学位論文

Analysis of high-temperature sensitive neural activity in the brains of Japanese honeybee workers during the formation of a ‘hot defensive bee ball’ using immediate early genes

（初期応答遺伝子を用いた熱殺蜂球形成時の
ニホンミツバチの脳における高温応答性神経活動の解析）

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Abstract

Animals exhibit various antipredator behaviors to survive. Some animals show characteristic behaviors against their natural enemies that are considered to be an evolutionary consequence of their adaptation to the enemies. The neural bases of such behaviors, however, remain largely unknown. Although honeybees commonly use their stingers to counterattack predators, Japanese honeybee (*Apis cerana japonica*) uses a different strategy to fight against their natural enemy, giant hornet (*Vespa mandarinia japonica*). Instead of stinging the hornet, Japanese honeybees form a 'hot defensive bee ball' by surrounding the hornet *en masse* and vibrating their flight muscles to produce heat. The temperature in the ball quickly rises to approximately 46°C, which is lethal to hornet but not to honeybees. The high temperature phase continues for approximately 20 min, and the hornet is killed in the ball by the heat. European honeybee (*A. mellifera* L.), on the other hand, does not exhibit this behavior, and their colonies are often destroyed by hornet attacks. Therefore, the hot defensive bee ball formation is considered to be characteristic of Japanese honeybee and a unique heat-generating behavior of insects.

In my master course studies, I attempted to identify the active brain regions of Japanese honeybee workers during the formation of a hot defensive bee ball, using a neural immediate early gene (IEG). I identified an *A. cerana* homolog (*Acks* = *Apis cerana kakusei*) of *kakusei*, an IEG previously identified from *A. mellifera* in our laboratory, and showed that *Acks* has characteristics similar to *kakusei* and can be used to visualize active brain regions in *A. cerana*. Using *Acks* as a neural activity marker, I demonstrated that neural activity in the mushroom bodies (MBs), especially in Class II Kenyon cells (KCs), one subtype of MB

intrinsic neurons, and a restricted area between the dorsal lobes and the optic lobes was increased in the brains of Japanese honeybee workers involved in the formation of a hot defensive bee ball. The types of sensory input that induced neural activity in these neurons and the possible role of the neural activity during formation of the hot defensive bee ball, however, remained unclear.

In my doctoral course studies, I analyzed the *Acks* expression pattern in the brains of the Japanese honeybees exposed to a high temperature or alarm pheromone. The *Acks* expression pattern observed in the brains of workers involved in the bee ball formation well resembled that observed in the brains of workers exposed to a high temperature (46°C), but not that of workers exposed to an alarm pheromone, suggesting that thermal information during the hot defensive bee ball formation is mainly processed in the brains of the Japanese honeybees. Because the inner temperature of a hot defensive bee ball is reported to be maintained at approximately 46°C, I hypothesized that the KCs activated by a high temperature during the formation of the bee ball selectively sense a temperature of ~46°C, acting as a kind of 'thermostat'. To determine the temperature threshold of the KCs, I analyzed the expression of *Acks* (and *kakusei*) in the MBs and found that the expression of *Acks* (and *kakusei*) was significantly increased at temperatures near 46°C, not only in Japanese honeybees but also in European honeybees, suggesting that the high-temperature responsive KCs have an activation threshold of ~46°C in both honeybee species. Interestingly, buff-tailed bumblebees (*Bombus terrestris*) also show high-temperature selective *kakusei* expression in the MBs, although there is some difference in the temperature threshold between honeybees and bumblebees. These findings suggested that the thermosensitive KCs are conserved, at least between honeybees and bumblebees.

Furthermore, I attempted to identify honeybee homologs of some well-known vertebrate IEGs, to confirm the above-mentioned thermosensitive response of some KCs. Expression of the *Egr-1* homolog of the honeybee (*AmEgr*) was transiently induced in the brains of European honeybees treated by injection of picrotoxin or awaken from CO_2 anesthesia, indicating that *AmEgr* has IEG characteristics. Finally, *AmEgr* was induced in both small- and middle-type KCs in the brains of foragers, and in Class II KCs of workers exposed to a high temperature, similar to the results obtained with *kakusei*, indicating that *AmEgr* is applicable as an IEG, like *kakusei* (and *Acks*).

Taken together, my findings with two distinct IEGs suggested that thermosensitive KCs are conserved, at least among European and Japanese honeybees, and bumblebees. While the thermoresponsive KCs are conserved among both honeybees and bumblebees, only the Japanese honeybees exhibit the 'hot defensive bee ball' formation. Then, I hypothesized that the Japanese honeybees utilize these neurons during bee ball formation to maintain the appropriate temperature inside of the ball. The IEGs, *kakusei* (and *Acks*), are only conserved in the honeybee and bumblebee genomes. Therefore, the conserved *Egr* homolog can be applicable to investigate evolutionary conservation of the thermosensitive KCs in insects in future studies.

Contents

Abbreviations

Acks	Apis cerana kakusei
AL	antennal lobe
AmEgr	Apis mellifera Egr
Arc	activity-regulated cytoskelton-associated protein
DL	dorsal lobe
ef-1α	elongation factor-1 alpha
Egr-1	early growth response protein-1
IAA	isoamyl acetate
IEG	immediate early gene
KC	Kenyon cell
lKC	large-type KC
MB	mushroom body
OL	optic lobe
PTX	picrotoxin
RACE	rapid amplification of cDNA ends
Rp49	ribosomal protein 49
RT-PCR	reverse transcription-polymerase chain reaction
s/mKC	small- and middle-type KC
TRP	transient receptor potential

General Introduction

In nature, animals threatened by predators exhibit a variety of adaptive behaviors to escape or actively defend themselves against the predators [Kavaliers and Choleris, 2001]. Some animals exhibit characteristic antipredator behaviors against their natural enemies that are considered to be an evolutionary consequence of adaptation to the threat of natural enemies [Rundus et al., 2007; Ratcliffe and Nydam, 2008]. The neural bases of the antipredator behaviors, however, are poorly understood.

Honeybees (Genus *Apis*) commonly use their stingers to counterattack an intruder [Winston, 1987]. Japanese honeybees (*Apis cerana japonica*), however, fight against giant hornet (*Vespa mandarinia japonica*), their most formidable natural enemy [Matsuura, 1988], by exhibiting a characteristic behavior. In autumn, giant hornets attack Japanese honeybee colonies to steal their larvae and pupae. If a foraging hornet tries to enter the beehive, a group of more than 500 workers quickly forms a spherical assemblage called a 'hot defensive bee ball', trapping the hornet inside the ball. In the ball formation, honeybees vibrate their flight muscles to produce heat (Fig. 1). The temperature in the ball quickly rises to around 46°C, which is lethal to the hornet but not to the honeybees. The high temperature phase continues for approximately 20 min. Within ~30 to 60 min after initiating the bee ball formation, the hornet is killed by the heat produced [Ono et al., 1995].

