Türk. entomol. derg., 2014, 38 (4): 431-441
ISSN 1010-6960

Orijinal araştırma (Original article)

Expression levels of glutamate and serotonin receptor genes in the brain of different behavioural phenotypes of worker honeybee *(Apis mellifera)*

Farklı davranış fenotiplerinde işçi bal arısı *(Apis mellifera)*, beyninde glutamat ve serotonin reseptör genlerinin ekspresyon düzeyleri

Qiu-Hong QIN[1,2] Xu HAN[1] Hao LIU[1]
Shao-Wu ZHANG[1, 3] Zhi-Jiang ZENG[1]*

Summary

Social insect colonies are known for their efficient system of task specialization. In this study, we analyzed the brain expression level of glutamate and serotonin receptor genes in different behavioural phenotypes of (*Apis mellifera*) workers by qRT-PCR. The glutamate receptor genes include the ionotropic glutamate receptor (iGluR) genes and metabotropic glutamate receptor (mGluRs) genes, and has 9 orthologous genes in honeybee, such as the N-methyl-D-aspartate receptor (NMDAR) genes, NMDAR1 and NMDAR2, the α-amino-3-hydroxy-5-methyl-4-isoxazole receptor (AMPAR) genes, AMPAR Δ2-a, AMPAR Δ2-b, AMPAR Δ2-c, AMPAR Δ2-d, and the mGluR1, mGluR4 and mGluR7. Our results showed that: the relative expression level of NMDAR genes was much higher in newly emerged workers (NW) than in young nurses (YN) and "old" foragers (OF) ($P < 0.001$); both NW and YN had a significantly higher relative expression level of AMPAR Δ2-b, AMPAR Δ2-c and AMPAR Δ2-d than in OF ($P < 0.05$). than in YN and OF; the relative expression level of mGluR7 gene in OF was significantly higher than in NW ($P > 0.05$), but there were no significant differences among NW, YN, and OF for the relative expression level of mGluR1 and mGluR4 gene ($P > 0.05$); in the case of Serotonin (5-HT), the relative expression level of the 5-HT1 gene showed no significant difference between YN and OF ($P > 0.05$), but was higher in YN and OF than in NW ($P < 0.001$). Above results indicate that some glutamate and serotonin receptor genes may play important roles in honeybee age-dependent role change.

Key words: *Apis mellifera*, age, brain, iGluRs, mGluRs, 5-HT

Özet

Sosyal böcekler görev dağılımının en bilinen sistemleridir. Bu çalışmada, qRT-PCR ile bal arası (*Apis mellifera*) işçilerinin farklı davranış fenotiplerinde beyinde bulunan glutamat ve serotonin reseptör genlerinin ekspresyon seviyeleri analiz edilmiştir. Glutamat reseptör geni; İyonotropik glutamat reseptörü (iGluR) ve metabotropik glutamat reseptörü (mGluR'ler) genlerini kapsar. Balarısında Glutamat reseptör geni, N-metil-D-aspartat reseptör (NMDAR) genleri, NMDAR1 ve NMDAR2, α-amino-3-hidroksi-5-metil-4-izoksazol reseptörü (AMPAR) genleri, AMPAR Δ2-a AMPAR Δ2-b AMPAR Δ2-c AMPAR Δ2-d ve mGluR1, mGluR4 ve mGluR7 olmak üzere 9 ortolog gene sahiptir. Sonuçlarımıza göre: NMDAR geninin ekspresyon düzeyi yeni çıkış yapmış işçilerde (NW), genç (YN) ve yaşlı bireylere (OF) oranla çok daha yüksek bulunmuşken ($p<0.001$); işçi arıların ve genç bireylerin her ikisinde yaşlı bireylerden belirgin bir şekilde daha yüksek oranda Ampar Δ2-b, Ampar Δ2-c ve Ampar Δ2-d gen ekspresyon düzeyi saptanmıştır ($P<0.05$). Yaşlı bireylerde mGluR7 geninin ekspresyon düzeyi işçi arılardan belirgin olarak daha yüksek iken ($P>0.05$); genç, yaşlı ve işçi arıların hepsinde mGluR1 ve mGluR4 genlerinin ekspresyon düzeyleri açısından ($p>0.05$) anlamlı bir fark bulunamamıştır. Serotonin (5-HT) söz konusu olduğunda, 5-HT1 geninin ekspresyon seviyesi bakımından genç ve yaşlı bireyler arasında anlamlı bir fark çıkmamıştır ($P>0.05$) ancak aynı genin ifadesi genç ve yaşlı bireylerde işçi arılara göre daha yüksek bulunmuştur ($P<0.001$). Yukarıdaki sonuçlar, bazı glutamat ve serotonin reseptör genlerinin arılarda yaşa bağlı görev değişikliğinde önemli roller oynayabileceğini göstermiştir.

