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Promoter analysis of the mushroom body-preferential genes of the honeybee

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Abstract

Mushroom bodies (MBs) are a higher center of the insect brain, and comprise interneurons called the Kenyon cells (KCs). There are four KC subtypes (class I large-, middle-, small-, and class II KCs) in the honeybee (*Apis mellifera* L.) MBs. Recent study indicated that parasitoidism, but not sociality, is associated with the evolution of the elaborate MBs in Hymenopteran insect brains. How each KC subtype contributes to the honeybee social behaviors, however, remains largely unknown. We have so far identified many genes expressed in a KC subtype-preferential manner in the honeybee brain. We expect that analyses of the function and regulatory mechanisms of genes expressed in a KC subtype-preferential manner may contribute to our better understanding of the molecular and neural bases underlying the honeybee social behaviors. In the present study, we aimed to clarify regulatory mechanism(s) of the KC subtype-preferential gene expression in the honeybee brain.

We previously used cDNA microarray to comprehensively search for candidate genes expressed preferentially in the honeybee MBs. In the present study, we focused on three genes, *phospholipase C epsilon* (*PLCe*), *Synaptotgmin 14* (*Syt14*), and *discs large 5* (*dlg5*), whose expression seemed highly enriched in the honeybee MBs, among the candidate genes identified. Quantitative RT-PCR and *in situ* hybridization revealed that *PLCe* is expressed almost selectively in all KC subtypes, while *Syt14* and *dlg5* are expressed almost selectively in the lKCs in the honeybee brain, suggesting that these three genes are most appropriate for the analysis of KC subtype-selective promoters. We used electroporation to introduce and express reporter genes, in which *gfp* is ligated downstream of the upstream regions of the above three genes, in the honeybee brain. So far, basic but not MB-selective promoter activities were detected in the genomic regions around the transcription start site of each gene.

Introduction; The mushroom body (MB) of the honeybee brain

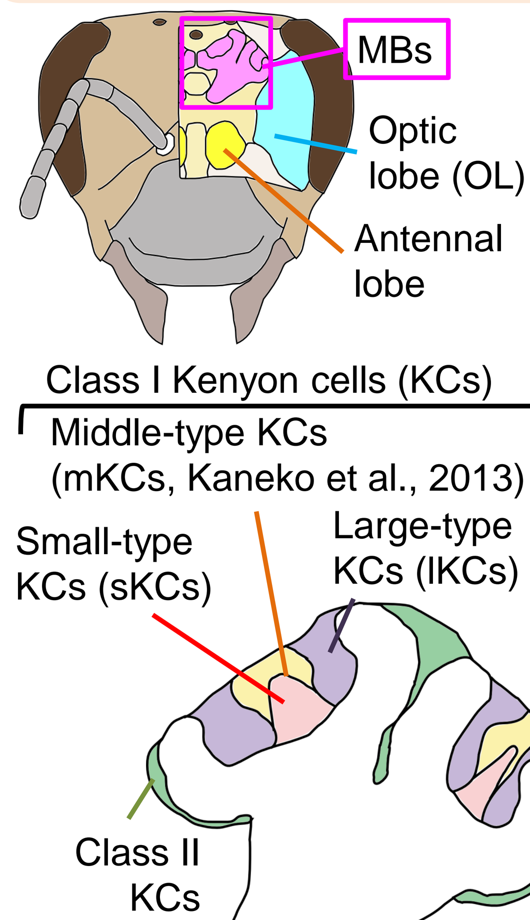

Fig. 1 The MBs of the honeybee brain

The MBs of the honeybee brain

- MBs are a higher center of the insect brain.
- The MB structure is more complex in Aculeata Hymenopteran insects.
- Complexity of the MB dendrites increases with foraging experiences (Farris et al., 2001).

→These suggest important roles of the honeybee MBs in regulating social behaviors.

KC of the honeybee MBs

- Both the sKCs and mKCs are active in forager brains (Kiya et al., 2007).
- Each KC subtypes have distinct gene expression profiles.

→ The functions and transcriptional regulation of each KC subtype remain unknown.

We expect analyses of the function and transcriptional regulation of each KC subtype could contribute our better understanding of the neural bases of the honeybee social behaviors and their evolution.

[Background] We previously performed a cDNA microarray analysis to comprehensively identify genes that are expressed in a MB-preferential manner in the honeybee brain.
[Present study] We focused on three genes: *Phospholipase C epsilon* (*PLCe*), *synaptotagmin 14* (*Syt14*) and *discs large 5* (*dlg5*), whose expression was most highly enriched in the MBs of the honeybee brain, and performed detailed expression analysis (Exp. 1).
In addition, we analyzed promoter activities of these genes (Exp. 2).

