学位論文 (要約)

Expression analysis of genes expressed preferentially in the honeybee mushroom bodies during metamorphosis and functional analysis of PLC in learning and memory.

(ミツバチ脳キノコ体で選択的に発現する遺伝子の変態期での発現と PLC の記憶学習における機能の解析)

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Abstract

The European honeybee (*Apis mellifera* L.) is a social insect, and its larvae cannot live alone, while the adult workers are engaged in various tasks to maintain colony activity. It is unknown how the brain structure and function underlying the honeybee social behaviors develop during its ontogeny. The mushroom bodies (MBs) are a higher order center of the insect brain and the honeybee MBs compromise four Kenyon cell (KC) subtypes (the class I large- [l], middle- [m], small- [s], and class-II KCs) that have distinct gene expression profiles. On the other hand, the larval honeybee MBs are consisted of only class-II KCs. However, it was unknown whether the larval and adult MBs are different in terms of gene expression. In my master course studies, I showed that *Phospholipase C epsilon* (*PLCe*), *Synaptotagmin 14* (*Syt14*), and *discs large 5* (*dlg5*) are expressed almost selectively in the MBs of the adult worker honeybee brains. Based on these results, in my doctoral course study, I first analyzed the expression of these genes in the larval and pupal brains.

In Chapter I, I used quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to examine the expression levels of *PLCe*, *Syt14*, and *dlg5* in the brains of the larval and pupal honeybees. Expression of *PLCe*, *Syt14*, and *dlg5*

increased from the larval to late pupal stage, suggesting that these genes play important roles in the later pupal and adult stages. *In situ* hybridization analysis revealed that *PLCe* was preferentially expressed in the class-II KCs in the late larval brain while the preferential expression of *PLCe* was detected in both of the class-II and differentiating lKCs in the early pupal brain, indicating that *PLCe* is expressed in all KC subtypes irrespective of the developmental stages. In contrast, while expression of *Syt14* and *dlg5* in the late larval brain was very low, *Syt14* and *dlg5* started to be selectively expressed in the lKCs at the late pupal stage, indicating that the lKCs-selective expressions of *Syt14* and *dlg5* are acquired during midpupal to adult stages. These results indicated that the larval and adult honeybee MBs have distinct gene expression profiles.

In Chapter II, I analyzed the role of PLC in learning and memory in the adult worker honeybee. For this, I planned to use pharmacologic inhibitors targeting PLC. Quantitative RT-PCR revealed that *PLCe* and the genes for other two PLC subtypes are expressed more strongly in the MBs than in other brain regions, proboscises and antennae, suggesting that PLC subtypes act mainly in the MBs in the worker brains. Furthermore, biochemical analysis revealed that two pharmacologic agents, edelfosine and neomycin, which are used as common PLC inhibitors in various animals, effectively inhibit PLC activity in the honeybee brain homogenate. Then I analyzed

possible involvement of PLC in olfactory associative learning of the honeybee using these two inhibitors. When the honeybees were injected with edelfosine or neomycin into their heads, memory formation in training was declined in comparison to the mock-injected control groups. On the other hand, memory maintenance at 1 h and 24 h after training was not different from the control groups, suggesting that PLC is involved in the early stage of memory formation but not memory maintenance till 24 h in the adult worker honeybees.

Although preceding studies showed that calcium/calmodulin-dependent protein kinase II (CaMKII) is involved in the maintenance of odor memory in the honeybee, my study first showed that PLC, an upstream factor in the calcium signaling, is involved in acquisition of memory in the honeybee. Considering that *PLCe* is also expressed in larval class-II KCs as well as adult MBs, it is possible that PLC also function in learning and memory even in the larval stages.

In summary, in my doctoral course study, I first indicated that larval and adult honeybee MBs have distinct gene expression profiles, and PLC functions in learning and memory in adult worker honeybees. I expect that these findings could contribute to give insights into the molecular and neural bases underlying honeybee social behaviors.

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Chapter II
Analysis of the function of PLC in olfactory learning and memory in the honeybee

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Abbreviations

AL	antennal lobe
CaMKII	calcium/calmodulin-dependent protein kinase II
DAG	diacylglycerol
dlg5	discs large 5
GPCR	G protein-coupled receptor
IP_3	inositol 1, 4, 5-trisphosphate
IP_3P	type I IP_3 5-phosphatase
IP_3R	IP_3 receptor
KC	Kenyon cell
lKC	large-type Kenyon cell
MB	mushroom body
mKC	middle-type Kenyon cell
Nb	neuroblast
OL	optic lobe
PKC	protein kinase C
PLC	phospholipase C
PLCe	phospholipase C epsilon
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RpL32	ribosomal protein L32
RTK	receptor tyrosine kinase
ryr	ryanodine receptor
sKC	small-type Kenyon cell
Syt14	Synaptotagmin 14

General Introduction

The European honeybee (*Apis mellifera* L.) is a social insect that shows highly integrated behavioral allocation in the colony [Winston 1986]. For example, honeybee reproduction is allocated to males and specialized females called “queens”, and the other female bees are engaged in labors to maintain colony activity and are called “workers”. This allocation of reproduction in females is called caste differentiation. Worker bees show age polyethism; young bees are mainly engaged in in-hive tasks such as taking care of the brood (“nurse bees”) while older bees collect food outside of the hive (“foragers”). Due to caste differentiation and the worker bees’ dedication to supporting reproductive individuals at the cost of their own reproduction, the honeybee colony is considered analogous to a multicellular organism: queens and males are the germ lines, while workers are the soma [Moritz and Fuchs 1997; Amdam and Seehuus 2006]. Based on this analogy, the honeybee colony is sometimes referred to as a “superorganism” [Moritz and Fuchs 1997; Amdam and Seehuus 2006]. Tautz and Heilmann wrote that the honeybee society also shares some features with mammals: nurse bees make and give “milk” to broods like the glands of mammals; in addition, the honeybee exhibits highly developed learning abilities that surpass those of some vertebrates [Tautz and Heilmann 2007]. This highly organized society was acquired in the evolution of Hymenopteran insects.

On the other hand, the evolution of sociality in the honeybee is associated with complete dependency of the larvae on workers. Honeybee larvae live at the bottom of honeycombs in the hive and must be fed by nurse bees, whereas the larvae of other holometabolous insects such as the fruit fly and butterfly search for their own food.

Thus, honeybee behavior is associated with its development as well as social evolution. How brain functions underlying the complex behaviors of the adult honeybee develop, however, remains unknown.

I began my doctoral course studies with the expectation that analysis of the development of the higher brain function of the honeybee would provide insight into the social evolution of Hymenoptera. This knowledge might also provide information on some of the evolutionary processes of the brain functions and sociality of higher animals.

The insect brain comprises the antennal lobes (ALs), optic lobes (OLs), and mushroom bodies (MBs). The MBs are the integration center for sensory information transmitted from primary sensory centers such as the ALs (olfactory centers) and OLs (visual centers; see Fig. 1A for an overview of the brain of the adult honeybee). The MBs comprise intrinsic neurons called Kenyon cells (KCs), which project their dendrites into the calyces and axons into the lobes via peduncles, respectively (Fig. 1B) [Strausfeld 2002]. The KCs of the honeybee are categorized into four subtypes (class-I large- [l], middle- [m], small-type [s], and, class-II KCs) based on the size and location of their somata (Fig. 1C) [Strausfeld 2002; Kaneko et al. 2013; Kaneko et al. 2016].

The MBs are involved in learning and memory [Davis 2011; Menzel 2012]. For example, *rutabaga*, *DC0*, and *dunce* (encoding type I calcium/calmodulin-stimulated adenylate cyclase, a regulatory subunit of protein kinase A, and cAMP phosphodiesterase, respectively) are critically required for learning and memory in *Drosophila melanogaster* and are preferentially expressed in the MBs [McGuire et al. 2005], and previous reports demonstrated that cAMP signaling in the MBs is important for *Drosophila* learning and memory [McGuire et al. 2005]. Involvement of the MBs in

honeybee learning and memory has also been analyzed [Hammer and Menzel 1998; Lozano et al. 2001; Szyszka et al. 2008]. Analysis of the expression of genes and proteins involved in cAMP signaling revealed, e.g., that expression of a gene encoding a catalytic subunit of protein kinase A is localized in the MBs [Eisenhardt et al. 2001], a protein corresponding to an adenylate cyclase subtype is expressed preferentially in the MBs as well as the ALs [Balfanz et al. 2012], and phosphorylated cAMP response element binding protein is enriched in the sKCs [Gehring et al. 2016]. Honeybee MBs are also thought to be involved in complex tasks, such as working memory, orientation, and foraging flight [Menzel 2009; Menzel 2012; Kiya et al. 2007; Lutz and Robinson 2013].

The honeybee KC subtypes have distinct gene expression profiles, suggesting functional diversification among subtypes [Kaneko et al. 2016]. For example, genes involved in calcium signaling, such as *inositol 1, 4, 5-trisphosphate receptor* (*IP_3R*), *type I IP_3 5-phosphatase* (*IP_3P*), *calcium/calmodulin-dependent protein kinase II* (*CaMKII*), *ryanodine receptor* (*ryr*), and *reticulocalbin* [Kamikouchi et al. 1998; Kamikouchi et al. 2000; Takeuchi et al. 2002; Uno et al. 2013], are expressed in an lKC-enriched manner, suggesting that calcium signaling enhancement in the lKCs contributes to learning and memory in the honeybee [Kaneko et al. 2016]. On the other hand, some genes involved in ecdysone signaling, such as *ecdysone receptor*, *hormonal receptor-like 38* (*HR38*), and *E74,* are enriched in the sKCs [Paul et al. 2005; Yamazaki et al. 2006; Takeuchi et al. 2007]. Because *HR38* expression is increased in foragers, sKCs are suggested to be involved in the division of labor [Yamazaki et al. 2006; Kaneko et al. 2016]. Moreover, the gene termed *kakusei*, whose expression reflects neural activity, is expressed selectively in the sKCs and part of the mKCs in the forager

brain, suggesting that these KCs are involved in sensory information processing during foraging [Kiya et al. 2007; Kaneko et al. 2013; Kaneko et al. 2016].

The knowledge of genes selectively expressed in each KC subtype allows for analysis of the development of the KC subtypes over the life of the honeybee using these genes as markers for each KC subtype. Therefore, in my doctoral studies, I first examined the expression of genes in honeybee brains during metamorphosis (larva and pupa) that seemed to be the most appropriate as marker genes for the KC subtypes and compared the expression levels of these genes with those in the adult brain. Among them, *Phospholipase C epsilon* (*PLCe*) was preferentially expressed in the MBs irrespective of developmental stages of the honeybee, suggesting that this gene regulate unique function of the MBs from the larval stages. Thus, in Chapter II, to gain insight into function of PLCe in behavior of the honeybee, I analyzed involvement of PLC in learning and memory in the adult honeybee.

Chapter I

Analysis of the expression of the adult MBs-preferential genes in the larval and pupal honeybee

Introduction

In the honeybee brain, Kenyon cells (KCs) are sequentially generated through the cell division of neuroblasts (Nbs) and exist as different subtypes in the adult brain depending on when they are born during metamorphosis (Fig. 2A) [Farris et al. 1999]. The KCs that differentiate into the class-II subtype in the adult honeybee are generated during the larval and prepupal stages; future class-I KCs are derived from Nbs in the later stages and large- (l) and small-type (s) KCs are generated in the early and midpupal stages, respectively (Fig. 2A) [Farris et al. 1999]. Therefore, the mushroom bodies (MBs) of larval and adult honeybees have a different composition of KC subtypes; larval MBs contain only the KCs that differentiate into class-II KCs in the adult while adult MBs are equipped with class-I KCs as well as class-II KCs (compare Fig. 1C and 2B). In addition to the different organization of the KCs, the calyces are absent in larval MBs and are acquired and compartmentalized during metamorphosis [Farris et al. 1999; Farris et al. 2004]. The peduncle is also reported to be thin in the larva [Farris et al. 1999].