Anahtar sözcükler: *Apis mellifera*, yaş, beyin, iGluRs, mGluRs, 5-HT

[1] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China
[2] Guangxi Liuzhou Animal Husbandry and Veterinary School, Liuzhou, China
[3] ARC Center of Excellence in Vision Science, Research School of Biology, The Australian National University, Canberra, ACT 0200, Australia
* Sorumlu yazar (Corresponding author) e-mail: bees1965@sina.com
Alınış (Received): 10.08.2014 Kabul ediliş (Accepted): 20.01.2015

Introduction

One of the most important characteristics of honeybee, *Apis mellifera*, is the age-related division of labor, based on a stereotyped pattern of behavioural development of adult worker bees (Winston, 1987). Adult worker bees perform in-hive jobs for the first 2-3 weeks, including feeding the brood ("nursing"), constructing nest, and processing honey, then switch to foraging for food outside the hive for the rest of their lives (about 4-6-week). Foraging is probably a more cognitively demanding task than activities that are performed exclusively in the hive (Fahrbach & Robinson, 1995) because foragers receive a vast input of environmental signals. Foragers leave the hive for food and return with nectar and pollen loads, during which their nervous system has to process, learn and memorize information, including light, landmarks, the color, shape and odors of flowers, which help them to navigate between hive and field, and find flower sources (Menzel et al., 1998; Zhang et al., 1999; Giurfa et al., 1994; Sanderson et al., 2006).

Glutamate is a main excitatory amino acid neurotransmitter in the central nervous system of vertebrates, which activate both metabotropic and ionotropic receptors (Hogner *et al*., 2002; Erreger et al., 2004). In honeybee adult brain, glutamate, GABA and acetylcholine are the most abundant neurotransmitters (Bicker, 1999). Mammalian glutamate receptors are classified into two groups based on a postsynaptic current caused by their activities. One group is called ionotropic glutamate receptors (iGluRs), which forms ion channel pores, and is divided into several subtypes named as N-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazole receptors (AMPARs) and Kainate receptors based on the chemicals binding to the receptors more selectively than glutamate. Another group, called metabotropic glutamate receptors (mGluRs), directly activates ion channels on the plasma membrane through a signaling cascade which involves G protein.

mGluRs are expressed in the mushroom bodies and the brain regions of honeybees, and their expression in the brain apparently overlaps, suggesting that they may interact with each other to modulate the glutamatergic neurotransmission (Funada et al., 2004). The results of Locatelli et al. (2005) provided the first direct evidence for a temporally and locally restricted function of glutamate in memory formation in honeybees and insects, and more and more evidence suggested that glutamate played key roles in honeybee learning and memory process (Si et al., 2004; Kucharski et al., 2007; El Hassani et al., 2012). Besides, the data from Ryzhova et al. (2010) indicated that in the mechanisms of the honeybee Central Nervous System (CNS) plasticity, an important role is played by heterogeneous of mGluRs affecting formation of the short-term and long-term memory. Furthermore, using immunohistochemistry, Démares et al. (2013) found that the glutamate-gated chloride channels (GluCl) subunit variants were localized in adult honeybee mushroom bodies and antennal lobes. Recently, a study represented the first successful attempt to differentiate two highly similar GluCl subunits, from a single gene, in olfactory and memory processes of an invertebrate species (Démares et al., 2014).