Interestingly, European honeybees (*A. mellifera* L.), which are a related but allopatric species and were introduced to Japan in the Meiji era (about 140 years ago) for apiculture, exhibit only stinging behavior against the hornet. The rigid exoskelton of the giant hornet renders the bee stings ineffective, however, and colonies of the European

honeybees are often destroyed [Okada, 1961]. Thus, the defensive bee ball formation is considered to be Japanese honeybee-characteristic highly adaptive behavior to avoid predation by giant hornets that inhabit East Asia, including Japan [Ono et al., 1995], and physiologically unique behavior in terms of intensive heat generation observed in insect generally thought to be poikilothermic. Since hot defensive bee ball formation by Japanese honeybees against giant hornet was first reported [Ono et al., 1995], several ecological studies have been performed [Ken et al., 2005; Abrol, 2006; Papachristoforou et al., 2007; Sugahara and Sakamoto, 2009; Baracchi et al., 2010; Sugahara et al., 2012]. The neural mechanism underlying this characteristic behavior, however, has remained totally unknown.

As a first step toward elucidating the neural mechanisms of this behavior, it is important to identify the brain regions that are specifically activated in bee ball-forming bees. Genes that show transiently induced expression in a neural activity-dependent manner are called neural immediate early genes (IEGs) [Hughes and Dragunow, 1995] and have been widely utilized as neural activity markers in neuroethological studies of vertebrates [Clayton, 2000; Guzowski et al., 2005; Kubik et al., 2007; Kovács, 2008]. Although the time resolution is not optimal due to the considerable delay (generally ~30 min) in the detectable expression change after neural activation, the use of IEGs allows identifying active brain regions in free-moving animals at the cellular level [Guzowski et al., 2005]. Because the hot defensive bee ball formation is a collective behavior (over 500 individual bees are involved in forming a bee ball) and is usually formed within the beehive, it is practically impossible to detect neural activity with current electrophysiology and Ca^{2+} imaging methods. In addition, as described above, bee ball formation continues for 30 to 60 min. Thus, I intended to investigate the neural activity pattern in the brains of the Japanese honeybee workers

involved in hot defensive bee ball formation using an IEG-mapping method.

In my master course studies, I identified a Japanese honeybee homolog (*Acks*: *Apis cerana kakusei*) of *kakusei*, an IEG previously identified from European honeybee [Kiya et al., 2007]. Expression analysis revealed that, like *kakusei, Acks* transcript expression levels transiently increase, peaking at 30 to 60 min after a seizure induced by awakening from anesthesia. The induced *Acks* expression can be broadly visualized in several brain regions. These findings suggested that the function of the *Acks* transcript is similar to that of the *kakusei* transcript as a nuclear non-coding RNA. Importantly, *Acks* signals were detected in the mushroom bodies (MBs), especially in the Class II Kenyon cells (KCs) of workers at 30 or 60 min after the bee ball formation, and in neurons located in a restricted area between the dorsal lobes and the optic lobes of workers at 60 min, whereas they were scarcely detected at 0 min, strongly suggesting that these neurons are highly activated in the brains of workers involved in forming the hot defensive bee ball. The type of sensory information that induces this neural activity, however, remains unclear.

In my doctoral course studies, aiming at determining the sensory information that induces the neural activity in the brains of bee ball-forming bees, I analyzed the *Acks* expression pattern in the brains of bees exposed to a high temperature or an alarm pheromone component, both of which are putative components of sensory input processing during the hot defensive bee ball formation. The *Acks* distribution patterns observed in the brains of workers involved in the bee ball formation resembled those observed in the brains of workers exposed to a high temperature (46°C), but not those of workers exposed to an alarm pheromone, suggesting that during the hot defensive bee ball formation, thermal information was mainly processed in the brains of Japanese honeybees. Quantitative reverse

transcription-polymerase chain reaction revealed that *Acks* or *kakusei* expression was strongly induced between 44°C and 46°C in the MBs of both Japanese and European honeybee workers, suggesting that both Japanese and European honeybees have thermosensitive KCs whose activation threshold is near 46°C. Furthermore, to confirm the high-temperature responsiveness of the KCs, I newly identified another IEG, *AmEgr*, which is the honeybee homolog of *Egr-1*, a well-known IEG in vertebrates, and demonstrated that expression of *AmEgr* was also increased in the MBs between 44°C and 46°C. Based on the conservation of thermoresponsive KCs in not only Japanese honeybees but also European honeybees, which do not form a hot defensive bee ball, I speculate that the Japanese honeybees utilize these neurons in bee ball formation to maintain the appropriate temperature inside of the bee ball, just like a ‘thermostat’.

Chapter I

Detection and analysis of high-temperature sensitive neural activity in the brains of Japanese honeybee workers forming a hot defensive bee ball

Introduction

Although an IEG-mapping method is widely used in vertebrates [Clayton, 2000; Guzowski et al., 2005; Kubik et al., 2007; Kovács, 2008], neural IEGs have not been identified in insects. In 2007, the first insect neuronal IEG, termed *kakusei* ('awakening' in Japanese), was identified in our laboratory from European honeybee [Kiya et al., 2007]. *kakusei* expression is transiently increased after a seizure induced by awakening from anesthesia. The *kakusei* transcript does not contain a long open-reading frame that encodes a protein, suggesting that it functions as a non-coding RNA, unlike vertebrate IEGs, which generally encode proteins [Loebrich and Nedivi, 2009]. In previous studies, my collaborators used *kakusei* as a neural activity marker to successfully identify the brain regions in the European honeybee that are active during foraging behavior [Kiya et al., 2007; Kiya and Kubo, 2010].

In Chapter I, using the *kakusei* homolog in Japanese honeybee (*Acks* = *Apis cerana kakusei*), I found that the neural activity of Class II Kenyon cells (KCs), which is one subtype of intrinsic neurons of mushroom bodies (MBs) was preferentially increased in the brains of Japanese honeybee workers during the formation of a hot defensive bee ball. I investigated the type of sensory information that induces the pattern of neural activity observed during hot defensive bee ball formation and found that the pattern of Class II KCs-preferential *Acks* expression in the brains of bees exposed to 46°C was similar to that of bee ball-forming bees. This finding suggests that neural activity in the MBs, especially the Class II KCs, during bee ball formation mainly reflects thermal information processing. Furthermore, I demonstrated that *Acks* or *kakusei* expression is significantly induced between 44°C and 46°C not only in Japanese honeybees but also in European honeybees,

although European honeybees do not exhibit hot defensive bee ball formation, suggesting that the temperature-selective responsiveness of some KCs is conserved in honeybees and that the Japanese honeybees employ these neurons during bee ball formation.

Materials and Methods

Bees

Japanese honeybees (*Apis cerana japonica*) maintained at the apiary in Tamagawa University (Tokyo, Japan) were used for all experiments. Both European honeybee (*Apis mellifera* L.) and buff-tailed bumblebee (*Bombus terrestris*) colonies were purchased from the Kumagaya bee farm (Saitama, Japan) and Agrisect (Ibaraki, Japan), respectively, and maintained at the University of Tokyo (Tokyo, Japan).