This increasing structural complexity from the larval to adult-stage MBs suggests that MB function also becomes more complex during honeybee development. Whether adult honeybee KC subtypes and larval KCs differ in terms of their gene expression, however, has remained unknown due to the lack of marker genes for the KC subtypes. It is possible that the larval KCs also express genes that are selectively expressed in each KC subtype in the adult MBs.

In a previous study in our laboratory, in order to gain insight into unique functions of the MBs in behavior of the adult honeybee, candidate genes which are

expressed in the adult MBs-selective manner were comprehensively searched using cDNA microarray and *in situ* hybridization [Paul 2004]. The study identified a total of 18 genes, including *Phospholipase C epsilon* (*PLCe*), *Synaptotagmin 14* (*Syt14*), and *discs large 5* (*dlg5*), as candidate genes that are preferentially expressed in the MBs in the adult honeybee brain. However, cDNA fragments identified there were sometimes annotated on intergenic regions in the honeybee genome database and/or small in length [Paul 2004]. Thus, in my master course study, I focused on three genes, *PLCe*, *Syt14*, and *dlg5*, which appeared to be expressed more selectively in the MBs than any other candidate genes, for the further analyses. To confirm the previous results, I used quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and *in situ* hybridization with probes corresponding to longer coding sequences. As a result, qRT-PCR analysis showed that all of three genes were expressed in the MBs higher than in the other brain regions. *In situ* hybridization analysis revealed that while *PLCe* is expressed preferentially in all KC subtypes, *Syt14* and *dlg5* are expressed preferentially in the lKCs in the adult honeybee brain (Fig. 3). Interestingly, MB-selective expression of these genes is not reported in other insect brains, such as the fruit fly [Kobayashi et al. 2006], suggesting that the MB-selective expression of these genes is unique to some Hymenopteran insects, including the honeybee. So I considered, if the MB-selective expression of these genes is not detected in the larval brain, the expression of these genes in the MBs could be related to behaviors unique to the adult honeybee. Therefore, in Chapter I, I used these genes to examine whether the larval class-II KCs have distinct molecular characteristics from those of adult MBs and if so, when the lKCs acquire the selective expression of *Syt14* and *dlg5* during pupal development.

Materials and Methods

Animals

The European honeybee (*Apis mellifera* L.) colonies were purchased from a local distributor and maintained at the University of Tokyo.

Subcloning of PLCe, Syt14, dlg5, Mblk-1, and RpL32, and synthesis of RNA probes

During my master course studies, I subcloned *PLCe*, *Syt14*, *dlg5* (for qRT-PCR and *in situ* hybridization), and *Ribosomal protein L32* (*RpL32*) (for qRT-PCR). I additionally subcloned *Actin related protein 1* (*Arp1*), *RpL32,* and *Mushroom body/large-type Kenyon cell-preferential protein-1* (*Mblk-1*) to prepare standard samples for qRT-PCR and RNA probes. cDNA from the pupal brain at day 9 after pupation (P9) stage was used as templates and PCR was performed with TaKaRa ExTaq and gene specific primers: *Arp1* (NM_001185146.1), 5'-TCCCCGAATCCCGAAAG-3' and 5'-CGGAGGAACCAAAGGACAA-3'; *RpL32* (XM_006564315.1), 5'-TCGTCACCAGAGTGATCGTT-3' and 5'- CGTAACCTTGCACTGGCATT-3'; and *Mblk-1* (AB047034.1), 5'- AATTTCAAATTTCGCCTCGA-3' and 5'-TTTTGGAACAACCCCACCAT-3'. The PCR products were cloned into a pGEM-T Easy vector (Promega). For preparation of RNA probes, after confirming the sequences of the cloned DNAs, PCR was again performed using TaKaRa ExTaq with M13 forward and reverse primers to obtain templates for *in vitro* transcription. Sense and antisense RNA probes were synthesized using a DIG labeling Kit (Roche) according to the manufacturer's instructions [Yamazaki et al. 2006; Kaneko et al. 2010; Kaneko et al. 2013].

Quantitative RT-PCR analysis

The stages of the developing honeybee were determined as described previously [Ganeshina et al. 2000; Moda et al. 2013]. Brains from worker honeybees at the larval fifth-instar feeding stage (L5F), and pupal day 2, 3, 7, and 9 days after puparium formation (P2, P3, P7, and P9, respectively) were dissected out under a binocular microscope and homogenized in TRIzol LS reagent (Ambion). Five L5F, three P2, two P3, and one each of P7 and P9 bees were used as a lot, respectively, and three lots of samples were analyzed for each stage.

Total RNA was extracted with TRIzol LS reagent (Ambion) and the same amount of RNA from each sample was reverse-transcribed using a PrimeScript RT reagent Kit with gDNA eraser (Perfect Real Time, TaKaRa). Quantitative RT-PCR was performed with LightCycler 480 Instrument II (Roche) and SYBR premix Ex Taq II (Tli RNase H Plus, TaKaRa) according to the manufacturer's instructions [Ugajin et al 2013]. The following primers were used: *PLCe*, 5'-GTTTCGCCAATCGAAAAACG-3' and 5-GATAGAGGTCAATGGAGCCA-3'; *Syt14*, 5'-TCCACCGCCGGATACTTTAAC-3' and 5'-CAATGCGTGGTAGAAGTGGG-3'; *dlg5*, 5'-GTGCTCGAATCTAGGCATCTC- 3' and 5'-CGTTGATCTCAGACAGGCAA-3'; *Mblk-1*, 5'-CAACACCAAATACGACCCAAAAC-3' and 5'-GACAACAGCGGCTTCAAC-3'; *RpL32*, 5'-AAAGAGAAACTGGCGTAAACC-3' and 5'-CTCGTCATATGTTGCCAACTG-3'; *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*, XM_393605.5), 5'-GATGCACCCATGTTTGTTTG-3' and 5'-TTTGCAGAAGGTGCATCAAC-3'. Although the expression of *RpL32*, *gapdh,* and

Arp1 as candidate reference genes was quantified, their expression levels changed significantly throughout metamorphosis. Thus, expression of transcripts was normalized by those at P9 but not by those of reference genes. Primers and standard samples for quantification of *Mblk-1* and *gapdh* were provided as a gift (see acknowledgements). The Kruskal-Wallis test and Steel-Dwass test were used for statistical analysis.

In situ hybridization analysis

Experimental procedures were essentially the same as described in previous studies [Kaneko et al. 2010; Kaneko et al. 2013; Ugajin et al. 2013].

Two brains from L5F and P3 worker bees were used to examine each gene. The brains were embedded in Tissue-Tek OCT compound (Sakura Finetek Japan) and frozen on dry ice. Frozen coronal sections of the brain (10 μm thick) was picked up onto slides and fixed in 4% paraformaldehyde in 0.1M phosphate buffer (4% PFA-PB) (pH 7.4) at 4℃ overnight, treated with 10 μg/ml proteinase K for 15 min, 4% PFA-PB for another 15 min, 0.2M HCl for 10 min, and 0.25% acetic-anhydride in 0.1M tri-ethanolamine HCl for 10 min. The sections were washed with PB between each step. The tissue was dehydrated in ethanol solutions (from 70% to 100%, in 10% increments), and hybridized with RNA probes at 50℃ overnight (>12h). RNA probes were diluted in hybridization buffer (50% formamide, 10 mM Tris-HCl at pH 7.6, 200 μg/ml tRNA, 1 ×Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA), and heated at 85℃ for 5 min before use. To prevent the probes spreading unevenly and drying out, the slides were covered with parafilm sheets cut to the size of the slides. Sections were washed with 5×saline sodium citrate (SSC) and 50% formamide in 2× SSC for 1 h at 50℃, and treated with TNE (10 mM Tris-HCl at pH 7.5, 0.5M NaCl, 1

mM EDTA) for 10 min at 37℃. RNase A was then added to the TNE at 10 μg/ml and sections were treated for 15 min and washed for 10 min with fresh TNE at 37℃. The slides were then treated with 2×SSC and twice with 0.2×SSC for 20 min at 50℃. The hybridized RNA probes were detected using a DIG Nucleic Acid Detection Kit (Roche). Sections were treated with DIG buffer I (100 mM Tris-HCl pH 7.5, 150 mM NaCl) for 5 min, 1.5% blocking reagent for 1 h, and anti-DIG antibody conjugated with alkaline phosphatase (diluted 1:1000) for 30 min. The sections were then washed in DIG buffer I twice for 15 min each, treated with DIG buffer III (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.005% Tween 20) for 3 min and NBT/BCIP solution (1:50) was used for staining. The detection procedure was performed at room temperature. Staining lasted for 3 h (*PLCe*), 6 h (*RpL32*), and >12h (the other genes). Images were reconstructed and processed using GIMP2.8 software (http://www.gimp.org/).

Results

Quantification of the expression of PLCe, Syt14, and dlg5 in the developing honeybee brain

First, to examine when *PLCe*, *Syt14*, and, *dlg5* are expressed in the honeybee brain during metamorphosis, I quantified their expression levels in the larval and pupal brains. Analysis of *RpL32*, *gapdh* and *Arp1*, which have been used as reference genes in the honeybee [Reim et al. 2013; Ugajin et al. 2013; Uno et al. 2013], revealed significant changes in their expression during metamorphosis. The expression of *RpL32* decreased with the progression of metamorphosis (Fig. 4A), whereas *gapdh* expression increased in later stages (Fig. 4B). Change in the expression of *Arp1* was more complex (Fig. 4C). Thus, I normalized expression of *PLCe*, *Syt14*, and *dlg5* by expression level of each gene at stage P9 but not by expression levels of the above reference genes. The expression of *PLCe*, *Syt14*, and *dlg5* generally increased with development of the brain. Compared to the L5F brain, the P9 brain had ~16.1-, 8.97-, and, 3.81-fold higher expression of *PLCe*, *Syt14*, and, *dlg5*, respectively (Fig. 5A-C), suggesting that *PLCe*, *Syt14*, and *dlg5* mainly function in the later pupal or adult stages. I also analyzed the expression of *Mblk*-1, a gene preferentially expressed in the lKCs at the pupal stage [Takeuchi et al. 2001] and used as a reference gene for MB development. The expression of *Mblk-1* was not detected in L5F brain, peaked at P3, and declined to nearly half at P7 and P9 (Fig. 5D). These findings suggested that *Mblk-1* has a role in the early pupal stage. Although the expression changes during metamorphosis were significant for all genes ($P<0.05$, Kruskal-Wallis test), differences between adjacent stages were not statistically significant ($P>0.05$, Steel-Dwass test), possibly due to the

small sample sizes.

Analysis of the spatial expression of PLCe, Syt14, and dlg5 in the developing brain by in situ hybridization

Based on qRT-PCR analysis, I performed *in situ* hybridization to examine changes in the expression of *PLCe*, *Syt14*, and, *dlg5* in larval and pupal brains of worker bees. I selected larval and pupal stages based on the expectation that the L5F brain has more differentiated KCs than brains in earlier stages (Fig. 2A) and that KCs may regulate larval behavior. The P3 pupal stage was also chosen on the basis that lKCs are born till the P2 stage and are expected to start differentiating at around the P3 stage (Fig. 2A). First, I analyzed the expression pattern of *RpL32* in the L5F brain to clarify the location of cell somata. Significant staining was detected only when the antisense probe was used, leading me to conclude that the antisense probe reflected the expression of *RpL32* (Fig. 6A and B). Whole brains, including the MBs, optic lobe primordium (OLp) [Roat and Landim 2010], and antennal lobes (ALs) [Moda et al. 2013], were intensely stained. Each MB at this stage has two clusters of Nbs and two peduncles extending from the bottom of each Nb cluster [Farris et al. 1999]. Based on this report, I concluded that cells with more intense staining in the MBs (arrows in Fig. 6C) corresponded to the Nbs (Fig. 6D). Higher expression of *RpL32* in the Nbs might reflect higher protein synthesis activity in these cells.