Research strategy
(1) Identification of genes expressed highly selectively in the MBs
(2) Identification of promoters regulating the MB-selective gene expression
(3) Loss of promoters in (2) and detection of behavioral change

Experiment 1 Quantitative reverse-transcription-PCR (qRT-PCR) and *in situ* hybridization (ISH) of the MB-selective genes

[Purpose] To confirmed the MB-preferential expression of the above three genes, we performed qRT-PCR and ISH.
[Methods] qRT-PCR; Expression of each gene was normalized with that of *ribosomal protein 49* (*rp49*). **ISH**; Probes were designed based on the coding sequences of each gene.

[Results]

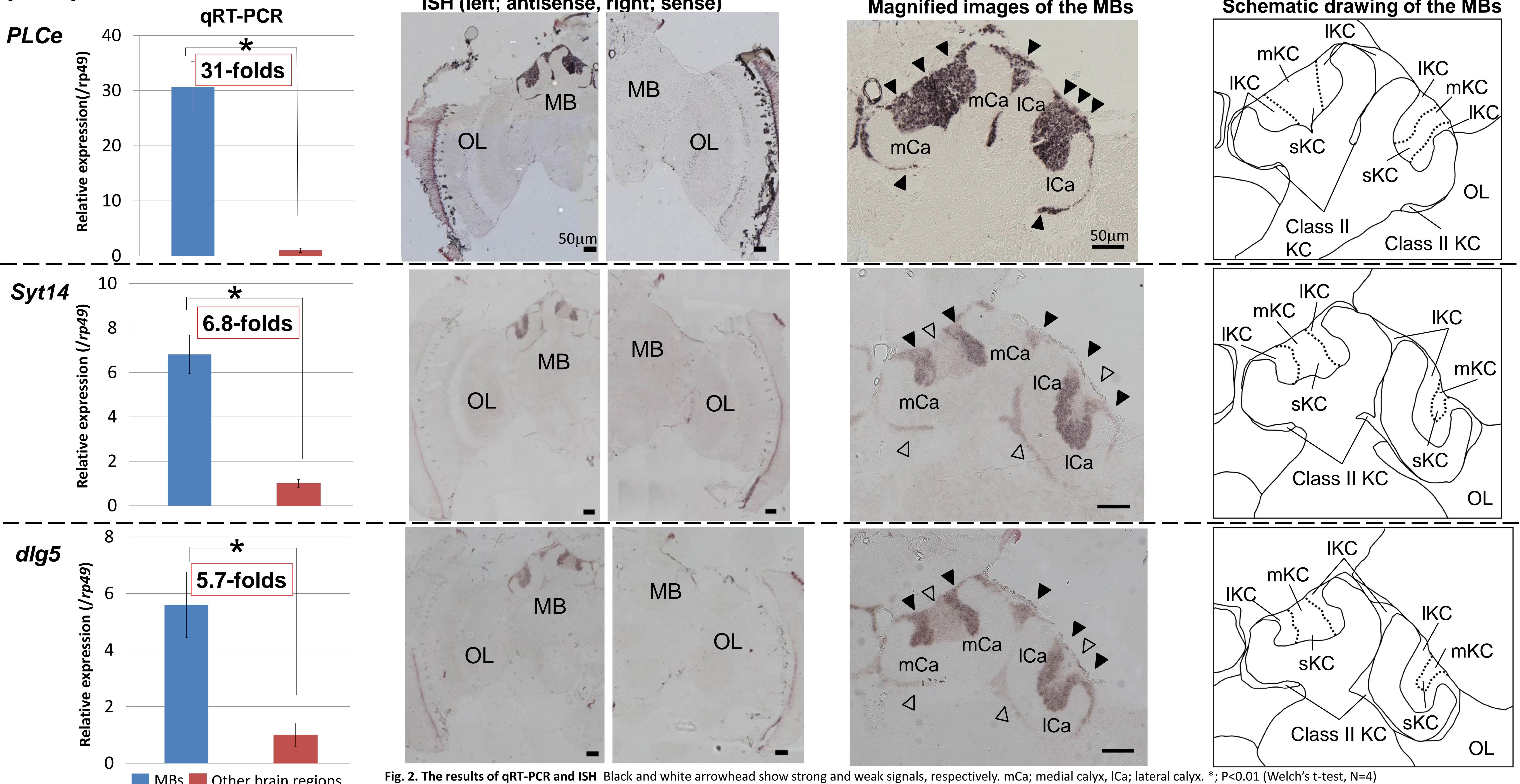

Fig. 2. The results of qRT-PCR and ISH Black and white arrowhead show strong and weak signals, respectively. mCa; medial calyx, lCa; lateral calyx. *; P<0.01 (Welch's t-test, N=4)

1. The expression of *PLCe*, *Syt14*, and *dlg5* in the MBs were 31-, 6.8- and 5.7-folds higher than those in the other brain regions, respectively. 2. *PLCe* was preferentially in all KC subtypes, whereas *Syt14* and *dlg5* were expressed preferentially in the lKCs.
[Discussion] PLCe has domains involved in hydrolyzing inositol phosphate . Syt14 is a subtype of synaptotagmin. *dlg5* is involved in polarization of neuronal precursors in fruit fly (Aranjuez et al., 2012). MB-selective expression of each gene suggests that the functions of molecular processes, in which each gene is involved: Ca^{2+}-signaling for *PLCe*, synaptic transmission for *Syt14* and maintenance of neural polarity for *dlg5*, are enhanced in the entire MBs (*PLCe*), or lKCs (*Syt14* and *dlg5*).

