeleton. The increase of the ankyrin mRNA level may indicate that the total number of membrane protein interactions with underlying cytoskeleton is increased in the old forager brains. *coracle (cora)* encodes a *Drosophila* Protein 4.1 homolog (Lamb *et al.*, 1998). The analysis of *Drosophila cora* mutants revealed that Cora is a necessary structural component of a septate junction required for the maintenance of trans-epithelial barrier and also involved in cell signaling events. *pumilio (pum)* encodes an RNA binding protein regulating mRNA translation. In *Drosophila, pum* is expressed in a subset of CNS cells and necessary for pathfinding of Bolwig nerve and motor neurons (Schmucker *et al.*, 1997). Thus, the honeybee Pum homolog would be expressed at higher level in the specific cells of the old forager brain and may function for the correct targeting of preexisting neurons during the neuronal reorganization. Human otoferlin was identified as the gene underlying an autosomal recessive, nonsyndromic prelingual deafness, DFNB9. It is a member of a mammalian gene family related to *Caenorhabditis elegans fer-1* and suggested to be involved in vesicle membrane fusion (Yasunaga *et al.*, 1999). The upregulation of honeybee otoferlin in the old forager brains may demonstrate that the synaptic vesicle fusion is more activated.

The honeybee genes homologous to *Drosophila* CG2611, 4702, 7781, 7886, 9259, 9487, and 9497 are expressed at higher levels in the old forager brains. The functions of these gene products remain to be established.

The expression of several genes identified by macroarray analysis was also analyzed by Northern blot hybridization. PolyA$^+$RNA was isolated from the brains of the bees at 0, 7, and 28 days after eclosion. The 7 days old bees perform the in-hive tasks and 28 days old bees are the pollen foragers. The relative levels of betaine/GABA transporter, Na^+/K^+-transporting ATPase α subunit, PI-PLC epsilon, orphan RTK, and *Drosophila GH11322* homolog mRNAs in three different bee populations are shown in Fig. 1. In these experiments, the honeybee *EF-1* α mRNA was used as a control to normalize the level of loaded RNA. The expression of the betaine/GABA transporter, Na^+/K^+-transporting ATPase α subunit, PI-PLC epsilon, and orphan RTK genes increases in the 7 and 28 days old bees relative to the 0 day old bees. There is a marked increase of betaine/GABA transporter and orphan RTK mRNAs in 28 days old bees. Meanwhile, *Drosophila GH11322* homolog mRNA is already decreased in 7 days old in-hive bees and remains constant in 28 days old pollen foragers. These results are consistent with the data obtained by the macroarray analysis. In summary, the gene expression patterns of the newly emerged bee brains and the old forager brains are quite different as described. The further experiments will be necessary to address whether the changes of the gene expression patterns in honeybee brains are causally related to the age or behavioral difference.

ACKNOWLEDGEMENTS

This study was supported by Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to YK and TK.

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(Received May 2, 2003 / Accepted June 28, 2003)