Next, to determine the location of the lKC somata, I analyzed the expression of *Mblk-1* in the P3 pupal brain. The staining pattern differed between experiments using the antisense and sense probes (Fig. 7A and B), indicating that the antisense probe represented *Mblk-1* expression. Expression was detected throughout the brain,

especially in the MBs. At this stage, the MBs have two Nb clusters and KCs, which differentiate into the s-, l-, and class-II KCs in the adult brain. Earlier born KCs are pushed away by newly born KCs [Farris et al. 1999], and thus sKCs are located between the Nbs and lKCs, and class-II KCs are located at the edge of the calyces [Farris et al. 1999]. *Mblk-1* was expressed most strongly in the cells sandwiched between cells with lower expression (Fig. 7C). According to previous reports [Farris et al. 1999; Takeuchi et al. 2001], I concluded that the cells with higher *Mblk-1* expression were lKCs (Fig. 7D). The sKCs could not be discriminated based on morphologic observations in this study.

Next, I examined the expression of *PLCe*, *Syt14*, and, *dlg5* in the L5F and P3 brains. Examination of *PLCe* in the larval and pupal brains revealed different staining patterns depending on the antisense or sense strand of the probes (Fig. 8A-D), indicating that the antisense probe represented *PLCe* expression. At both stages, *PLCe* expression was highly preferentially localized in the MBs. In the L5F brains, *PLCe* was expressed in cells around the Nbs, which correspond to the class-II KCs in a previous report [Farris et al. 1999]. Thus, I concluded that this gene is expressed preferentially in the class-II KCs in the L5F brain (Fig. 8E and F). At the P3 stage, a strong signal was detected in the cells next to the Nbs and cells at the edge of the calyces, indicating that *PLCe* is expressed preferentially in both the class-II KCs and lKCs in the P3 brain (Fig. 8G and H).

In contrast to *PLCe*, no significant expression of *Syt14* was detected in the L5F brain (Fig. 9A, B, E and F), suggesting that *Syt14* is not highly expressed in the larval brain. The P3 brain showed staining only when the antisense probe was used (Fig. 9C and D), suggesting that this staining reflected *Syt14* expression. At this stage, the

staining intensity was moderate in the MBs and weak in the other brain regions. In the MBs, higher staining intensity was detected in the cells around the Nbs and cells at the edge of the calyces, indicating that the class-II KCs and lKCs selectively express *Syt14* (Fig. 9G and H).

As for *dlg5*, use of the antisense probe in the L5F brain produced a different pattern compared to that of the sense probe (Fig. 10A and B), indicating that the antisense probe detected *dlg5* expression. The staining was weak all over the brain, however, suggesting that *dlg5* is not significantly expressed in the larval brain. At the P3 stage, the MBs showed higher expression of *dlg5* than the other brain region (Fig. 10C and D). In the MBs, although some expression was detected in both the KCs and Nbs, cells at the tip of the calyces showed higher expression than the other cells (Fig. 10G). Because the outer halves of the lKCs are located in this region of the calyces (Fig. 10H), this result suggested that a population of the lKCs, which was born earlier than the remaining lKCs, selectively expresses *dlg5* at the P3 stage. The results of *in situ* hybridization including expression in the adult brain are summarized in Fig. 11.

Discussion

In this chapter, I analyzed the expression of genes which are expressed preferentially in the adult MBs/lKCs during metamorphosis. First, I used qRT-PCR to examine when each gene was expressed in the brain during metamorphosis of the honeybee. Expression of *PLCe*, *Syt14*, and *dlg5* was detected as early as the L5F stage, and increased with the progression of metamorphosis. I therefore performed *in situ* hybridization and identified the expression patterns of all the genes in the L5F and P3 brains. *PLCe* was preferentially expressed in the MBs, both in the larval and pupal brains. MB-selective expression of *Syt14* and *dlg5* was detected at the pupal stages, but not in the larval brain. The expression patterns of these two genes, however, differed from those in the adult brain. In the P3 brain, *Syt14* was expressed at a similar intensity in the class-II KCs and lKCs, whereas *dlg5* was selectively expressed in the outer halves of the lKCs.

Quantitative RT-PCR analysis revealed that expression of *PLCe*, *Syt14*, and, *dlg5* generally increased. This was in contrast to *Mblk-1*, whose expression was peaked at P3. *Mblk-1* is an ortholog of *Drosophila E93*, which is a transcription factor required for apoptosis of the larval salivary gland downstream of ecdysone [Lee et al. 2000]. Because the ecdysone titer increases at pupation, the increase in *Mblk-1* expression as early as the P2 pupal stage of the honeybee may also be downstream of ecdysone. *Mblk-1*, *Syt14,* and *dlg5* are expressed in the lKCs in the adult brain, and thus it is possible that *Syt14* and *dlg5* expression is regulated by *Mblk-1*, and that the increase in *Syt14* and *dlg5* expression in later pupal stages is affected by *Mblk-1* expression during the early pupal stages. I cannot exclude the possibility that *Broad-Complex* (*BR-C*),

which is another transcriptional factor expressed in the adult lKCs [Paul et al. 2006], affects the expression of *Syt14* and *dlg5*, because I did not analyze *BR-C* expression.

In situ hybridization of all the genes revealed distinct expression patterns in the larval and pupal brains. First, I analyzed expression of *RpL32* and *Mblk-1,* and identified cells with higher levels of these genes. High *RpL32* expression was detected in some cells in the OLp as well as in the MBs (Fig. 6A arrow in OLp). Although the cell types in the honeybee OLp have not been characterized in detail, it is reported that Nb clusters in the optic primordium of *Drosophila melanogaster* produce neurons that are organized into the OLs [Togane et al. 2012]. Because the Nbs are thought to actively synthesize proteins needed for cell division, cells with intense expression of *RpL32* in the OLp of the honeybee might correspond to the Nbs. *Mblk-1* expression was not only enriched in the MBs, it was also observed in some cell populations in the OLs and ALs (Fig. 7A arrows). *Mblk-1* expression outside of the MBs has not been reported [Takeuchi et al. 2001]. *Mblk-1* is suggested to be involved in neuronal remodeling based on the finding that a nematode homolog, *MBR-1,* functions in neurite pruning in the larval stage [Kage et al. 2005]. Given that neural remodeling occurs all over the brain during metamorphosis, it is plausible that *Mblk-1* regulates the morphology of some neurons in the OLs and ALs, as well as the lKCs. *Mblk-1* was also expressed in Nbs in the MBs, suggesting that *Mblk-1* functions before generation of the KCs. In fact, the activity of *Mblk-1* as a transcription factor is upregulated by the Ras/mitogen activated protein kinase pathway [Park et al. 2003], suggesting its role in the proliferation of Nbs.

PLCe was selectively expressed in the class-II KCs in the L5F brain. Its expression in the pupal brain was detected at a similar intensity in the lKCs and class-II KCs. This suggests that *PLCe* is preferentially expressed in all KC subtypes irrespective

of the developmental stage, consistent with its preferential expression in all KC subtypes in the adult brain. Although no neuronal function of *PLCe* has yet been revealed, *PLCe* expression is observed in cells committed to neuronal lineage in the mouse brain [Wu et al. 2003]. This is similar to the results obtained in my study, suggesting that *PLCe* functions similarly in the differentiation of neurons and KCs in the mouse and honeybee, respectively. Greenberg et al. (2012) reported that miR-2796, which is a microRNA nested by the *PLCe* gene in the honeybee genome, is abundantly expressed in the adult honeybee brain, and suggested that this miRNA regulates *PLCe*. Although miR-2796 expression in the MBs was not analyzed, it is possible that miR-2796 is already expressed in the larval brain and regulates *PLCe* expression.

The pupal expression patterns of *Syt14* and *dlg5*, which were identified as genes selectively expressed in the lKCs in the adult brain, were unexpected based on analyses in the adult brain. In the early pupal brain, moderate expression of *Syt14* was detected in the class-II KCs and lKCs, suggesting that *Syt14* expression is restricted to the lKCs during midpupal to adult stages. In contrast, selective expression of *dlg5* was detected in the outer halves of the lKCs. Because KCs that are born earlier are pushed away to distal part of the MBs by the newly generated KCs [Farris et al. 1999], it is possible that the outer halves of the lKCs are more differentiated, thus expressing *dlg5,* whereas the inner halves of the lKCs do not express this gene yet because they are immature.

Syt14 is suggested to be involved in membrane trafficking [Fukuda 2003; Doi et al. 2011], while *dlg5* is reported to regulate spine formation through localization of N-cadherin [Wang et al. 2014] in mammals. If these genes have similar functions in the honeybee, membrane trafficking and spine morphology unique to the lKCs are likely to

be acquired later than P3. They were not expressed in an MB-selective manner in the L5F brain, suggesting that membrane trafficking and spine formation are less enhanced, consistent with previous reports that larval MBs lack calyces and have only thin peduncles [Farris et al. 1999; Farris et al. 2004]. Although expression of *Syt14* and *dlg5* was detected in qRT-PCR, *in situ* hybridization signals were detected at very low level. This might be because the whole brain broadly expresses *Syt14* and *dlg5*, and the signal intensity was too low to detect through *in situ* hybridization. Therefore, it is likely that synaptic transmission and spine formation in the larval brain are regulated by other Syt subtypes and postsynaptic proteins.

In the honeybee brain, many genes that have neural functions are enriched in the MBs. Among them, expression of dopamine receptors (Am*dop1*, Am*dop2*, Am*dop3*) and a tyramine receptor, Am*tyr1,* has been examined in the adult brain as well as during metamorphosis. Am*dop1* is widely expressed in the adult brain [Blenau et al. 1998]. In the pupal stages, it is expressed in all KC subtypes with more intense expression in newborn KC subtypes. It is also expressed in some other brain regions than the MBs [Kurshan et al. 2003]. Am*dop2* is preferentially expressed in the sKCs in both newly emerged workers and foragers [Humphries et al. 2003]. It is expressed in class-II KCs, sKCs, and cells in brain regions outside the MBs in pupa [Kurshan et al. 2003]. Am*dop3* is broadly expressed in both the adult and pupal brain [Beggs et al. 2005]. Am*tyr1* is also widely detected over the brain with a probe corresponding to a coding region [Blenau et al. 2000]. Use of another probe targeting the 3'-untranslated region revealed somewhat higher expression in the MBs [Mustard et al. 2005]. In the pupal brain, Am*tyr1* is expressed in all KC subtypes as well as in other cells in the brain [Mustard et al. 2005].

Compared to Am*dop1*, Am*dop3,* and Am*tyr1*, which are expressed in all KC subtypes and in some brain regions, *PLCe* is unique in that it is preferentially expressed in all of the KC subtypes at all stages analyzed. Compared to Am*dop2*, which is expressed selectively in the sKCs, *Syt14* and *dlg5* are unique because of their preferential expression in the lKCs of the adult brain. Additionally, *Syt14* and *dlg5* were selectively expressed in the pupal MBs, unlike Am*dop2*. These findings suggest that *PLCe* can be used as marker gene for all of the KC subtypes while *Syt14* and *dlg5* mark the lKCs.

The results obtained in this chapter suggest that the class-II KCs function in honeybee behavior through *PLCe*. Direct analysis of the behavior of the larval bee, however, is extremely difficult because the honeybee larva is very fragile and does not move actively. Therefore, to gain insight into the function of *PLCe* in honeybee behavior, I analyzed the possible involvement of PLC in olfactory associative learning and memory in the adult worker honeybee in Chapter II. Part of the results in Chapter I was published in Suenami et al. 2016.