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is involved in regulation of mood, appetite and sleep in humans. The 5-HT receptorsare a group of G protein-coupled receptors (GPCRs) and ligand-gated ion channels (LGICs) found in the central and peripheral nervous systems in different organisms. They are activated by the neurotransmitter serotonin, which acts as their natural ligand. The 5-HT receptors influence various biological and neurological processes such as pain, feeding, sleep, sexual behavior and cognition by means of mediating both excitatory and inhibitory neurotransmission (Barnes & Sharp, 1999; Hoyer et al., 2002).

In recent years, many studies on 5-HT have been carried out in the honeybee. Meltzer et al. (1998) showed that 5-HT system is involved in learning and memory processes in honeybees. The study by Schlenstedt et al. (2006) marked the first comprehensive characterization of a serotonin receptor, Am5-HT7, in the honeybee and should facilitate further analysis of the role(s) of the receptor in mediating the various central and peripheral effects of 5-HT. And growing evidence indicate that 5-HT is involved in the

control of motor function of PER in honeybees (Wright et al., 2010; Wright, 2011). Furthermore, recent report explored function and distribution of 5-HT2 receptors in the honeybee, and it marked the first molecular and pharmacological characterization of 5-HT2α and 5-HT2β receptor subtypes in this insect (Thamm et al., 2013). Moreover, the role of 5-HT in feeding and gut contractions in the honeybee has been reported by French et al. (2014). Even so, it is not clear what role 5- HT systems have in the process of age-dependent role change.

With the recent release of its genome sequence, the honeybee has emerged as an excellent model for molecular studies of social behavior. Denison & Raymond-Delpech (2008) made some insights into the molecular basis of social behavior by examined three genes in particular, foraging, malvolio and vitellogenin, all implicated in the striking behavioural change in the life of the honeybee. However, there was no study has characterized the pattern of expression of glutamate and serotonin receptor genes in the brain of the honeybee in term of age-related tasks. In the present study, gene expression of 13 glutamate and serotonin receptors in honeybee brains were investigated, which included three age-related task groups: newly emerged honeybee workers, nurses and foragers.

Material and Methods

Insect

The honeybees used for the experiment were the Western honeybee, *A. mellifera*. Colonies were raised according to standard beekeeping techniques at the Honeybee Research Institute, Jiangxi Agricultural University, Nanchang, China (28.46 °N, 115.49 °E). In this study, honeybees were sampled in three groups: newly emerged workers (NW), young nurses (YN) and “old” foragers (OF). Newly emerged workers were collected within 1 h after they emerged. Nurses were caught when they entered into the uncapped brood cells to nurse larvae. Returning pollen foragers, easily identified by the brightly colored pollen loads on their hind legs (Robinson, 1987), were collected during peak foraging hours at the entrance of the hives. Independent biological replicates were performed on three colonies to gather sufficient data. 100 newly emerged workers, nurses and foragers were respectively collected from each of the three colonies. All samples were collected alive and immediately frozen in liquid nitrogen and then stored in the -80ºC until further processing.

Brain dissection

Brain tissues were dissected in 1xPBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4), rinsed with DEPC-treated water, and then stored in liquid nitrogen. Each sample consisted of 25 brains pooled from each behavioural group per colony for subsequent differential expression analysis (n = 4 samples per age (behavioural) group per colony).