Cloning of Acks cDNA

Total RNA was extracted from the whole brains of Japanese honeybee workers with seizures induced by awakening from anesthesia, using TRIzol Reagent (Invitrogen, Carlsbad, CA). To obtain cDNA fragments of the *kakusei* homolog in Japanese honeybee, PCR was performed using a set of primers designed based on the nucleotide sequence of the European honeybee *kakusei*, 5'-GGGGAAGCCAGGAGCCGCGGGTTTACAT-3' and 5'-AGGCAACAGCACACCATGGGCCTTGGAT-3', with Ex Taq (Takara, Tokyo, Japan). After sequencing the PCR products, we performed the rapid amplification of cDNA ends (RACE) method with a SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA) following the manufacturer's instruction. Amplification was performed with two sets of gene-specific primers for 5'-RACE and 3'-RACE. These amplified products were subcloned into pGEM-T vectors (Promega, Madison, WI) and sequenced.

Quantitative RT-PCR of the Acks transcript in the brains of workers after seizure induction

Japanese honeybee workers kept in a plastic chamber were maintained overnight in a dark incubator (LH-70CCFL; NK System, Osaka, Japan) at 25°C. The bees were then anesthetized in CO_2 for 3 min. To induce seizures, the bees were then left in normal air at room temperature. The bees were sampled at 0, 15, 30, 45, 60, 90, 120, 150, or 180 min after seizure induction. Control bees were maintained continuously in CO_2 and collected at the same time points (bees at 0 min were identical to seizure induced bees at 0 min). Bees used for the high temperature experiment were anesthetized and placed in an incubator set to 46°C. I sampled the bees at the same time points as in the previous experiments under room temperature for up to 150 min after seizure induction (at 180 min, the workers died). After anesthetizing workers by immersing them in ice-cold water, I dissected two brains each from five lots of workers at each time point. After homogenizing these samples with a bead cell crusher (MS-100; Tomy, Tokyo, Japan), total RNA was extracted using TRIzol reagent. RNA was reverse transcribed with a PrimeScript RT reagent Kit (Takara) and quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with LightCycler (Roche, Nutley, NJ) using SYBR Premix Ex TaqII (Takara) and gene-specific primers (*Acks*; 5'-AGTGATGTCTGACCGAGCA-3' and 5'-CGAACGCACTTTGGTTAGTC-3' *elongation factor-1 alpha*: *ef-1α*; 5'-TTGGTTTAAGGGATGGACTG-3' and 5'-CCATACCTGGTTTCAACACA-3' [Kawakita et al., 2008]). PCR products of *Acks* and *ef-1α* of known concentrations were used as standards. The amount of *Acks* transcript was normalized with that of *ef-1α* and calculated as the expression level relative to the value of the samples at 0 min in the room temperature experiment. Tukey-Kramer's test was

performed to examine the significant difference in the relative expression of *Acks* among the time points using JMP software (SAS, Cary, NC). There was no significant difference in the expression of e*f-1α* between control and seizure-induced workers irrespective of experimental temperature (data not shown).

Sampling of worker honeybees from a hot defensive bee ball

To continuously collect Japanese honeybee workers from the same bee ball, I used the following sampling procedure. A worker hornet with its stinger cut off and hung by a wire around its thorax, was presented to the bees at the entrance of the beehive. As soon as a bee ball was formed in the hive, we placed the bee ball in a beaker to separate the workers forming the bee ball from the other nestmates. The bees were collected from the surface of the bee ball with long tweezers at 0, 30, and 60 min after separation of the bee ball. I sampled the workers from four bee balls collected from three colonies. After anesthetizing the bees in ice-cold water, their brains were dissected, embedded in TissueTek O.C.T. Compound (Sakura Finetek, Torrance, CA), and rapidly frozen in dry ice and stored at -80°C until use.

Exposure to high temperature and quantification of Acks (and kakusei) transcript

Honeybee (approximately 15 individuals per a chamber) or bumblebee (8 individuals per a chamber) workers kept in plastic chambers were maintained overnight in a dark incubator (M-210F; TAITEC, Saitama, Japan) at 33°C. The workers were then placed into another incubator (LH-70CCFL; NK System) set to 38, 40, 42, 44, 46 or 48°C. After 30 min, they

were anesthetized in ice-cold water and their brains were dissected. To validate the tissue specificity of high temperature-induced *Acks* expression, total RNA was extracted from the brains, thoraxes, and abdomens of 46°C-exposed or control Japanese honeybee workers and then subjected to quantitative RT-PCR. To deprive appendages, antennae, proboscis, legs and wings were cut with fine scissors at the base of the scapus. As MB samples used for quantitative RT-PCR, optic lobes (OLs), subesophageal ganglion and dorsal lobes (DLs) were cut off from the dissected brains and the remaining MBs were used for the RNA extraction. Quantitative RT-PCR was performed with gene-specific primers (for Japanese honeybee: *rp49*; 5'-AAAGAGAAACTGGCGTAAACC-3' and 5'-TGGCAACATGTGACGAGTTT-3', for European honeybee: *kakusei*; 5'-CAAGACACTTCTACTGCGAACC-3' and 5'-CGAACGCACTTTGGTTAGTC-3', *rp49*; 5'-AAAGAGAAACTGGCGTAAACC-3' and 5'-CAGTTGGCAACATATGACGAG -3', for buff-tailed bumblebee: *kakusei*; 5'-ACACTTCTACTTACGAACCATT-3' and 5'-GATTATACTATTGATTGCTGTATTAC-3', *rp49*; 5'-GAAGAGAAACTGGCGTAAACC-3' and 5'-CAGTTGGTAACATGTGTCGAG-3'). PCR products of *Acks* (and *kakusei*) and *rp49* of known concentrations were used as standards. The amount of *Acks* (and *kakusei*) transcript was normalized with that of *rp49*. The *rp49* expression levels did not differ significantly among groups in any experiment (data not shown). Statistical analyses were conducted by Student's *t*-test, Tukey-Kramer's test, Dunnett's test, or two-way ANOVA using Statcel2 (OMS, Saitama, Japan). A P value less than 0.05 was regarded as significant. All data are shown as means ± standard error (SEM).

Exposure to isoamyl acetate (IAA)

Japanese honeybee workers maintained in the condition similar to those used for the heat-exposure experiments were placed into a Ziplock bag (Lion, Tokyo, Japan). The workers were then presented with a filter paper, on which 5 µl of IAA (Wako, Osaka, Japan) was spotted, for 10 min. After the workers were removed from the Ziplock bag and left in normal air for 20 min, they were anesthetized in ice-cold water and their brains were dissected.

Exposure to 4°C

European honeybee workers (approximately 80 individuals per a plastic chamber) were kept in plastic chambers were maintained overnight in a dark incubator at 33°C. The workers were then placed into a cold room (MCU-R1510; SANYO, Osaka, Japan) set to 4°C. After 30 min, they were anesthetized in ice-cold water and their brains were dissected into three parts, the MBs, the OLs and the other brain regions. A thermography (i3; FLIR Systems, Wilsonville, OR) was used for measurement of the surface temperature of the bees.