Chapter II

Analysis of the function of PLC in olfactory learning and memory in the honeybee

本章については、**5** 年以内に雑誌等で刊行予定のため、非公開。

General discussion

In my doctoral studies, I analyzed the larval and pupal Kenyon cells (KCs) with regard to the acquisition of selective gene expression in the class-II and large-type (l) KCs during metamorphosis. *Phospholipase C epsilon* (*PLCe*) was preferentially expressed in larval class-II KCs and pupal class-II and lKCs, suggesting that this gene marks all KC subtypes irrespective of the developmental stage. In early pupa, *Synaptotagmin 14* (*Syt14*) was also selectively expressed in the class-II and lKCs, while *discs large 5* (*dlg5*) was expressed in the outer half of the lKCs at this stage, suggesting that selective expression of these genes in the lKCs is acquired during the midpupal to adult stages. I also analyzed the involvement of PLC in olfactory associative learning and memory to gain insight into the possible functions of the mushroom bodies (MBs) in larval and adult honeybees. The results suggested that PLC is involved in the early stage of memory formation, but not in memory maintenance till 24 h. Possible functions of *PLCe*, *Syt14,* and *dlg5* in the MBs of larval and adult honeybees are discussed below.

Possible involvement of larval MB-selectively expressed PLCe in learning and memory

MB-preferential *PLCe* expression was detected already at the larval stage, suggesting that *PLCe* is involved in the differentiation and/or function of the class-II KCs at this early stage. This study is the first to identify the gene expression preferentially in the MBs of the honeybee larva.

What role does *PLCe* play in larval MBs? One possible function of the larval MBs is associative learning and memory. Although *PLCe* is lost in the *Drosophila*

genome, larval *Drosophila* MBs are suggested to have a role in associative learning and memory [Honjo and Furukubo-Tokunaga, 2005; Honjo and Furukubo-Tokunaga 2009; Pauls et al. 2010]. Therefore, MB-preferential expression of *PLCe* might be involved in some form of learning and memory in the honeybee larva. A recent study reported that young adult honeybees easily learn an odor presented with food during the larval stage [Ramírez et al. 2016]. Although the learning and memory abilities of larval honeybees were not directly examined in that study, honeybee larvae may also be able to learn an association between an odor and reward. Associative learning in *Drosophila* larvae requires embryonic γ-KCs [Pauls et al. 2010], which are suggested to be homologous to the honeybee class-II KCs [Strausfeld 2002]. Considering that PLC is involved in associative learning and memory in the honeybee, as demonstrated in my study, *PLCe* may regulate larval olfactory learning through the class-II KCs. It is difficult to examine larval behavior, however, and thus imaging techniques may be useful for detecting changes in neural activity or neural network structures in the larval MBs after simultaneous presentation of odor stimulation and food reward. In the study mentioned above, [Ramírez et al. 2016] hypothesized that odor experience in the larval stages helps young adult workers in their colony, such as in the unloading of nectar and pollen from foragers with a familiar scent. I speculated that knowledge of the colony circumstances experienced in the larval stage would help young adult bees to judge other individuals as nestmates because associative learning is immature in young adults [Ray and Ferneyhough 1997; Behrends et al. 2007]. Precise detection of nestmates is critical for integration and maintenance of the colony.

Speculated function of PLCe in learning and memory, and dance communication in

the adult honeybee

The learning and memory abilities of the adult honeybee are especially important because foragers who successfully find good food sources communicate the location and quality of the food source to their nestmates through dance communication. A previous study proposed that repeated learning increases the intracellular calcium concentration and facilitates the transfer of information from short- to long-term storage in the honeybee [Perisse et al. 2009]. *Inositol 1, 4, 5-trisphosphate receptor* (*IP_3R*) and *ryanodine receptor* (*ryr*), whose products are calcium channels activated downstream of PLC, are selectively expressed in the lKCs [Kamikouchi et al. 1998; Uno et al. 2013]. Moreover, *PLCe* expression overlaps with expression of these genes in the lKCs [Suenami et al. 2016; Kaneko et al. 2016]. Although it is unknown whether IP_3R and ryr are involved in transferring information from short- to long-term memory and whether these receptors are activated by PLCe, it is possible that high activation of PLCe by repetitive learning induces these calcium channels to open and the intracellular calcium concentration to increase, thus facilitating the switch from short- to long-term memory.

Which neurotransmitter activates PLCe? Although the protein activated by octopamine receptors is unknown in insects, activation of octopamine receptors ectopically expressed in a cell line increases the intracellular calcium concentration [Grohmann et al. 2003], and it is suggested that at least some octopamine receptor subtypes activate PLC [Farooqui 2007]. Moreover, octopamine receptors expressed in *Drosophila* MBs and honeybee antennal lobes (ALs) are known to be involved in learning and memory [Farooqui et al. 2003; Kim et al. 2013]. Although it is not clear whether octopamine receptors expressed in honeybee MBs are involved in learning and memory, PLCe activation by octopamine may lead to memory formation. Octopamine

fed to honeybees is also reported to modulate dance communication [Barron et al. 2007]. PLCe might be involved in dance communication through learning and memory or, more directly, by regulating dance itself.

Possible involvement of PLCe in the division of labor and sociality of the honeybee

Age polyethism is another aspect of honeybee life in which PLCe may be involved. Previous studies reported that octopamine and insulin, which potentially activate *PLCe*, have functions in foraging onset [Schulz et al. 2002; Ament et al. 2008], although it is unknown whether these signals are received by the KCs. Furthermore, worker bees express more *PLCe* in the brain than males and queens [Grozinger et al. 2007; Zayed et al. 2012]. The female paper wasp (*Polistes metricus*), another social Hymenopteran, exhibits differential expression of *PLCe* in the brain in association with certain behaviors [Toth et al. 2010]. These findings imply the involvement of *PLCe* in behaviors unique to worker honeybees.

While social Hymenopterans express *PLCe* in their brain, it is absent in the genome of *Drosophila melanogaster*, a solitary insect [Greenberg et al. 2012]. Moreover, *PLCe* mutant mice that cannot generate IP_3 exhibit increased memory performance and social interaction, suggesting that *PLCe* is involved in social interactions and/or cognition [Quan et al. 2012]. If *PLCe* functions similarly in mice and Hymenopterans, this gene might be involved in decision-making behavior regarding social interaction, such as whether the honeybee interacts with nestmates or forages alone. If so, *PLCe* expression in the MBs might have been acquired in the evolution of sociality in Hymenopteran insects.

Reevaluation of proposed functions of the KC subtypes and evolution of the MBs in Hymenopteran insects

Previously, the functions of each KC subtype were assumed based on gene expression analyses [Kubo 2012; Kaneko et al. 2016]: the lKCs are involved in learning and memory, and information processing in orientation flight; the mKCs are related to information processing in orientation and foraging flights; the sKCs are suggested to function in the division of labor and in orientation and foraging flights. On the other hand, the present and previous studies [Pauls et al. 2010; Ramírez et al. 2016] suggest that the larval honeybee likely learns and memorizes odors before differentiation of the lKCs in metamorphosis. How do the findings of my study relate to the conventional hypothesis? Possible explanations include: (1) different combinations of brain structures might be involved in learning and memory depending on the developmental stage in the honeybee. For example, it is possible that while the MBs and ALs are involved in learning and memory in the adult honeybee as previously demonstrated [Hammer and Menzel 1998], only the ALs function in larval learning and memory. (2) The larval class-II KCs might be involved in learning and memory like the lKCs. This would be possible if genes expressed selectively in the adult lKCs were also expressed in the larval class-II KCs. This is unlikely, however, because my findings did not indicate preferential expression of *Syt14* and *dlg5* in the larval class-II KCs. (3) It is possible that both the class-II and lKCs are important for learning and memory in both the larval and adult stages despite potential differences in the contribution of the KC subtypes to learning and memory. For example, in adult *Drosophila melanogaster*, KCs are divided into three subtypes according to their projections, and short-term and long-term memories are processed in parallel in different KC subtypes [Trannoy et al. 2011].

Moreover, considering that the lKCs share some genes with the mammalian hippocampus and cerebellum, e.g., *IP_3R*, *type I IP_3 5-phosphatase*, *Syt14*, *protein kinase C*, *ryr*, *calcium/calmodulin-dependent protein kinase II*, *PLC* [Brandt et al. 1987; Burgin et al. 1990; Mailleux et al. 1992; De Smedt et al. 1994; Watanabe et al. 1998; Mori et al. 2000; Doi et al. 2011], the lKCs might be involved not in simple associative learning but in complex forms of learning and memory, such as motor learning, which appears to be less important in larval honeybee. Farris (2011) previously described similarities between the MBs and the mammalian cerebellum in terms of the neural network architecture.

The MBs of Hymenopteran insects are structurally complicated in association with behavioral evolution: insects such as the sawfly and some parasitic wasps, which diverged from the honeybee before the emergence of sociality, have MBs with a simpler calyceal composition than the honeybee [Farris and Schulmeister 2011; Kaneko et al. 2016] (Fig. 20). The lKCs and sKCs extend their dendrites into distinct compartments in the calyces in the honeybee [Strausfeld 2002], suggesting that different calyceal regions correspond to different KC subtypes. This implies that the composition of the KC subtypes is simpler in solitary Hymenopteran insects and that the acquisition of the KC subtypes led to the emergence of sociality [Kaneko et al. 2016] (Fig. 20).

Conclusion of my doctoral course studies and future direction

My studies suggested that characteristics of the honeybee MBs with respect to their gene expression profile are largely acquired in two steps: the first step is the MB-preferential expression of *PLCe* from the larval stage. PLC is involved in memory formation in the adult honeybee, and the larva might also learn and memorize some

aspect of their experience, such as the scent of their colony or nestmates. The second step is the acquisition of gene expression selective to the class-I KCs in metamorphosis. If the adult class-I KCs and larval class-II KCs function in different types of learning and memory, one candidate memory in which the adult class-I KCs are involved is that formed in foraging flight. I speculate that acquisition of these gene expression profiles was needed for the social evolution of the honeybee (Fig. 20).

In future studies, I will analyze the functions of *PLCe*, *Syt14*, *dlg5*, and the KC subtypes in relation to four questions: (1) What molecular and neural mechanisms work in associative learning and memory? In particular, which precise step of learning and memory is PLC involved? (2) What molecular and neural mechanisms underlie learning and memory acquired during a foraging trip? These mechanisms may differ from those involved in associative learning. (3) Is acquisition of the expression of *PLCe*, *Syt14*, and *dlg5* or the KC subtypes related to the evolution of sociality? For example, *PLCe* expression in the MBs might not be detected in solitary species and honeybees lacking *PLCe* would not be expected to leave the colony to forage. The recently reported genome editing methods [Kohno et al. 2016] will facilitate and accelerate gene function analyses, and help to clarify the function of each KC subtype by knocking out master genes controlling the differentiation of KC subtypes. I believe that clarifying the molecular mechanisms underlying the development and evolution of higher brain function will enhance our understanding of the evolution of this “superorganism”.

Figures

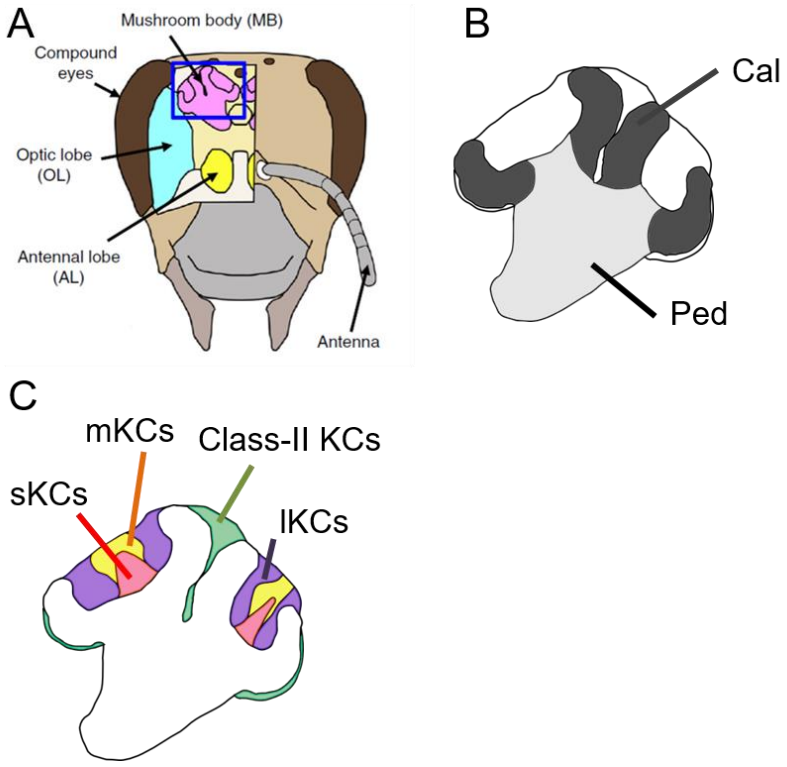


Figure 1. Schematic drawing of the brain and MBs of the adult worker honeybee.