RNA extraction and cDNA synthesis

Trizol reagent (Invitrogen, USA) was used to isolate total RNA from brain tissue from each sample following the manufacturer’s instructions. RNA concentration and quality were measured using a spectrophotometer, NanoPhotometer^{TM} P300 (IMPLEN). Purity of the total RNA was determined as the 260 nm/280 nm ratio with expected values between 1.8 and 2.0. RNA integrity was determined by agarose gel (1.0 %) electrophoresis and ethidium bromide staining. The amount of total RNA was standardized to 1 μg/μL for reverse transcription. cDNA was synthesized by mixing 400ng RNA with 3μL (0.5 μg/μL) oligo-dT 18 primer (Invitrogen, USA) in 24μL DEPC-treated water, and incubated for 10 min at 70ºC. Then 10μL dNTPs (2.5 mM each), 1.5 μL Ribonuclease inhibitor (50 U/μL, TransGen), 10μL 5×RT M-MLV buffer (Takara, Japan) and 1.5μL M-MLV Revertase (50 U/μL, Takara, Japan), were added and incubated at 42ºC for 1h and extended 15min at 70ºC. Lastly, the synthesized cDNA was diluted 1:10 with DEPC-treated water for subsequent qRT-PCR reactions.

Primer design and real-time PCR assays

Primer 5.0 was used to design primers for glutamate and 5-HT receptor primers. The gene IDs were listed in Table 1. The resulting cDNA templates were used for quantitative measurement by real-time quantitative PCR (Bio-Rad IQ5, USA) for the expression levels of the 13 specific genes, with GAPDH-1 as an internal control. According to the study of Lourenco et al. (2008) and Scharlaken et al. (2008), GAPDH is one of the most stable expressed reference genes in the honeybee head, and it can be used in the same bioligical context and tissue solely.

Table 1. Description of genes and primer sequences used for qRT-PCR assays

Gene name	Accession number	Primer (5' to 3')
NMDAR1	NM_001011573.1	F: ACTGACGGTACCGAAGAGGA R: CCCATACCATGCCCAACACT
NMDAR2	XM_396271.3	F: GATCTCAGAGTCGAAGCCCG R: ACAGCCTTGGTGTATTCCCG
AMPAR Δ2-a	XM_003249192.1	F: TTTTGAACAGAATAACGGAACA R: AAAGCGGAGTAAACATCGGC
AMPAR Δ2-b	XM_624086.2	F: GAGAAGATGCCGATGAAAGATAAA R: AAAGAGAAAGAAGAAAGCCAACG
AMPAR Δ2-c	XM_003250237.1	F: GCCCTTACCTCCACCACCAT R: GCGACCACTAATCTCCTCTGTTC
AMPAR Δ2-d	XM_624093.2	F: CAAAGTTGTCATAGGTGGATAC R: AAATAGAAAAGCAGAAGGAGTT
mGluR1	AY463910.1	F: GGATGAAAGAAGGAAAAGGATA R: ACAGTAACAATAACAACAGCGAT
mGluR4	XM_395227.4	F: TTTCCGCGTCAGTAGCTCTC R: CGCATGCTGTATGTTCCACG
mGluR7	AB161182.1	F: AGCAAAGAGGCGAGGGATAAT R: CACTCTGATTTATAGGTCCGTTTCC
5-HT1	NM_001171108.1	F: GCCGTCTGGGTGGTGTCCT R: TCCTCCCTCGGTCTTTTTGTGA
5-HT2α	FR727107.1	F: GCAAGTGTTCCAGGTCAGCA R: AAAGCGGAGTAAACATCGGC
5-HT2β	FR727108.1	F: TTTCCAGCGACACGATGAG R: AAGAACACCACCCCGAGC
5-HT7	AM076717.1	F: GCTTTCATAGTTTGTTGGTTAC R: AATCCCTGTTCAGAGTCGCATAG
GAPDH-1	AF023666.1	F: GCTGGTTTCATCGATGGTTT R: ACGATTTCGACCACCGTAAC

1μL of diluted cDNA was mixed with 0.4μL of specific gene primer (10 mM), 5 μL of SYBR *Premix Ex Taq* II and 3.2 μL of DEPC-treated water for qRT-PCR assay. The qRT-PCR assay began with an initial phase of 95ºC for 30s, followed by the temperature cycle: 95ºC for 10s; 60ºC for 1 min for 40 cycles. Finally, melting curves were recorded by increasing the temperature from 50ºC to 90ºC. The specificity of the PCR products was verified by melting curve analysis for each sample.