Experiment 2 Detection of promoter activity of the MB-selective genes

[Purpose] To identify promoters that regulate MB-preferential (*PLCe*) or lKC-preferential (*Syt14* and *dlg5*) expression of the above three genes in the honeybee brain, we analyzed expression of reporter genes introduced in the honeybee brain using *in vivo* electroporation.
[Methods] Reporter genes, in which *egfp* was ligated with the upstream regions of the above three genes (Fig. 3), were introduced into the brain of the worker bees by *in vivo* electroporation (Kunieda and Kubo, 2004, Fig. 4). RNAs extracted from the honeybee brain after electroporation was detected by RT-PCR followed by electrophoresis.

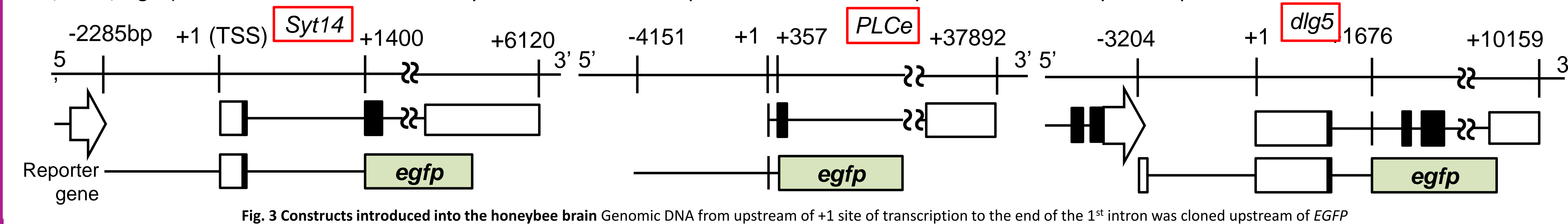

Fig. 3 Constructs introduced into the honeybee brain Genomic DNA from upstream of +1 site of transcription to the end of the 1st intron was cloned upstream of *EGFP*

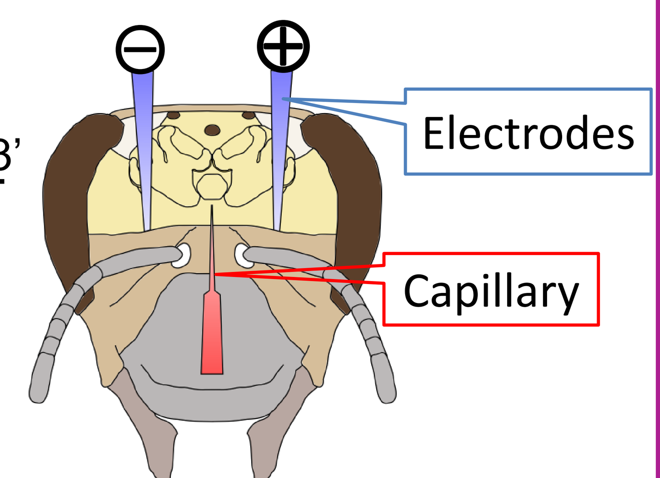

Fig. 4 ***in vivo*** **electroporation**
Constructs were injected by capillary over the brain and voltage was applied.

[Results]

	Syt14				*PLCe*				*dlg5*			
	MB		Other		MB		Other		MB		Other	
RT	+	-	+	-	+	-	+	-	+	-	+	-
	*		*		*		*		*		*	

Fig. 5 Detection of promoter activity
RNA showed bands stronger when reverse-transcribed (RT+) than not done (RT-) in whole brain (asterisks).

Bands corresponding to the RNAs for the reporter genes (RT+) were detected for all above three genes, and their intensities were stronger than those of the control experiments (RT-). However, there was no significant difference in the band intensities between the MBs and the other brain regions.
[Discussion] These results suggest that the upstream regions that we use in the present experiments contain minimal promoters of the above three genes. To detect MB- or lKC-preferential expression, the detection method of the transcripts needs to be improved.

Conclusion

(1) We Identified 3 genes expressed highly selectively in the MBs
(2) Promoter activity of genomic DNA around the TSS of genes was detected in whole brain

Future Plans

(1) Identification of genomic region regulating the MB- selective gene expression by qRT-PCR
(2) Knockdown of the expression of the above three genes using CRISPR/Cas9 system.