(A) Overview of the head and brain of the adult worker honeybee. The brain is shown at the depth near the MB peduncle. The MBs (magenta), OL (light blue), and ALs (yellow) in the brain are highlighted. This figure was reproduced from [Kaneko et al. 2016] with the publisher's permission. (B and C) Structure of the MBs of the adult worker honeybee. (B) Neuropil such as the calyces (dark grey) and peduncle (light grey) is colored. White regions correspond to the location of the somata of KCs. (C) Locations of the somata of each KC subtype are differentially colored. The adult MBs comprise the lKCs (purple), mKCs (yellow), sKCs (red), and, class-II KCs (green). MB, mushroom body; OL, optic lobe; AL, antennal lobe; Cal, calyx; Ped, peduncle; lKCs, large-type Kenyon cells; mKCs, middle-type Kenyon cells; sKCs, small-type Kenyon cells; Class-II KCs, Class-II Kenyon cells.

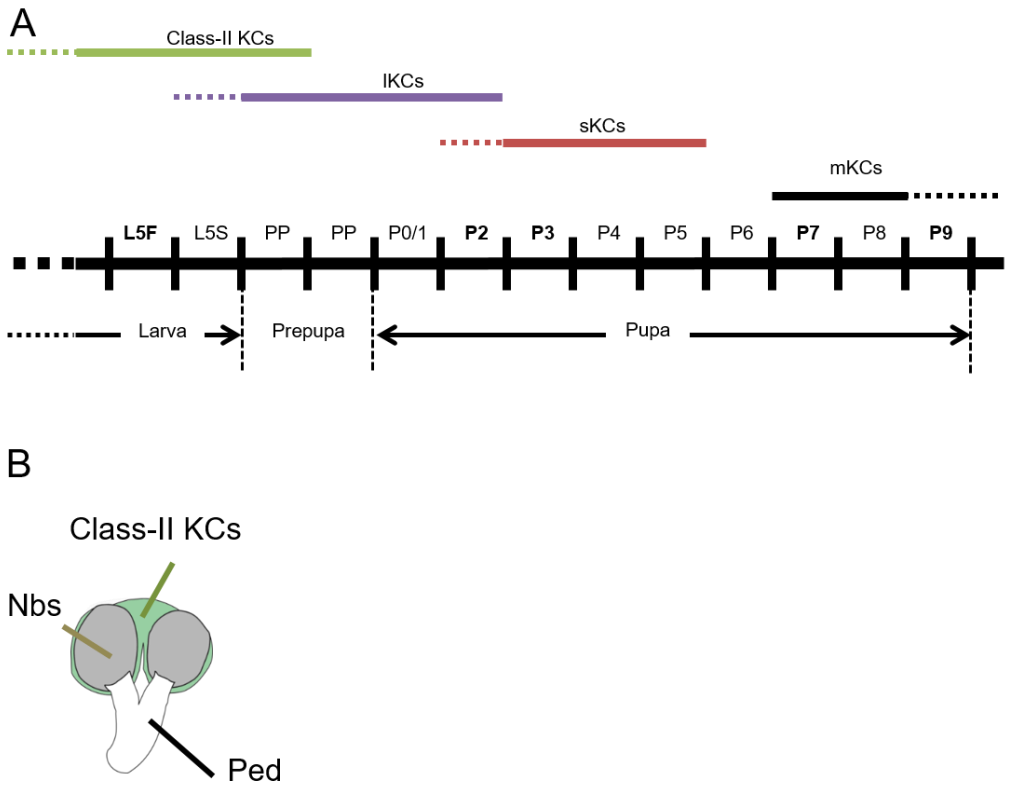


Figure 2. Development of the MBs of the worker honeybee.

(A) Proliferation of KC subtypes and expression of marker genes for mKCs during the larval and pupal stages [Farris et al. 1999; Kaneko et al. 2013]. The lower black bar depicts the developmental stages of the worker honeybee. Development progresses from larva to prepupa to pupa. Stages analyzed in this study are described in bold letters. The green, purple, and red bars above indicate the stages at which the class-II, l-, and sKC subtypes proliferate, respectively. Black bar above the lower black bar indicates the period when *middle-type Kenyon cell-preferential arrestin-related gene-1* (*mKast*), which is the only known marker gene for the mKCs, is expressed. Dotted lines indicate that generation of the class-II, l-, and sKCs is ambiguous, and that *mKast* expression was predicted, but not analyzed. L5F, larval fifth-instar feeding stage; L5S, larval fifth-instar spinning stage; PP, prepupa. The number after 'P' in pupal stages indicates

the number of days after pupation. (B) Structure of the MB of worker larva. Larval MBs have only Nbs (light grey) and class-II KCs (green). White region corresponds to peduncle. Ped, peduncle; Class-II KCs, Class-II Kenyon cells; Nbs, neuroblasts.

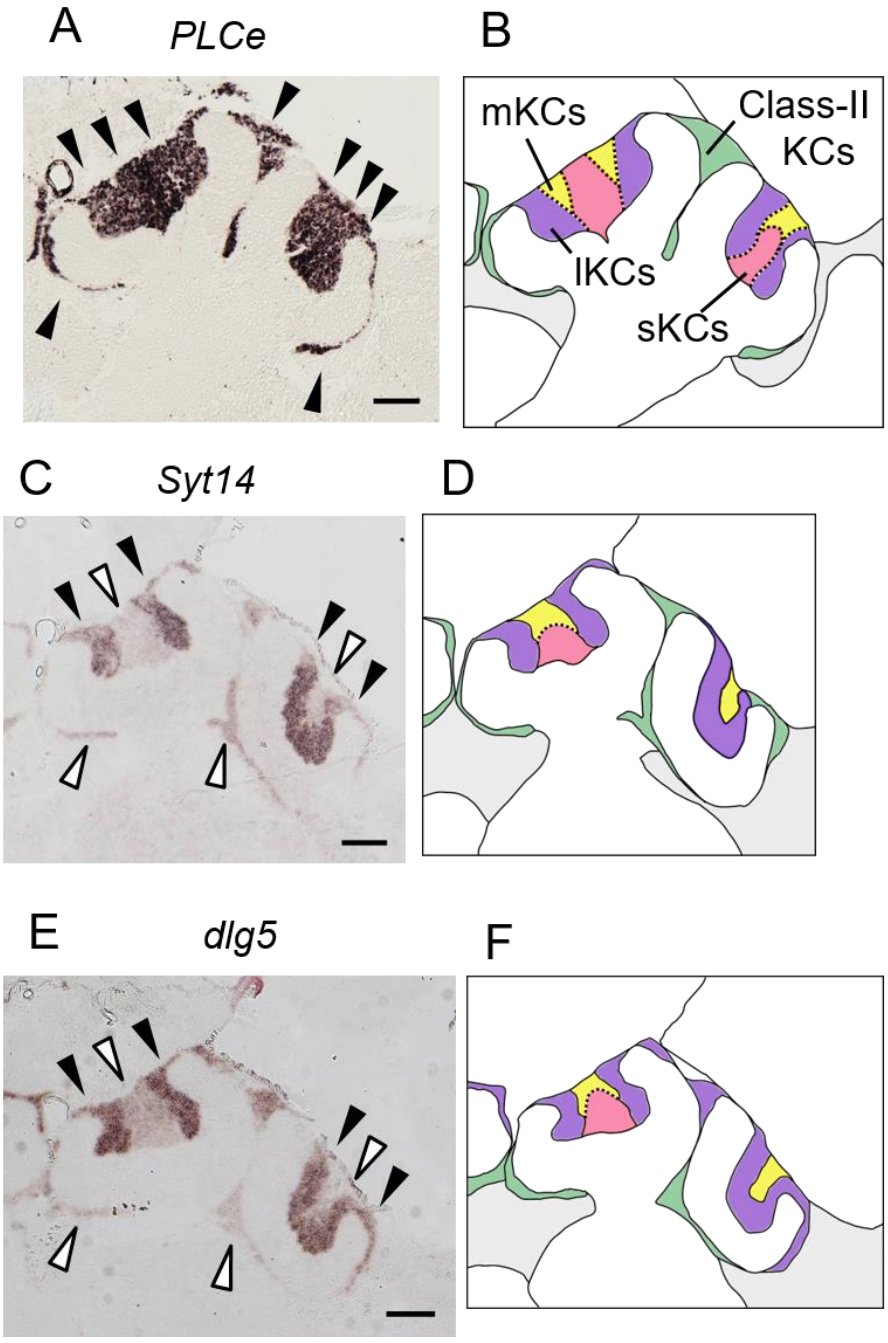


Figure 3. *In situ* hybridization analysis of the three MB-preferential genes in the adult worker honeybee brain.

(A and B) Expression of *PLCe* in the MBs identified in a previous experiment (A) and its schematic drawing (B). *PLCe* was expressed in all KC subtypes (black arrowheads in A). (C and D) Expression of *Syt14* in the MBs (C) and schematic illustration. *Syt14* was

selectively expressed in the lKCs (black arrowheads in C). White arrowheads indicate weak signals in the other KCs. (E, F) Expression of *dlg5* in the MBs (E) and its schematic illustration (F). *dlg5* was also selectively expressed in the lKCs (black arrowheads in E). White arrowheads indicate weak signals in the other KCs. (A, C, and E) Frontal section of the MBs. In illustrations (B, D, and F), boundaries between the lKCs (purple), mKCs (yellow), and sKCs (red) are represented by dotted lines when they were not discriminated by morphologic observation. The class-II KCs and the other cells are colored green and light gray, respectively. lKCs, large-type Kenyon cells; mKCs, middle-type Kenyon cells; sKCs, small-type Kenyon cells; Class-II KCs, class-II Kenyon cells. Scale bar, 100 μm.

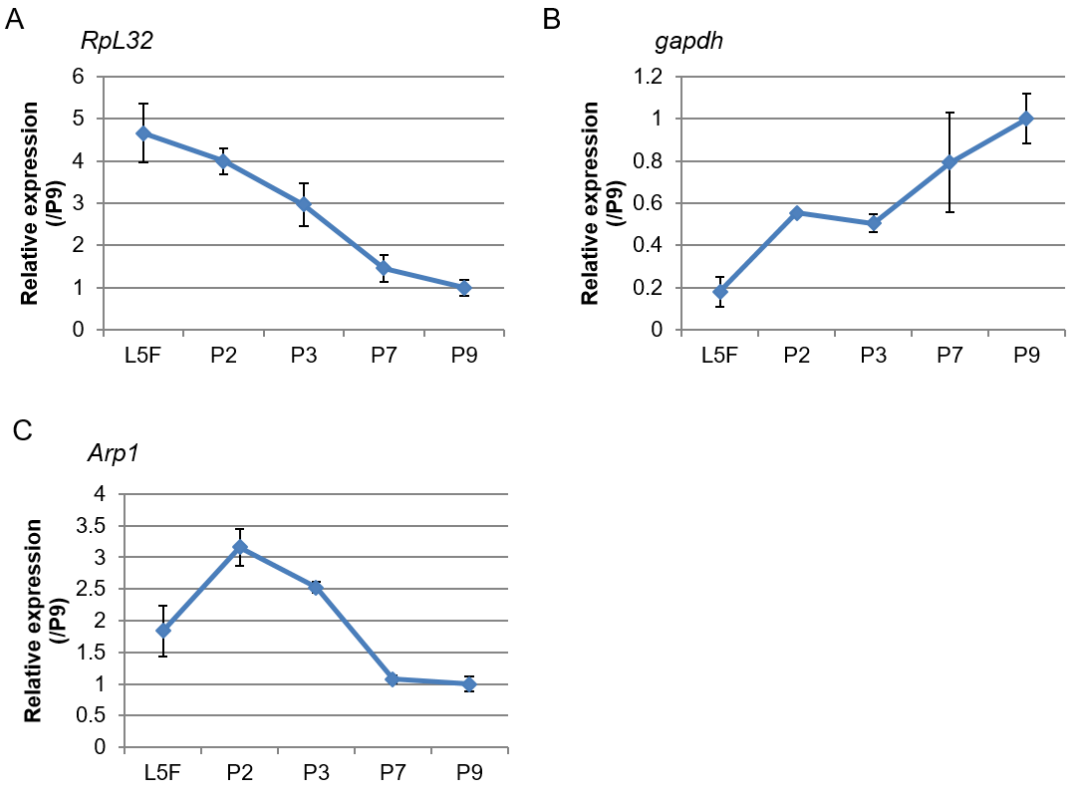


Figure 4. qRT-PCR analysis of *RpL32*, *gapdh*, and *Arp1* in worker brains during metamorphosis.