Data analysis

The Bio-Rad iQ5 2.1 Standard Edition Optical System Software was used to calculate the C_t values and the qpcR package (Spiess & Ritz, 2010; Hornik, 2011) was used to calculate the PCR amplification efficiency of each gene. The relative gene expression levels were calculated according to Liu & Saint's (2002) formula and then square root transformed to attain normality. We tested for statistical significance between relative expression levels by ANOVA, using StatView (v 5.01, USA). Differences between groups were considered to be significant at the probability level of 0.05 %.

Results

Expression levels of glutamate receptor genes

Results from Fig 1 indicated that when compared to young nurses (YN) and "old" foragers (OF), the relative expression levels of NMDAR genes in newly emerged workers showed much higher levels (Df=2, F=14.97, P < 0.001, Fig 1a; Df=2, F=82.10, P < 0.001, Fig 1b). Moreover, YN had a significantly higher expression level of NMDAR2 gene than OF (Df=2, F=82.10, P < 0.05, Fig 1b). However, the relative expression levels of NMDAR1 gene showed no significant difference between YN and OF (Df=2, F=14.97, P > 0.05, Fig 1a).

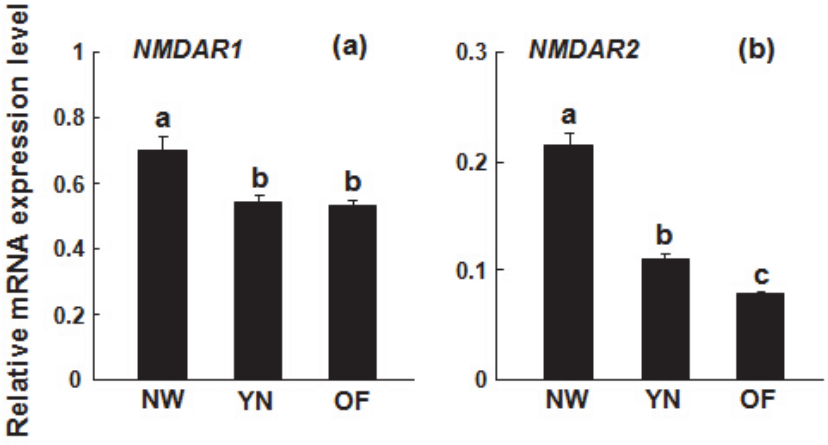

Figure 1. Gene expression levels of NMDA receptor genes relative to a reference gene GADPH-1 in the three groups. a, b. The relative expression levels of NMDAR1 and NMDAR2 in NW, YN and OF. Groups: newly emerged workers (NW), young nurses (YN) and "old" foragers (OF). Different letters on top of bars indicate significant difference (P<0.05) between the groups. Relative expression data were transformed by square root transformation, and are presented here after transformation. Each bar corresponds to a single group represented as the mean ± S.E. of its biological replicates.

The relative expression level of AMPAR Δ2-a has no significant difference among the three groups (Df=2, F=0.33, P > 0.05, Fig 2a). As shown in Figs 2b, c, d, both NW and YN had a significantly higher relative expression level of the other three AMPAR genes, AMPAR Δ2-b, AMPAR Δ2-c and AMPAR Δ2-d than OF (Df=2, F=6.07, P < 0.05, Fig 2b; Df=2, F=3.56, P < 0.05, Fig 2c; Df=2, F=1.65, P < 0.05, Fig 2c); but there were no significant difference of gene expression level of AMPA genes between NW and YN (Df=2, F=6.07, P > 0.05, Fig 2b; Df=2, F=3.56, P > 0.05, Fig 2c; Df=2, F=1.65, P < 0.05, Fig 2d).