In situ hybridization and quantification of the density of the Acks signals

Digoxigenin (DIG)-labeled sense or anti-sense riboprobes corresponding to +4530b to +4806b were synthesized by *in vitro* transcription with a DIG RNA Labeling Mix (Roche). Frozen coronal brain sections (10 µm thick) were fixed in 4% paraformaldehyde in phosphate buffer (PB; pH 7.4) overnight at 4°C, treated with proteinase K (10 µg/ml) for 15 min and then with HCl (0.2 N) for 10 min, followed by acetic-anhydride solution for 10 min

at room temperature. The slides were washed with PB between each step. After dehydration through an ascending series of ethanol solutions, brain sections were hybridized with the riboprobes overnight at 60°C (>16 h). The riboprobes were diluted in hybridization buffer (50% formamide, 10 mM Tris-HCl, 200 μg/ml tRNA, 1×Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA at pH 7.6), heat-denatured at 85°C for 10 min, and then added to each slide. After hybridization, slides were washed in 50% formamide and 2×SSC at 60°C for 30 min, treated with 10 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO) in TNE (10 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl at pH 7.6) at 37°C for 30 min, and washed at 60°C in 2×SSC for 20 min and twice in 0.2×SSC for 20 min. DIG-labeled riboprobes were detected immunocytochemically with Anti-DIG Peroxidase (1:500; Roche), TSA Biotin System (PerkinElmer, Salem, MA), Alkaline Phosphatase Streptavidin (1:1000; Vector Laboratories, Burlingame, CA) and NBT/BCIP stock solution (Roche) according to the manufacturer's protocol.

To quantify *Acks* signal density, I randomly selected *in situ* hybridization sections that contained the MB pedunculus and DLs (as a schematic drawing shown in Fig. 3B) for each experiment. The square measure of each brain area containing *Acks*-positive neurons (specific soma area) was measured using ImageJ analysis software (NIH, http://rsb.info.nih.gov/ij). At the same time, the number of *Acks* signals in the selected area was manually counted and divided by the square measure to calculate the *Acks* signal density. The density of signals is presented as the value relative to 10,000 μm^2. Micrographs were numbered and an investigator (Prof. Takeo Kubo) blind to the bee type or treatment assignment counted the signals. Statistical analyses were conducted using Statcel2. Data are shown as means ± SEM.

Results

Identification of the kakusei homolog in Japanese honeybee

I first amplified parts of *kakusei* cDNA from Japanese honeybee using primers designed from the European honeybee *kakusei* cDNA sequences. RACE method was then used to identify the *Acks* cDNA of approximately 7.8kb in length. The nucleotide sequences of *Acks* cDNA of approximately 7.1 kb in length shared approximately 85% sequence identity with the European honeybee *kakusei* cDNA, and had additional unique sequences of approximately 700b at its 3' end. No 5'-upstream or 3'-downstream cDNA sequence was obtained by the RACE method, leading me to conclude that I obtained a full-length *Acks* cDNA.

Transient induction of Acks in response to seizures

To determine whether *Acks* has properties as a neural IEG, I used quantitative RT-PCR to analyze the time course of *Acks* expression in response to seizures. I induced seizure by awakening Japanese honeybee workers from CO_2-induced anesthesia at room temperature (25°C) as described previously [Kiya et al., 2007], and measured the *Acks* expression levels in the whole brain using quantitative RT-PCR. The relative *Acks* expression was approximately 5 to 7-fold higher from 30 to 60 min after seizure induction, and then decreased to basal levels (Fig. 2A). In control bees kept under anesthesia, there was no significant change in the expression level at any time-point sampled. This finding suggests that *Acks* shares similar properties with the European honeybee neural IEG, *kakusei*. In

addition, I also examined whether *Acks* upregulation after neural activation begins earlier under a higher temperature, because the temperature in the hot defensive bee ball rises to almost 46°C [Ono et al., 1995; Sugahara and Sakamoto, 2009; Sugahara et al., 2012]. In the bees awakened from anesthesia under the high temperature (46°C), *Acks* expression also peaked at 30 to 60 min (Fig. 2B). The expression levels during peak times at the higher temperature were similar to those at room temperature. The *ef-1α* expression levels did not differ significantly between control and seizure-induced workers at any sampling time, irrespective of the experimental temperature (25°C or 46°C; data not shown). Based on these findings, I concluded that *Acks* has neural IEG properties, even at the high temperature the bees are exposed to during the formation of a hot defensive bee ball.

Visualizing neural activity in Japanese honeybee brain with detection of Acks expression

I then examined whether *Acks* can be used as a neural activity marker in Japanese honeybee brain by visualizing the spatial distribution of the *Acks* transcript in the brain after seizure induction using *in situ* hybridization. Bees at 30 min after seizure induction and control bees without anesthesia or seizures were analyzed. In the seizure-induced bees, a large number of *Acks* signals was observed in most brain regions, including the MBs (higher brain center), OLs (primary visual center), and a restricted area between the DLs and the OLs, as spots localized in the somata (Fig. 3C-H). A previous study using fluorescent *in situ* hybridization demonstrated that *kakusei* transcript signals are localized exclusively in the nuclei, suggesting that the *kakusei* transcript functions as a nuclear non-coding RNA [Kiya et al., 2007]. Thus, my findings of the spotted *Acks* signals in the somata coincided with the previous notion. On the other hand, the brains of the control bees contained very few signals

(Fig. 3I-N). These findings suggest that neural activity was induced in various brain regions, including the MBs, OLs, and the area between the DLs and the OLs, in the Japanese honeybees soon after induction of the seizure. I concluded that *Acks* could be utilized as a marker of neural activity that occurred 30 to 60 min before in the brains of Japanese honeybees.

Identification of brain regions active in Japanese honeybees during the formation of a hot defensive bee ball

Using *Acks* as the neural activity marker, I next attempted to find brain regions of the Japanese honeybee workers that were active during the formation of a hot defensive bee ball. Because the hot defensive bee ball is usually formed within the beehive [Ono et al., 1995], it is difficult to collect only the workers involved in forming the bee ball. Therefore, I attempted to induce Japanese honeybee workers to form a bee ball artificially using giant hornets as a decoy (Fig. 4). First, the bees were presented with a giant hornet that was hung by a wire at the entrance of the beehive. After a short time (approximately 5 min), when the decoy giant hornet was inserted into the beehive through the entrance, the Japanese honeybee workers immediately crowded around the decoy hornet to form a hot defensive bee ball. The bee ball was then recovered from the upper site of the hive and transferred to a glass beaker to collect only workers that formed the bee ball. I sampled the bees from the surface of the bee ball at 0, 30, and 60 min after formation of the bee ball and examined the *Acks*-expressing brain regions using *in situ* hybridization (n=5, 7 and 7 for 0, 30 and 60 min, respectively).

The previous experiments indicated a 30- to 60-min time lag between neural

activation and *Acks* upregulation in the worker brain (Fig. 2). Therefore, I considered that the *Acks* signals detected at each sampling time mainly reflect neural activity involved in the following phases of the bee ball formation; 0 min: normal phase before presentation of the hornet; 30 min: early phase of bee ball formation, such as the recognition of the hornet and/or formation of the bee ball; 60 min: late phase of the bee ball formation, such as the maintenance of the bee ball (Fig. 4). In several brain regions, characteristic *Acks* expression was observed in a region-preferential and sampling time-dependent manner (Fig. 5B).