(A-C) Changes in expression of three reference genes, *RpL32* (A), *gapdh* (B), and *Arp1* (C). The same amount of total RNA from each developmental stage was analyzed. Expression levels were normalized by those at the P9 stage, and average and standard deviation are shown. Three lots of samples were analyzed in each stage. A significant difference among stages was detected for all genes by the Kruskal-Wallis test ($P<0.05$).

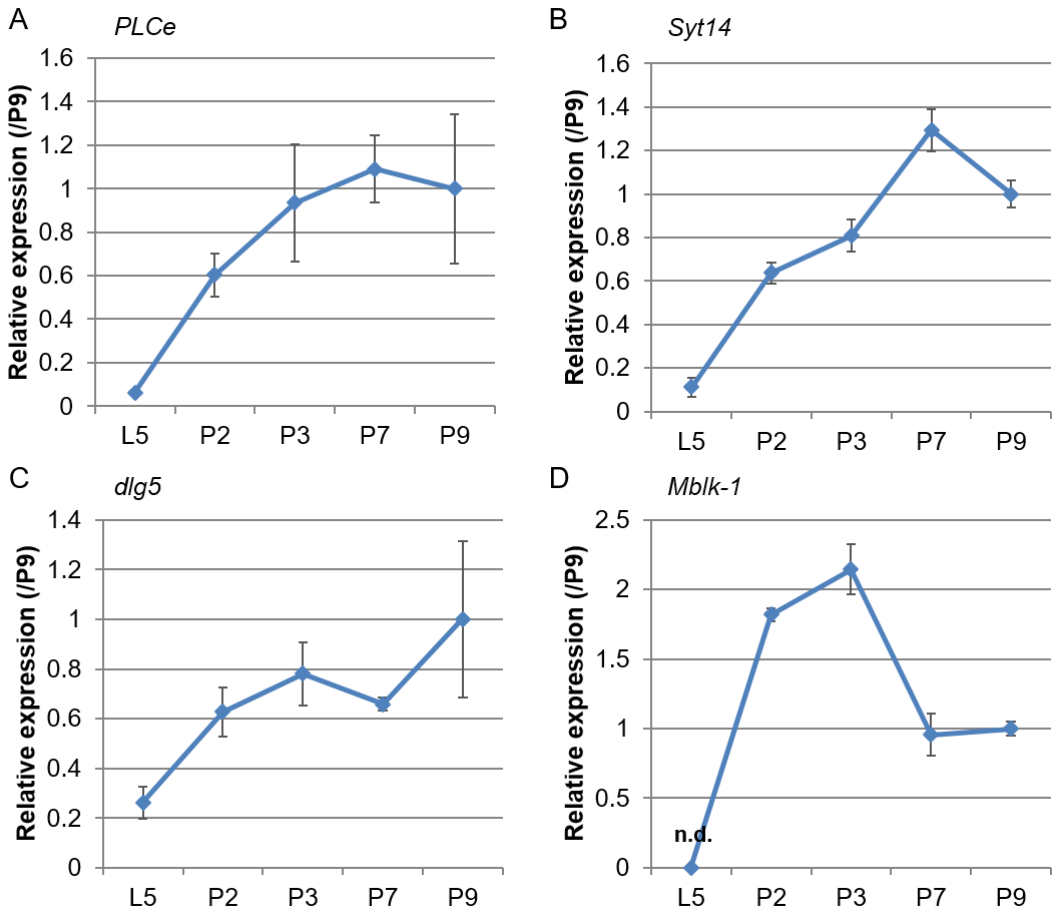


Figure 5. qRT-PCR analysis of *PLCe*, *Syt14*, *dlg5,* and *Mblk-1* in worker brains during metamorphosis.

Changes in expression of *PLCe* (A), *Syt14* (B), *dlg5* (C), *Mblk-1* (D). The same amount of total RNA from each stage was used in this analysis. Expression is normalized to that at the P9 stage. Three lots of samples from each stage were analyzed. n.d., not detected. Significant differences among stages were detected for all genes by the Kruskal-Wallis test ($P<0.05$), whereas no significant difference in expression was detected when each pair of stages was considered due to small size of samples (Steel-Dwass test, $P>0.05$).

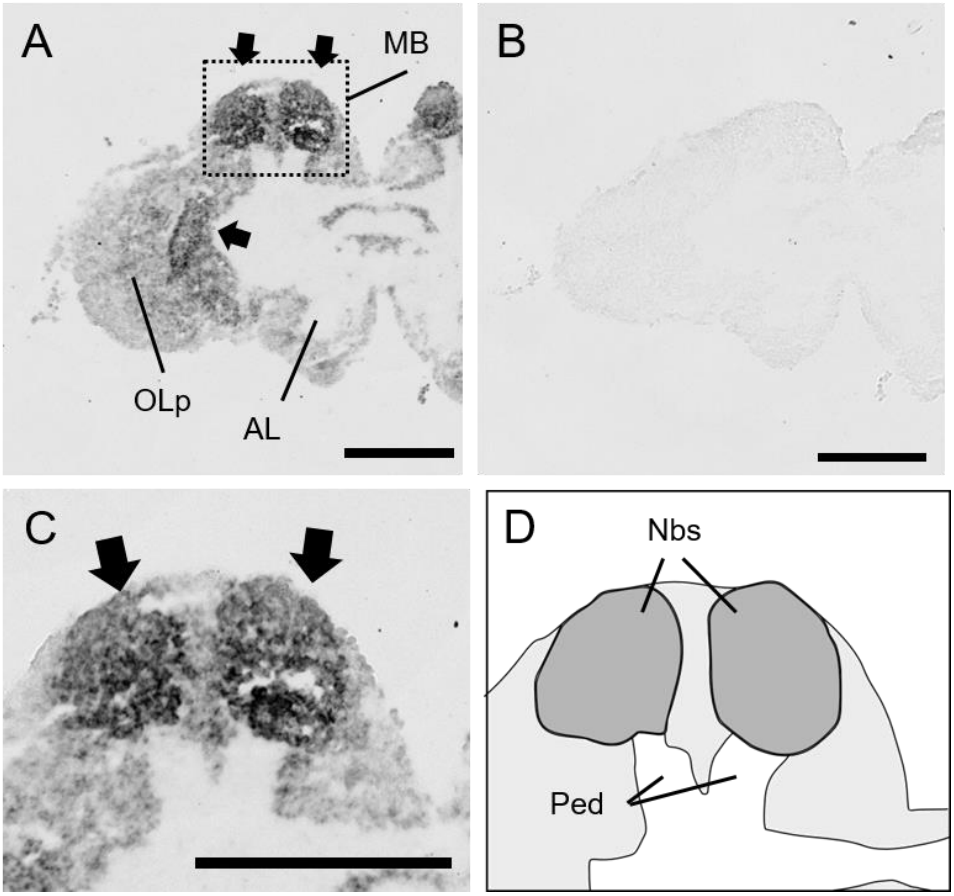


Figure 6. *In situ* hybridization analysis of *RpL32* in the L5F larval brain.

(A, B) Frontal sections of a brain hemisphere stained with antisense (A) and sense (B) probes. Arrows indicate cell populations with higher signals. Dotted square shows the MBs. (C, D) Magnified view of the MB in (A) and its illustration. Arrows in (C) indicate Nbs (dark grey in D). In (D), the regions colored light gray contain the other cells. MB, mushroom body; OLp, optic lobe primordium [Roat and Landim, 2010]; AL, antennal lobe [Moda et al. 2013]; Nbs, neuroblasts; Ped, peduncle. Scale bar, 200 μm.

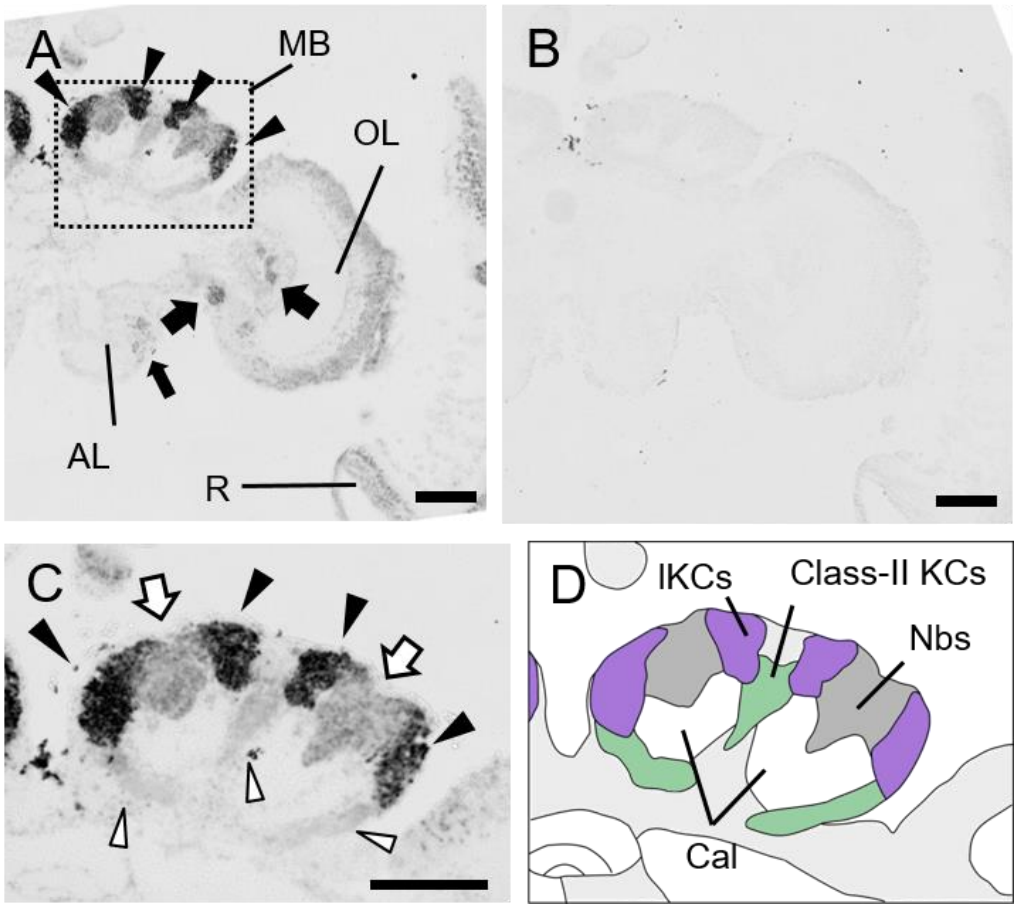


Figure 7. *In situ* hybridization analysis of *Mblk-1* in the P3 pupal brain.