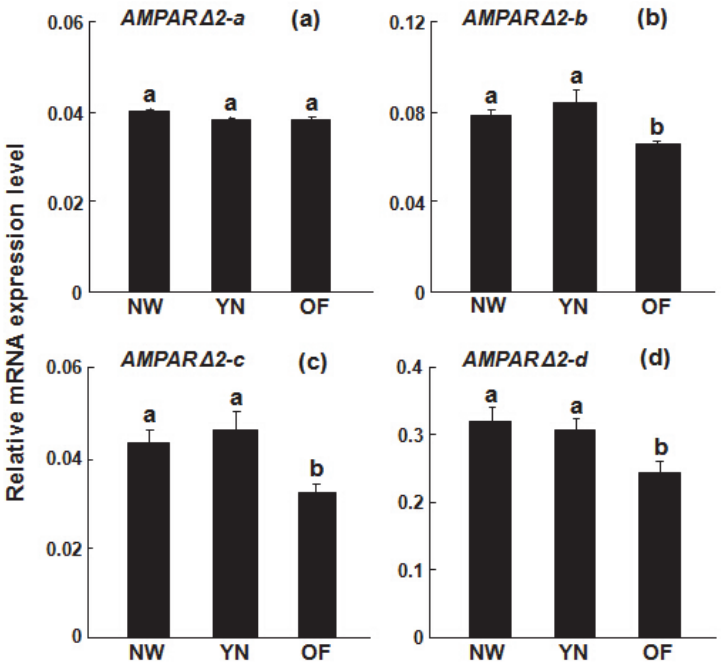

Figure 2. Gene expression levels of AMPA receptor genes relative to a reference gene GADPH-1 in the three groups. a-d. The relative expression level of AMPAR Δ2-a-d. Other details as in Figure 1.

The relative expression level of mGluR7 gene in OF was significantly higher than in NW (Df=2, F=3.41, P < 0.05, Fig 3c). There were no significant differences among NW, YN, and OF for the relative expression level of mGluR1 and mGluR4 gene (Df=2, F=1.50, P > 0.05, Fig 3a; Df=2, F=1.06, P > 0.05, Fig 3b).

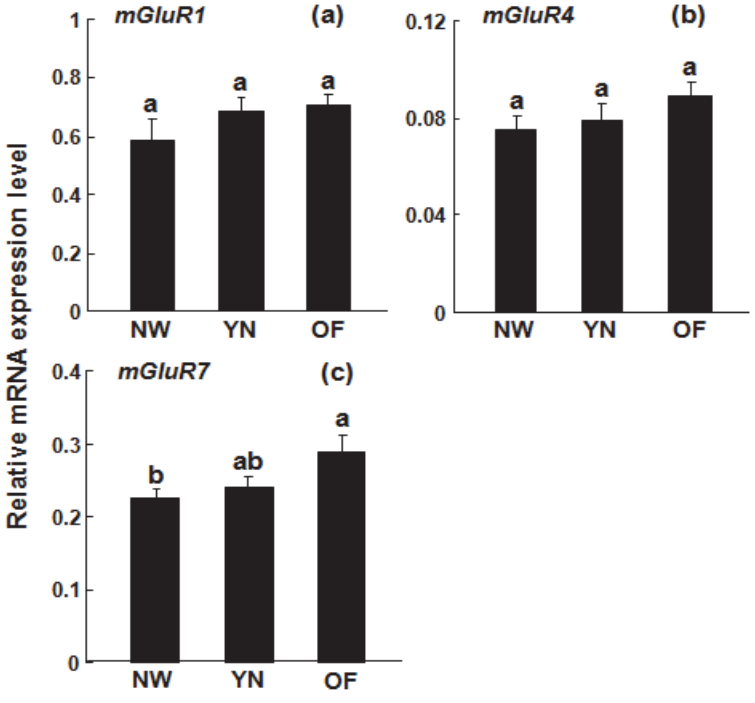

Figure 3. Gene expression levels of mGluR genes relative to a reference gene GADPH-1 in the three groups. a-c. The relative expression level of mGluR1, mGluR4 and mGluR7. Other details as in Figure 1.

Expression levels of serotonin receptor genes

As shown in Fig 4a, the relative expression level of the 5-HT1 gene showed no significant difference between YN and OF (Df=2, F=10.44, P > 0.05), but was higher in YN and OF than in NW (Df=2, F=10.44, P < 0.001). For the other three 5-HT genes, 5-HT2α, 5-HT2β and 5-HT7, the relative expression level were not significantly different among NW, YN, and OF (Df=2, F=0.17, P > 0.05, Fig 4b; Df=2, F=1.23, P > 0.05, Fig 4c; Df=2, F=0.24, P > 0.05, Fig 4d).