Honeybee MBs are a paired structure comprising many thousands of intrinsic neurons called Kenyon cells (KCs) and their dendrites form two cup-shaped neuropiles, calyces, which receive sensory inputs [Strausfeld, 2002; Fahrbach, 2006]. The KC axon terminals and their postsynaptic targets form the lobes in which integrated sensory information is transferred. KCs are classified into two distinct types, Class I and Class II KCs [Mobbs, 1982; Strausfeld, 2002; Fahrbach, 2006]. Whereas the somata of Class I KCs are located inside of the MB calyces, the somata of Class II KCs are located outside of the calyces (Fig. 5C). Interestingly, the *Acks* signals were most densely detected in the Class II KCs at 30 and 60 min after the bee ball formation (Fig. 5H and I), whereas they were scarcely detected at 0 min (Fig. 5G). In contrast, much less *Acks* signals were detected in the OLs, during sampling time (Fig. 5M-O). *Acks* signals were also detected in some neurons whose somata formed a cluster in a restricted region between the DLs and the lobula of the OLs at 60 min after the bee ball formation (Fig. 5L). Scarce *Acks* signals were detected at 0 min in this restricted region between the DLs and the lobula of the OLs (Fig. 5J), indicating that the *Acks* signals were detected in a sampling time-dependent manner. No significant signal was detected in any of the brain regions hybridized with sense probe (data not

shown).

Statistical analyses of the number of *Acks* signals detected in *in situ* hybridization experiments using sections corresponding to the middle brain parts (Fig. 5A and B) (n=5, 5, and 4 for 0, 30, and 60 min, respectively) revealed that there was a significant increase in the number of *Acks* signals at 30 and 60 min after the bee ball formation in both the Class I and the Class II KCs, and at 60 min after the bee ball formation in the brain area between the DLs and the OLs, and in the OLs (Fig. 5P). The *Acks* signal density, however, was highest in the Class II KCs at both 30 and 60 min after the bee ball formation. These findings suggested that neural activity of the KCs, especially the Class II KCs and a certain subpopulation of neurons located between the DLs and the OLs, was increased in the brains of workers during formation of the hot defensive bee ball. It is also plausible that some visual input was processed in the OLs of the workers after the bee ball formation, because I picked up the bee ball outside the hives to sample the workers.

The MBs are believed to be a higher-order center of the insect brain that processes complex multimodal information [Li and Strausfeld, 1997 and 1999; Strausfeld, 2002; Fahrbach, 2006]. In current opinion, sophisticated roles in adaptive behaviors such as predictive motor actions [Mizunami et al., 1998], short- and long-term associative memory [Zars et al., 2000; Lozano et al., 2001; Menzel, 2001; Pascual and Préat, 2001; Blum et al., 2009; Akalal et al., 2010] and temperature preference behavior [Hong et al., 2008; Bang et al., 2011], are ascribed to the MBs. Possible involvement of the MBs in adaptive antipredator behavior, however, has not been reported in any insects, including the honeybee.

Exposure to 46°C heat but not to honeybee alarm pheromone reproduced neural activity in the KCs observed during hot defensive bee ball formation

During the formation of a hot defensive bee ball, Japanese honeybee workers are exposed to high temperature typically around 46°C [Ono et al., 1995]. In addition, 3-methylbutyl acetate (also known as isoamyl acetate: IAA), which is the major component of the honeybee alarm pheromone [Free, 1987], is emitted from a hot defensive bee ball [Ono et al., 1995]. Therefore, to investigate what kind of information processing induces the observed neural activity in the brains of bees from the defensive bee ball, I examined *Acks* expression patterns in the brains of Japanese honeybee workers exposed to high temperature (42°C or 46°C) or IAA. In the brains of the workers exposed to 46°C for 30 min, a lot of spotted *Acks* signals were preferentially detected in Class II KCs and sparsely distributed in Class I KCs (Fig. 6A and D). *Acks* signals were also detected in some neurons whose somata formed a cluster in a restricted region between the DLs and the lobula of the OLs (Fig. 6G).

Statistical analyses of the number of *Acks* signals detected in *in situ* hybridization experiments indicated that the numbers of *Acks* signals significantly increased in the Class I and Class II KCs and in the brain area between the DLs and the OLs in the heat-exposed workers compared with those of control workers, whereas there were no significant differences of the number of *Acks* signals in the OLs (Fig. 6J). The highest *Acks* signal density, however, was observed in the Class II KCs. This *Acks* signal distribution pattern in the brains of workers exposed to heat well resembles that observed in the brains of workers involved in the formation of the hot defensive bee ball (Fig. 5P).

In contrast, no significant increase in the density of *Acks* signal was detected in the MBs or the restricted region between the DLs and the OLs in the brains of workers exposed

to IAA (Fig. 6C, F and I). Interestingly, there were also few significant signals the MBs or the restricted region between the DLs and the OLs in the brains of workers exposed to 42°C (Fig. 6B, E and H). These findings suggest that information processing of alarm pheromone does not contribute mainly to the neural activity of Class II KCs, which is observed in the brains of workers involved in the formation of the hot defensive bee ball, and that some KCs show temperature selective response and have threshold between 42°C and 46°C to induce neural activity.

Acks induction by heat-treatment was selective in the brain

Next, I analyzed the tissue specificity of *Acks* induction under a high temperature. Quantitative RT-PCR using RNAs extracted from brain, thorax and abdomen of workers revealed that statistically significant and prominent *Acks* induction was observed only in the brain when the bees were exposed to high temperature, and scarce *Acks* expression was detected in the thorax and abdomen under both room and high temperatures (Fig. 7), supporting that *Acks* induction under a high temperature reflects neural activity and does not result from systemic 'heat shock responses' that could occur independently of neural activity, although I cannot completely exclude the possibility of some brain specific stress responses.

Determination and comparison of the temperature threshold for KC neural activation in Japanese and European honeybees, and buff-tailed bumblebees

1) Japanese honeybees and European honeybees

In the former experiment, I found that some KCs in Japanese honeybees selectively respond to high temperature (Fig. 6). Because there is only a 3°C to 5 °C difference in the lethal

temperature between Japanese honeybees and giant hornets [Ono et al., 1995; Sugahara and Sakamoto, 2009; Sugahara et al., 2012], accurate monitoring and precise control of heat generation during the formation of a hot defensive bee ball would be critical for the Japanese honeybee workers. Therefore, I attempted to determine the temperature threshold for activation of these KCs and examined whether the temperature selectivity of KCs is specific for Japanese honeybees.