(A,B) Frontal sections of a brain hemisphere stained with antisense (A) and sense (B) probes. Arrowheads and arrows in (A) indicate cells with higher signals in the MBs, and the ALs and OLs, respectively. (C, D) Magnified view of the MB indicated by dotted square in (A) and its illustration. Arrows and arrowheads in (C) indicate Nbs (dark grey in D) and KCs. Black and white arrowheads indicate strong signals in the lKCs (purple in D) and weak signals in the class-II KCs (green in D), respectively. In (D), the regions colored light gray contain the other cells. MB, mushroom body; OL, optic lobe; AL, antennal lobe; R, retina; Nbs, neuroblasts; lKCs, large-type Kenyon cells; Class-II KCs, class-II Kenyon cells; Cal, calyces. Scale bar, 200 μm.

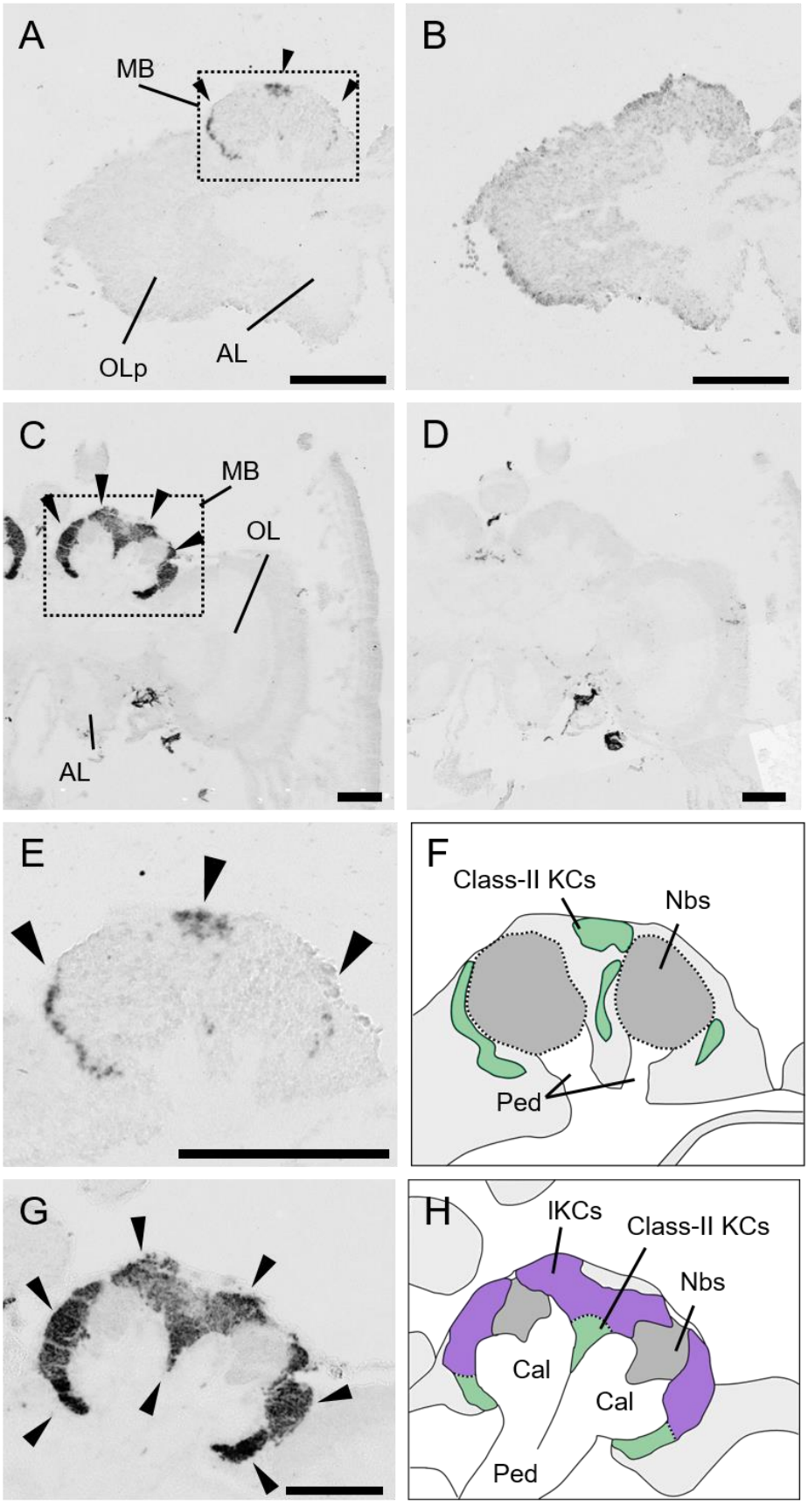
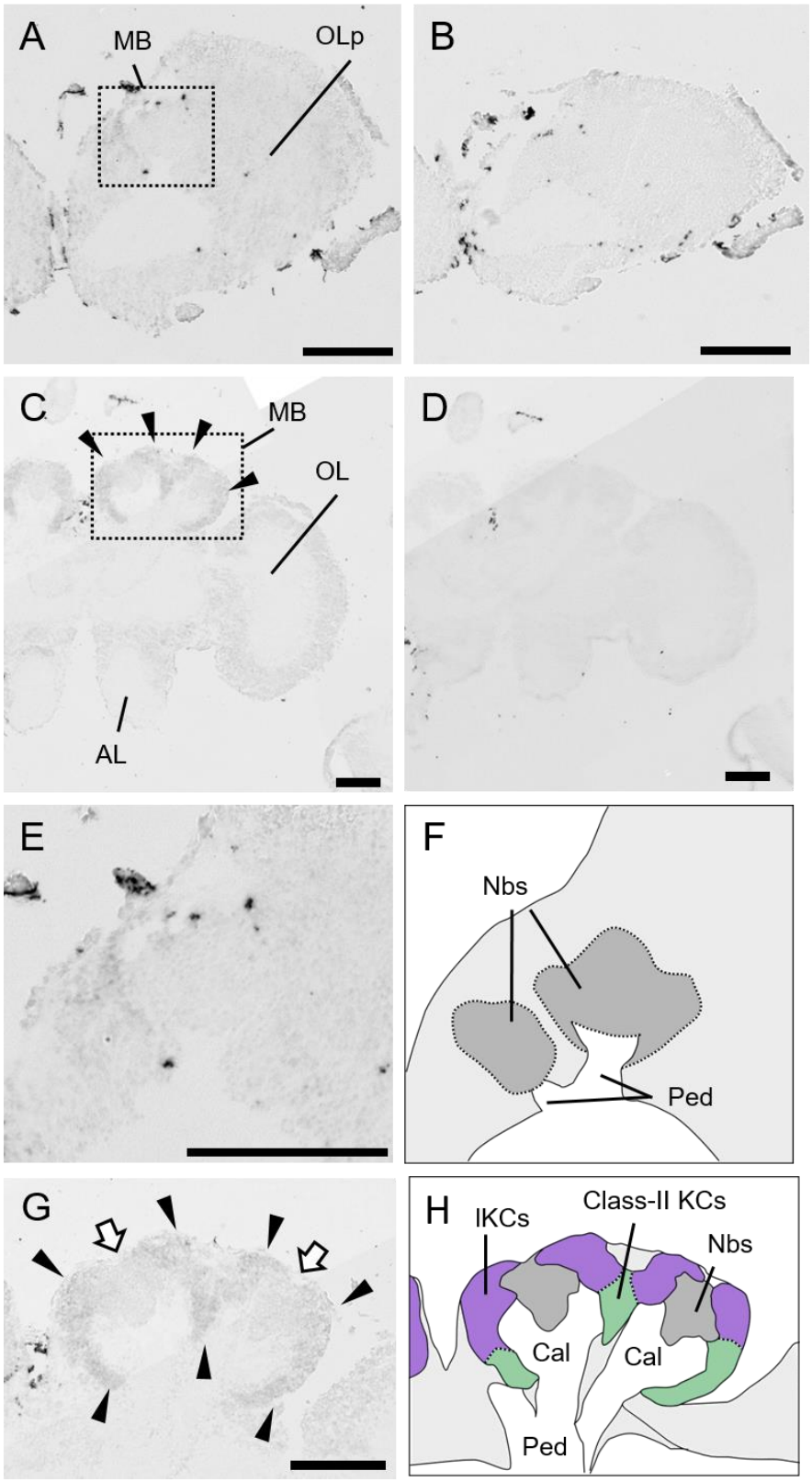

A
MB
OLp
AL
B
C
MB
OL
AL
D
E
F
Class-II KCs
Nbs
Ped
G
H
lKCs
Class-II KCs
Nbs
Cal
Cal
Ped

Figure 8. *In situ* hybridization analysis of *PLCe* in the L5F and P3 brains.

(A-D) Frontal sections of brain hemispheres stained with antisense (A, C) and sense (B, D) probes. (A) and (B) are the results in the larval brain while (C) and (D) are those in the pupal brain. Arrowheads in (A) and (C) indicate prominent signals in the MBs. (E-H) Magnified view of the MBs represented in (A) and (C) and their illustrations. Arrowheads in (E) indicate prominent signals in the class-II KCs (green in F) in the larva MBs, while those in (G) represent prominent signals in the class-II- and lKCs (green and purple in H, respectively) in the pupal MBs. Because boundaries of the Nbs (dark grey in F) in the larval MBs and those between the class-II- and lKCs in the pupal MBs were not discriminated by morphologic observation, they are depicted by dotted lines in (F) and (H). In (F) and (H), the regions colored light gray contain the other cells. MB, mushroom body; OLp, optic lobe primordium [Roat and Landim, 2010]; AL, antennal lobe [Moda et al. 2013]; OL, optic lobe; Nbs, neuroblasts; Class-II KCs, class-II Kenyon cells; Ped, peduncle; lKCs, large-type Kenyon cells; Cal, calyces. Scale bar, 200 μm.


A
MB
OLp
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Nbs
Ped
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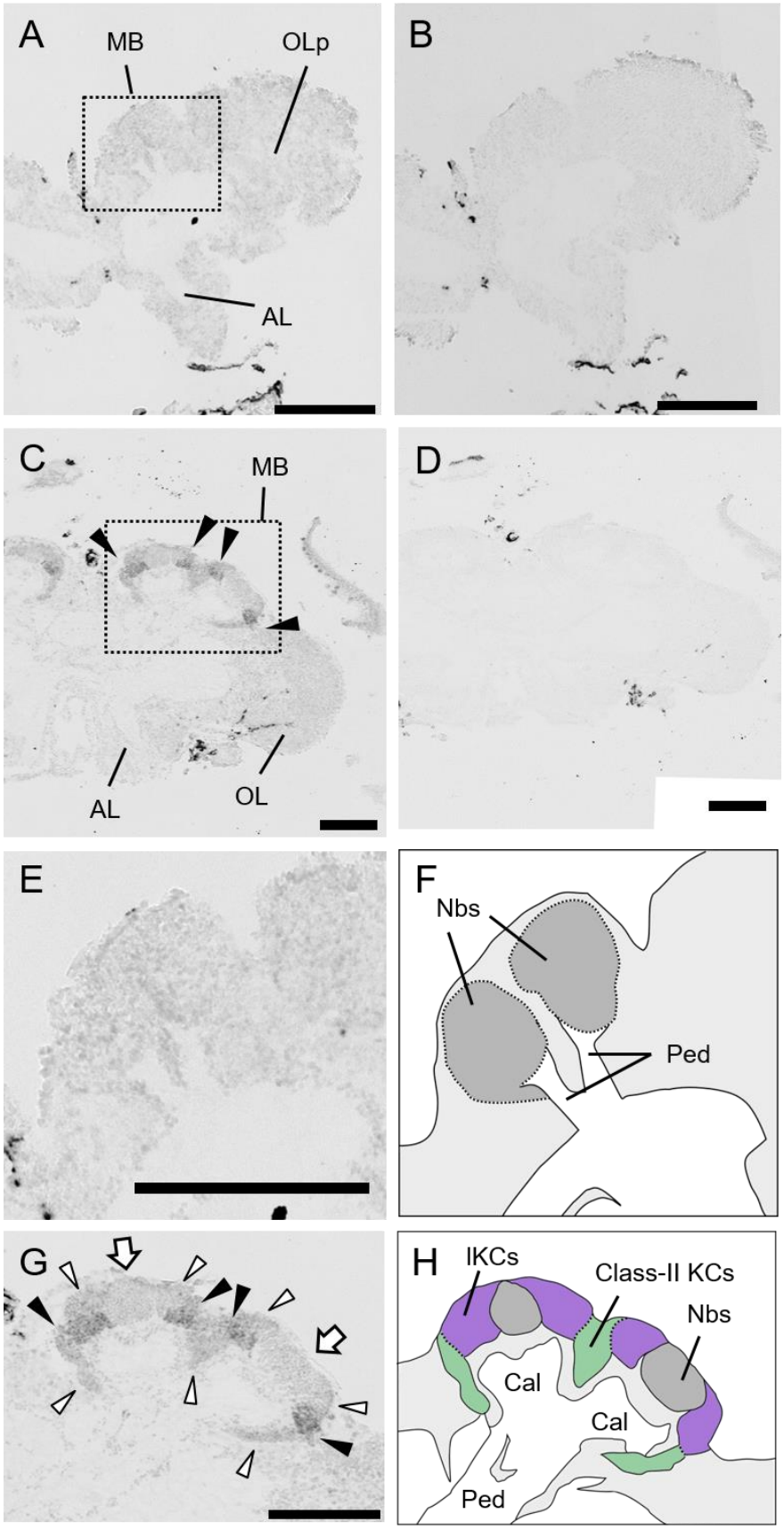
lKCs
Class-II KCs
Nbs
Cal
Cal
Ped