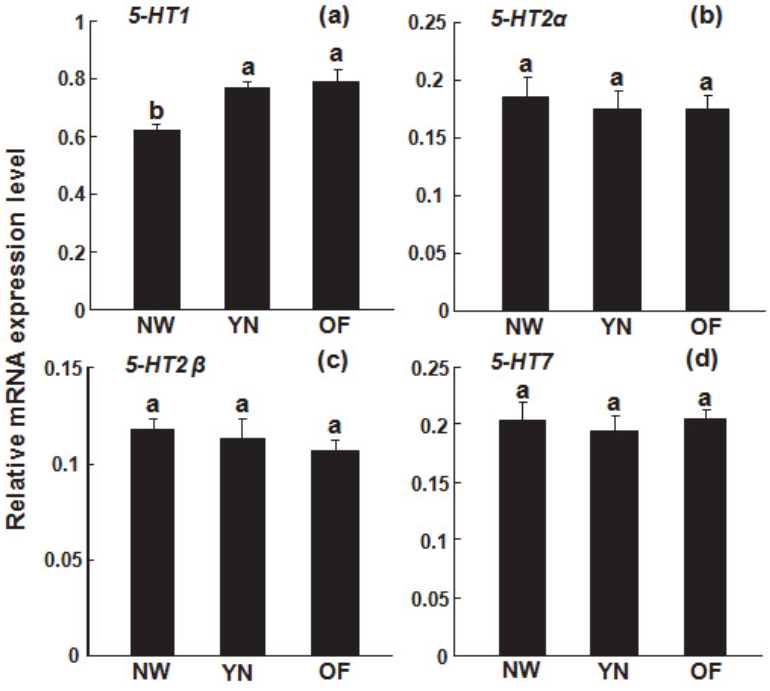

Figure 4. Gene expression levels of 5-HT receptor genes relative to a reference gene GADPH-1 in the three groups. a-d. The relative expression level of the 5-HT1,5-HT2α, 5-HT2β and 5-HT7. Other details as in Figure 1.

Discussion

Glutamate is considered to be the major excitatory neurotransmitter in vertebrate brain regulating learning and memory, but also plays crucial roles in cell differentiation and synapse formation during development of the nervous system (Danbolt, 2001). N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors which can bind to an agonist NMDA regulating synaptic plasticity also in learning and memory (Milner et al., 1998). It has been demonstrated that disruption of NMDARs in the hippocampus leads to blockade of synaptic plasticity and memory malfunction (reviewed by Morris et al., 1991; Rawlins, 1996). Recently, molecular genetic tools provided evidence that the NMDARs are involved in learning and memory in Drosophila (Xia et al., 2005). The localization of the expression sites at the mRNA and the protein levels indicated that the NMDAR1 is expressed throughout the brain, in neurons and in glial cells of the honeybee (Zannat et al., 2006). However, in contrast to vertebrates, the involvement of NMDARs in brain functions in insects is also poorly understood. In our present study, the results showed that when compared to YN and OF, the relative expression levels of NMDAR genes in NW showed higher levels. Besides, YN has a higher expression level of NMDAR2 gene than OF. This suggests that the NMDAR genes are probably involved in the maturation of brain functions of honeybee at different age.

The ability to change behavior likely depends on the selective strengthening and weakening of brain synapses. AMPA-type glutamate receptors (AMPARs) mediate a majority of excitatory synaptic transmission in the brain. A change in AMPAR-mediated transmission underlies several developmental and adult forms of synaptic plasticity (Bliss & Collingridge, 1993; Linden & Connor, 1995; Nicoll & Malenka, 1995; Cline et al., 1996; Bear, 1999) that may play important roles in learning and memory (Martin et al., 2000). While AMPAR trafficking is likely not the only molecular mechanism for behavioural plasticity, a number of studies suggest that monitoring and manipulating AMPAR trafficking is an attractive approach to identify synapses that undergo experience-dependent changes to modify behavior (reviewed by Kessels & Malinow, 2009). Our study showed that all three homologous genes of AMPAR Δ2 have higher relative expression in both NW and YN, when compared with OF. It may suggest that low expression of AMPAR Δ2 genes regulate specific brain functions that facilitate foraging in honeybees.