Japanese and European honeybees were exposed to 40°C, 42°C, 44°C, 46°C or 48°C for 30 min. At each temperature, both honeybee species kept in distinct chambers were exposed simultaneously. After that, the MBs were dissected out and the expression level of *Acks* and *kakusei* was analyzed in Japanese and European honeybees using quantitative RT-PCR. Statistical analyses revealed that these IEGs were significantly upregulated at temperatures between 44°C and 46°C, and showed maximal expression at 46°C and 48°C in both honeybee species (Fig. 8A and B). *In situ* hybridization using the brains of European honeybee workers exposed to 46°C revealed that the signal distribution pattern of *kakusei* in the MBs (Fig. 8C and D) was similar to that of *Acks* observed in the previous experiments using Japanese honeybees exposed to 46°C (Fig. 6A and D). These findings suggest that the high-temperature responsive KCs have a threshold between 44°C and 46°C, even in European honeybees, which do not exhibit hot defensive bee ball formation.

2) Buff-tailed bumblebees

To examine whether bee species other than honeybees (Genus *Apis*) have high-temperature responsive KCs, I performed a comparative analysis using buff-tailed bumblebees (*Bombus terrestris*). Buff-tailed bumblebees are eusocial Hymenopteran insects belonging to the same

family (*Apidae*) but a different genus (*Bombus*) as honeybees, and whose genomic information is available [Munoz-Torres et al., 2011]. Thus, I cloned a cDNA fragment of the *kakusei* homolog in the bumblebee and verified that the bunblebee *kakusei* also showed increased expression after the seizure induction (Fig. 8E), and exposed the bumblebee workers to high temperatures (38°C, 40°C, 42°C, 44°C, or 46°C). Quantitative RT-PCR and statistical analyses revealed that the expression of bumblebee *kakusei* in the MBs was increased at temperatures between 38°C and 40°C and peaked at 40°C and 42°C (Fig. 8F). Taken together with the findings from the honeybees, although there is an apparent difference in temperature threshold for neural activation between honeybees and bumblebees, the temperature-selective responsiveness of the KCs is conserved, at least in the family *Apidae*.

Evaluation of the contribution of thermal input from appendages to neural activation in the MBs

A previous study in honeybees reported that ablation of the antennal flagella impairs warmth avoidance behavior in European worker honeybees [Kohno et al., 2010]. Thus, I next evaluated the contribution of sensory input from the appendages, including the antennae, to the activity of brain neurons under high temperature (46°C). Quantitative RT-PCR and statistical analyses revealed that there was no significant difference in *kakusei* expression levels in the MBs between heat-exposed workers whose appendages (antennae, proboscis, legs, and wings) were ablated and heat-exposed intact workers (Fig. 9). This finding suggests that the contribution of sensory input from the peripheral appendages to the neural activity in the whole MBs of heat-exposed workers is, at most, partial.

Analysis of neural activity in the brains of honeybees exposed to 4°C

When honeybees are exposed to an extremely low temperature, such as in winter, they also form a bee ball to generate heat and avoid hypothermia [Stabentheiner et al., 2002]. Therefore, I used this bee ball formed under the extremely low temperature as a control to examine whether the neural activity observed in the brains of hot defensive bee ball forming bees reflects responses to high temperature rather than other factors related to the bee ball formation, such as gathering and flight muscle vibration. For this, I exposed European honeybee workers to 4°C in a plastic chamber. After 30 min, the bees gathered in the bee ball formation and the temperature of the bee ball core increased over 35°C (Fig. 10A). The brains were then dissected out and the *kakusei* expression level in the MBs, the OLs and the other brain regions was analyzed by quantitative RT-PCR. There was no increase in *kakusei* expression compared to control bees (left at 33°C for 30 min) in any brain regions (Fig. 10B). These findings support that the neural activity during hot defensive bee ball formation is induced by high temperature rather than simply by gathering or by vibrating their flight muscles.

Discussion

In this chapter, I identified *Acks*, a Japanese honeybee homolog of *kakusei*, which is a neural IEG in the European honeybee and first demonstrated that *Acks* signals were detected in the MBs, especially in the Class II KCs of workers at 30 or 60 min after the bee ball formation, and neurons located in a restricted area between the DLs and the OLs of workers at 60 min whereas they were scarcely detected at 0 min (Fig. 5), strongly suggesting that these neurons are highly activated in the brains of workers involved in forming the hot defensive bee ball. Further *in situ* hybridization experiments for *Acks* revealed that the *Acks* distribution patterns observed in the brains of workers involved in the bee ball formation were clearly mimicked by those observed in the brains of 46°C-exposed workers but not by those observed in 42°C-exposed or IAA-exposed workers (Fig. 6). These results strongly suggested that the neural activity detected in the brains of workers involved in the formation of the hot defensive bee ball was induced by high temperature but not by IAA generated during the formation of the bee ball.

Quantitative RT-PCR analyses using bees exposed to various high temperatures revealed that *Acks* expression in the MBs was significantly increased between 44°C and 46°C (Fig. 8A), suggesting that some KCs have an activation threshold between 44°C and 46°C. Previous studies on the time-course for the temperature shift in a hot defensive bee ball reported that once Japanese honeybee workers start to form the bee ball, the temperature inside of the bee ball quickly rises and reaches 46°C within 5 min [Sugahara and Sakamoto, 2009; Sugahara et al., 2012]. After that, the inner temperature no longer rises [Sugahara and Sakamoto, 2009; Sugahara et al., 2012] and is maintained at ~46°C for more than 15 min

[Ono et al., 1995]. Therefore, it is plausible that Japanese honeybees have neural mechanisms for selectively detecting 46°C, allowing the bees to avoid exposure to unnecessary high (near lethal) temperatures and to maintain the inner temperature of the bee ball at an appropriate level. I speculate that some temperature-selective KCs, most likely Class II KCs, are involved in thermal information processing, to appropriately regulate the duration of flight muscle vibration and to control heat generation during formation of the bee ball. Interestingly, the temperature-selective responsiveness of the KCs was also observed in European honeybee and buff-tailed bumblebee based on the quantification of *kakusei* transcripts (Fig. 8B and F). The nest temperature of both Japanese and European honeybees is usually maintained at ~33°C [Okada, 1975; Yoshida, 2005] whereas buff-tailed bumblebees prefer a lower temperature (~27°C) [Weidenmüller, 2004]. Thus, in the family *Apidae*, the high-temperature responsive KCs might originally be related to the regulation of body or nest temperature when the bees are exposed to a much higher temperature, and Japanese honeybees might have applied this mechanism to maintain the appropriate temperature during bee ball formation.

Quantitative RT-PCR revealed that the amount of *Acks*-transcript in the whole MBs of the heat-exposed workers was not significantly affected by appendage ablation (Fig. 9). Therefore, it might be that the contribution of the peripheral sensory input to the neural activity of the MBs of heat-exposed workers is partial or minor. In *Drosophila melanogaster*, a small set of warmth-activated neurons located in anterior part of brain was critical for ambient temperature sensing and regulation of temperature preference behavior [Hamada et al., 2008; Tang et al., 2013] although the higher projection pattern of these warmth-activated neurons is not well characterized [Shih and Chiang, 2011]. It is possible that some MB

neurons that receive thermal information from other brain neurons and/or MB neurons directly activated by high temperature are responsible for the increased neural activity in the MBs of heat-exposed workers.