Figure 9. *In situ* hybridization analysis of *Syt14* in the L5F and P3 brains.

(A-D) Frontal sections of brain hemispheres stained with antisense (A, C) and sense (B, D) probes. (A) and (B) are the results in the larval brain while (C) and (D) are those in the pupal brain. Arrowheads in (C) indicate prominent signals in the MBs. (E-H) Magnified view of the MBs represented in (A) and (C) and their illustrations. Arrowheads in (G) represent weak signals in the class-II- and lKCs (green and purple in H, respectively) in the pupal MBs. Arrows indicate weaker signals in the Nbs (dark grey in H). Because boundaries of the Nbs in the larval MBs and those between the class-II- and lKCs in the pupal MBs were not discriminated by morphologic observation, they are depicted by dotted lines in (F) and (H). In (F) and (H), the regions colored light gray contain the other cells. MB, mushroom body; OLp, optic lobe primordium [Roat and Landim, 2010]; AL, antennal lobe; OL, optic lobe; Nbs, neuroblasts; Ped, peduncle; Class-II KCs, class-II Kenyon cells; lKCs, large-type Kenyon cells; Cal, calyces. Scale bar, 200 μm.


A
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Nbs
Ped
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lKCs
Class-II KCs
Nbs
Cal
Cal
Ped

Figure 10. *In situ* hybridization analysis of *dlg5* in the L5F and P3 brains.

(A-D) Frontal sections of brain hemispheres stained with antisense (A, C) and sense (B, D) probes. (A) and (B) are the results in the larval brain while (C) and (D) are those in the pupal brain. Black arrowheads in (C) indicate selective signals in the MBs. (E-H) Magnified views of the MBs represented in (A) and (C) and their illustrations. In (G), black arrowheads represent the moderate signals in the outer halves of the lKCs, while white arrowheads indicate weaker signals in the remaining lKCs and class-II KCs. White arrows indicate weak signals in the Nbs. Because boundaries of the Nbs (dark grey) in the larval MBs and those between the class-II and lKCs (green and purple, respectively) in the pupal MBs were not discriminated by morphologic observation, they are depicted by dotted lines in (F) and (H). In (F) and (H), the regions colored light gray contain the other cells. MB, mushroom body; OLp, optic lobe primordium [Roat and Landim, 2010]; AL, antennal lobe [Moda et al. 2013]; OL, optic lobe; Nbs, neuroblasts; Ped, peduncle; Class-II KCs, class-II Kenyon cells; lKCs, large-type Kenyon cells; Cal, calyces. Scale bar, 200 μm.

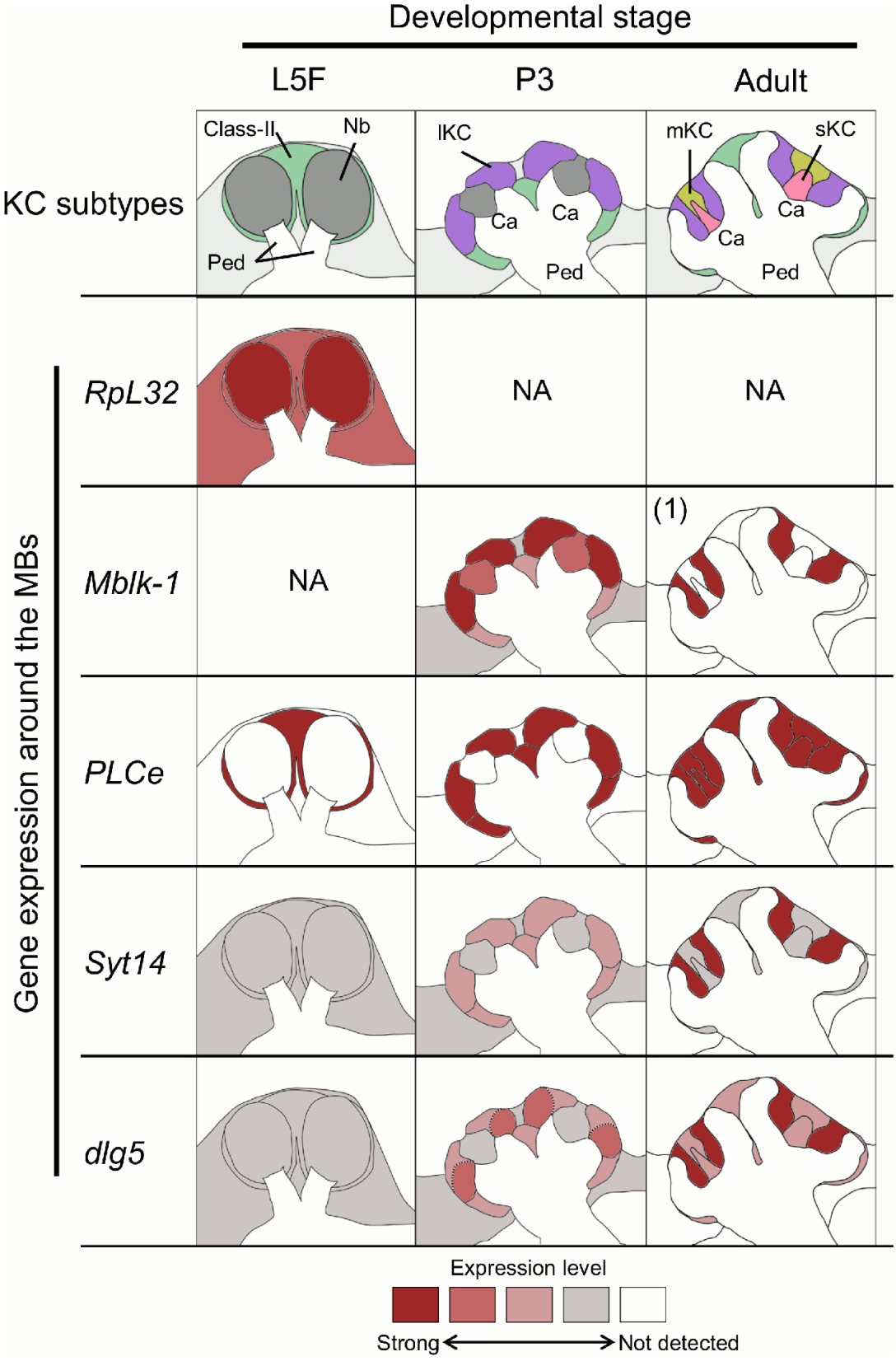

Figure 11. Summary of the changes in gene expression profiles during

metamorphosis.

Changes in the profile of the KC subtypes and expression patterns of genes that occur with metamorphosis. This figure was reproduced from [Suenami et al. 2016] with permission from the publisher. Signal intensities of genes are indicated by the intensity of the red color. (1), *Mblk-1* expression in the adult MBs was previously reported [Takeuchi et al. 2001]. Data on expression of *PLCe*, *Syt14* and *dlg5* in the adult brain was obtained in my master course studies. In the illustration of *dlg5* expression at the P3 stage, boundaries of the outer and inner halves of the lKCs are depicted by dotted lines because they were not discriminated by morphologic observation. NA, not analyzed. In the uppermost figures of the KC profiles, the Nbs, class-II-, l-, m-, and sKCs are represented by dark grey, green, purple, yellow, and red colors, respectively. The other cells are colored light grey. Nb, neuroblasts; Ped, peduncle; Class-II, class-II Kenyon cells; lKCs, large-type Kenyon cells; Ca, calyces; mKCs, middle-type Kenyon cells; sKCs, small-type Kenyon cells.

Figure 12. から **Figure 19.** は第 **2** 章の内容に対応する。

第 **2** 章と同様の理由で非公開。

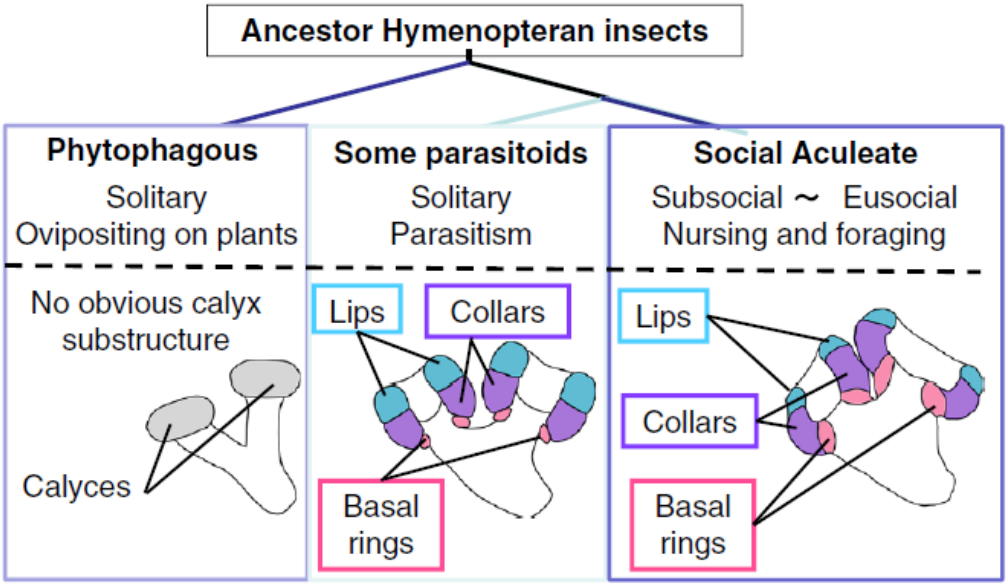

Figure 20. Evolution of the MBs of the Hymenopteran insect.

Hypothesis regarding the evolution of the MBs in Hymenopteran insects is illustrated. Schematic phylogenetic tree of the Hymenopteran insects is shown on top. Phytophagous species, parasitoids, and social Aculeate diversified from the common ancestral species. Characteristics of life history and illustrations of the MBs in each lineage are shown in the middle and lower panels. Lips, collars, and basal rings (colored light blue, purple and pink, respectively), which are compartments of the calyces observed in some parasitoids and social Aculeate, do not exist in the MBs of Phytophagous species. This figure was obtained from [Kaneko et al. 2016] with permission from the publisher.

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