In vivo, metabotropic glutamate receptor antagonists have been shown to block and agonists to facilitate, induction and maintenance of LTP. As demonstrated in behavioural investigations, mGluRs apparently play an important part in hippocampus-dependent learning paradigms. As numbers of group III metabotropic glutamate receptors, age-dependent changes in the expression of mGluR4 and mGluR7 were studied by Simonyi et al. (2000). In our study, the relative expression level of mGluR7 gene in OF was significantly higher than in NW, while the relative expression level of mGluR4 gene was not significantly different among NW, YN, and OF. mGluR7 is expressed in brain regions implicated in emotional learning and working memory. Previous behavioural experiments indicated contributions of mGluR7 to various complex behaviors (Holscher et al. 2004; Callaerts-Vegh et al., 2006). Therefore, it is possible that mGluR7 is also related to learning and working memory involved in division of labor of the honeybee.

Using both invertebrates and mammals, recent studies have revealed that endogenous 5-HT modulates plasticity processes, including learning and memory (Meneses, 1999; Barbas et al., 2003, 2005; Schmitt et al., 2006). It has been shown that pharmacological manipulation of 5-HT1-7 receptors or 5-HT re-uptake sites might modulate memory consolidation, which is consistent with the emerging notion that 5-HT plays a key role in memory formation (reviewed by Meneses, 2002; Meneses, 2007). In our study, our homology search suggested that the orthologous genes of 5-HT1, 5-HT2 and 5-HT7 receptor genes are present in the honeybee. QRT-PCR results showed that the relative expression level of 5-HT1 gene was not significantly different between YN and OF, but was higher in both YN and OF when compared with NW. However, for the 5-HT2 and 5-HT7 genes, the relative expression level was not significantly different among NW, YN, and OF. These results indicated that 5-HT1 receptor may play a more important role in age-dependent division of labor of honeybee than other receptors.

As stated before, these candidate genes are the orthologous genes of glutamate and serotonin receptor genes, which are related to learning and memory in other organisms. However, not all of them showed significantly different expression among the NW, YN, and OF, although behavioural development in honeybees is thought to reflect differences in learning and memory. This may be the case for several reasons. As we know, although many pathways of gene expression regulation are conserved among species, species specificity still exists, e.g. juvenile hormone affected the expression of vitellogenin and life span in opposite directions in Drosophila and A. mellifera (Corona et al., 2007). Some specific single genes like vitellogenin even affect multiple physiological processes (Nelson et al., 2007). That is to say, some of these genes reported to be related to learning and memory in other organisms may have weak or even no involvement in the learning and memory of honeybees. On the other hand, before foraging outside, young honeybees (nurses) will have performed a variety of tasks in the hive such as building comb, receiving nectar from incoming foragers and storing it in combs, guarding the nest entrance, or removing corpses from the nest (Fahrbach & Robinson, 1995). In addition, young workers will fly around outside the hive in order to gradually become familiar with the surrounding environment at the age of about 4-14 days (Chen, 2001). As a result of this, the development in learning and memory capacity of young nurses may have started well before they were captured or our experiment as pollen foragers.

Acknowledgment

We thank Dr. Ying WANG, Dr. Aung Si and Dr. Zi-Long WANG for helpful suggestions that improved the manuscript, Fei ZHANG and Zhen-Xiu ZENG for help with beekeeping, Run SHEN for help with dissection of samples. This work was supported by the Earmarked Fund for the National Natural Science Foundation of China (No.31260524) and the China Agricultural Research System (No.CARS-45-KXJ12).

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