Statistical analyses indicated that the number of *Acks* positive cells in the restricted area between the DLs and OLs, in addition to Class II KCs, also increased significantly compared to the control bees upon exposure to 46°C (Fig. 6G and J). In European honeybee, somata of a GABAergic recurrent neuron cluster called A3v are located in this restricted brain portion between the DLs and lobula of the OLs and these neurons receive input from the lobe of the MBs and provide inhibitory feedback to the calyces [Rybak and Menzel, 1993; Grünewald, 1999a; Ganeshina and Menzel, 2001]. A previous electrophysiological study demonstrated that individual feedback neurons showed multimodal sensitivity [Grünewald, 1999b]. It might be that the neurons located in this area are also involved in processing thermal information in the worker honeybees. To test this possibility, it will be necessary to examine whether the *Acks*-expressing neurons are A3v neurons by double staining for *Acks* expression and immunoreactivity to a GABA-synthesizing enzyme.

Kiya et al. (2007) previously demonstrated that the expression of the *kakusei* transcript was induced specifically in the brain, but not in any other body part, when European honeybee workers were awaken from anesthesia. In the present study, I also demonstrated that *Acks* was induced almost exclusively in the brain even under high temperature (Fig. 7). These results argue against the possibility that *Acks* induction in the brains of workers during formation of a hot defensive bee ball or heat-exposed workers merely represents a ‘heat-shock response’. This possibility cannot be completely excluded,

however, because some vertebrate heat-shock proteins are induced in specific body parts [Kawazoe et al., 1999], although other heat-shock protein genes are expressed ubiquitously, irrespective of the body part and cell type [Lindquist, 1986]. This ambiguity is partly attributed to the lack of detailed studies of the induction mechanisms of *kakusei*. It will be important to examine whether these findings are reproducible by using homologs of well-characterized IEGs utilized in vertebrates, although there has been no report of conserved IEGs available for detecting neural activity between vertebrates and insects. In next chapter, I address this problem.

Chapter II

Identification and characterization of an *Egr* ortholog as a neural immediate early gene in European honeybee and confirmation of the high-temperature responsiveness of Kenyon cells

Introduction

In the study described in Chapter I, I used *Acks* and *kakusei* as neural activity markers, and found that the neural activity observed in the brains of Japanese honeybee workers involved in hot defensive bee ball formation was evoked by thermal stimuli from the bee ball (Fig. 5 and 6) and that some Kenyon cells (KCs) in the mushroom bodies (MBs) exhibit a temperature-selective response in honeybees and bumblebees (Fig. 8).

The *Acks* and *kakusei* transcripts, however, do not contain a long open-reading frame that encodes a protein [Kiya et al., 2007; Ugajin et al., 2012], unlike vertebrate immediate early genes (IEGs), which generally encode proteins [Loebrich and Nedivi, 2009], and their expression was detected as dotted signals localized in neuron somata by *in situ* hybridization, suggesting that *Acks* and *kakusei* transcripts function as a long nuclear RNA in the nervous system, although their role is totally unknown. It is important to examine whether the findings of thermosensitive neurons are reproducible using other well-characterized IEGs. In addition to the unconventional characteristics of *kakusei*, homologs of *kakusei* are found in the genome database of only honeybees and bumblebees. Because, as mentioned above, bumblebees also have thermosensitive KCs, as evidenced by the increased expression of *kakusei* in the MBs at high temperatures (Fig. 8F), conserved IEGs are needed for comparative studies aimed at elucidating the evolutionary origin of thermosensitive KCs in various insect species.

In Chapter II, with the expectation that an IEG with similar characteristics in both mammals and honeybees shares conserved properties within insects, I attempted to identify the homolog of a well-characterized mammalian IEG in the European honeybee and to

examine whether the high-temperature responsiveness of the KCs in the bee brain is reproduced using homologous genes. After the initial report of the identification of *kakusei*, some studies reported neural IEGs of insect origin: i.e., *c-fos*/*fra* (*fos related antigen*) in the cricket (*Acheta domesticus*) [Ghosal et al., 2010] and *c-jun* [Alaux and Robinson, 2007; Alaux et al., 2009] in the honeybee. The actual applicability of *c-fos*/*fra* and *c-jun* to activity mapping, however, is unclear, because in most cases, the induction of these genes upon neural excitation has not been prominent. Indeed, in previous experiments using European honeybees, my collaborators detected no increased expression of *c-fos* or *c-jun* homologs in the brains in response to seizure induction, suggesting that these homologous genes do not have the properties of neural IEGs in honeybees [Kiya et al., unpublished]. Therefore, in the present study, I focused on finding homologs of other widely used neural IEGs in vertebrates, *Egr-1* (*early growth response protein 1*) and *Arc* (*activity-regulated cytoskeleton-associated protein*).

Egr-1 (also known as *zif268*, *Krox-24*, *NGFI-A*, *TIS8*, and *ZENK* in vertebrates [Knapska and Kaczmarek, 2004]) encodes a transcription factor with a DNA-binding domain comprising unique three tandem Cys_2His_2 zinc finger motifs [O'Donovan et al., 1999]. *Egr-1* was originally identified as an IEG based on its response to serum treatment in mouse fibroblasts [Lau and Nathans, 1987], as well as to nerve growth factor treatment in rat PC12 cells [Milbrandt, 1987]. Subsequent studies revealed that *Egr-1* is also induced in the nervous system by neural activity [Beckmann and Wilce, 1997]. Since then, *Egr-1* has been widely utilized as a neural activity marker to identify brain regions relevant to specific behaviors and learning processes in vertebrates [Mello and Clayton, 1994; Guzowski et al., 2001; Tokuyama et al., 2002; Burmeister and Fernald, 2005; Avey et al., 2008; Chakraborty

et al., 2010; Harvey-Girard et al., 2010; Lonergan et al., 2010]. *Arc* (also known as *Arg3.1*) was first described in 1995 as a nontranscription factor IEG upregulated after electrically induced seizure in rat brain [Lyford et al., 1995] and nerve growth factor treatment in PC12 cells [Link et al., 1995]. Uniquely, newly transcribed *Arc* mRNA is trafficked rapidly to dendrites where the RNA accumulates at sites of synaptic activity and is translated locally [Steward et al., 1998]. As well as *Egr-1*, *Arc* also utilized to map active brain regions involved in adaptive behaviors and memory storage [Guzowski et al., 1999, 2000 and 2001; Bramham et al., 2010; Lonergan et al., 2010].

In the first part of Chapter II, I show that an *Egr-1* homolog but not an *Arc* homolog is significantly induced in the honeybee brain after both pharmacologically evoked neural activity by picrotoxin treatment and seizure induction. Furthermore, I used *in situ* hybridization to show an increased signal for the *Egr-1* homolog (*AmEgr*) in forager brains in a certain MB neuron subtype. These findings indicate that an *Egr* homolog gene can be utilized as a neural activity marker even in the honeybee. In the latter part of Chapter II, I analyzed the expression pattern of *AmEgr* in the brains of bees exposed to a high temperature and demonstrated that the distribution pattern of *AmEgr* signals in the MBs of bees exposed to 46°C was similar to that of *kakusei*, and that *AmEgr* expression was dramatically increased at temperatures over 46°C. These findings strongly supported the high-temperature selective responsiveness of the KCs in bee brains and the validity of *kakusei* as a neural IEG.

Materials and methods

Bees

European honeybee (*Apis mellifera* L.) colonies were purchased from the Kumagaya bee farm and maintained at the University of Tokyo. Nurse bees and foragers were collected according to their behaviors, as described previously [Kubo et al., 1996].

Isolation of AmEgr cDNA

The cDNA sequence of *AmEgr* (GB15421) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) with total RNA isolated from seizure-induced worker brains and primers designed based on predicted 5' and 3' untranslated region of *AmEgr*: 5'-AAAAGCTGCGGAATTCGTTTTTCCTTTCTCCTCCT-3' and 5'-ACTAGTCTAGCGGCCCTGGTGGAGATCGACGGTAT-3'. Three zinc finger motifs were identified by a SMART (http://smart.embl-heidelberg.de/) search. Alignments of protein sequences of EGR homologs were generated using ClustalX software.

Quantification of the AmEgr transcript

Under ice-cold anesthesia, worker honeybees randomly collected from a beehive and kept in a plastic insect cage overnight at room temperature were fixed to small corkboards with staples to immobilize their heads. The bees were left at room temperature for 3 h to allow them to completely recover from ice-cold anesthesia. Picrotoxin (Tocris Bioscience, Bristol,

UK) (1 μl of 500 μM dissolved in honeybee Ringer solution [Vergoz et al., 2007]) or Ringer solution alone (control) was injected into the brain through the median ocellus using a fine glass capillary. The bees were then set free and kept at room temperature. After 30 min, they were anesthetized in ice-cold water and the MBs were dissected out. Quantitative RT-PCR was performed with Light Cycler (Roche, Nutley, NJ) and SYBR Premix Ex TaqII (Tli RNaseH Plus) (TaKaRa, Tokyo, Japan), according to the manufacturer's protocol with gene-specific primers (*AmEgr*; 5'-GAGAAACCGTTCTGCTGTGA-3' and 5'-GCTCTGAGGGTGATTTCTCG-3', GB11926; 5'-TGGAAAAGGTGAACGAGGAC-3' and 5'-CCATGCTCCAAGACTCGTTC-3' *rp49*; 5'-AAAGAGAAACTGGCGTAAACC-3' and 5'-CAGTTGGCAACATATGACGAG-3'). The seizure-induction experiment was performed as described previously [Ugajin et al. 2012].

To test whether *AmEgr* expression observed in the forager brain is neural activity (foraging experience)-dependent, I first collected nurse bees and foragers from a hive based on their behaviors [Kubo et al., 1996]. The foragers and nurse bees were then divided into two groups, and the MBs were immediately dissected out of half of each group. The second half of each group was maintained in a plastic insect cage in a dark incubator for 5 h to suppress *AmEgr* expression and then their MBs were dissected out. To quantify *AmEgr* pre-mRNA, two primers were designed within the intron (5'-ATCCCTTGCGTACACACCTC-3' and 5'-AATCGCACCAGATTCCACTC-3'). The amount of *AmEgr* transcript was normalized with that of *rp49*. Statistical analyses were conducted by Student's *t*-test, Tukey-Kramer's test, Dunnett's test, or two-way ANOVA using Statcel2 (OMS, Saitama, Japan). The *rp49* expression levels did not differ significantly among groups in any experiment (data not shown). All data are shown as

means ± SEM.

In situ hybridization and quantification of the density of AmEgr signals in the MBs

The *AmEgr* intron fragment was amplified with two primers; a forward primer, the same as that used in quantitative RT-PCR, and a reverse primer; 5'-GCTTGTCGCGGATCAATACT-3' using genomic DNA extracted from the thorax of the worker honeybee. *In situ* hybridization was performed with digoxigenin (DIG)-labeled riboprobes and TSA Biotin System (PerkinElmer, Salem, MA), as described previously [Ugajin et al., 2012]. For quantification, we randomly selected *in situ* hybridization sections that contained the MB pedunculus and DLs (as in the schematic drawing shown in Fig. 3B) for each experiment. The number of *AmEgr* pre-mRNA signals in the lKCs or sKCs was manually counted and normalized to the total number of the lKCs or sKCs somata in each section, respectively. Data are shown as means ± SEM.

Results

Exploration of honeybee homologs of Egr-1 and Arc

I searched for an *Egr-1* and *Arc* homologs in the European honeybee genome database and found that GB15421 could encode a protein (LOC726302, XP001122050.2) with three tandem zinc finger domains that has significant amino acid sequence identity (83%) with the corresponding region of mouse EGR-1. As for *Arc*, GB11926 could encode a protein (LOC 725123, XP001121008.1), which showed 22% amino acid sequence identity with the C-terminus half of mouse ARC. Then, I tested whether these candidate genes show increased expression in response to neural activation.

In mammals and birds, expression of many neural IEGs is induced in brains 30 to 60 min after treatment with pentylenetetrazol or picrotoxin (PTX) [Saffen et al., 1988; Murphy et al., 1991; Mello and Clayton, 1995], both of which are $GABA_A$ receptor antagonists [Olsen and DeLorey, 1999]. PTX also acts as an ionotropic GABA receptor blocker in the honeybee MBs [Grünewald and Wersing, 2008]. Therefore, to examine whether these candidate genes are induced in the brains of worker honeybees treated with PTX, I injected 1 μl of 500 μM PTX into the brains of awake immobilized honeybees through the median ocellus (Fig. 11A and B) and used quantitative RT-PCR to quantify the transcripts of these genes in the MBs 30 min after PTX injection. The amount of GB15421 transcript increased approximately 3-fold in PTX-injected bees in comparison with Ringer solution-injected bees (Fig. 11C). Kiya et al. (2007) previously reported that *kakusei* is induced approximately 4-fold in bees 15 to 30 min after seizures induced by awakening from CO_2-induced anesthesia. Expression of GB15421 transcript in the honeybee brain was

induced approximately 6-fold upon seizure induction (Fig. 11D). On the other hand, GB11926 showed neither PTX injection nor seizure induction-dependent upregulation (Fig. 11E and F). These results strongly suggest that *Egr-1* homolog is increased in response to neural activation but *Arc* homolog is not in honeybee.

Determination of Egr-1 homolog sequence

To determine the amino acid sequence of the GB15421 product, its putative open reading frame was isolated by RT-PCR using primers designed based on the 5' and 3' untranslated regions. The GB15421 product was 584 amino acids in length. The zinc finger domains of this protein had significant sequence identity (more than 75%) with corresponding regions of EGRs from mouse (EGR-1, NP031939.1; EGR-2, NP034248.2; EGR-3, NP061251.1; EGR-4, NP065621.1), fruit fly (STRIPE, NP001262693.1), and nematode (EGRH-1, NP510462.2) (Fig. 12). I named this gene *AmEgr* (*Apis mellifera Egr*; GenBank: KC986376). As there are no other genes that encode proteins that exhibit sequence similarity with *AmEgr* in the honeybee genome database, I concluded that *AmEgr* was the only *Egr* family member in the honeybee.

Time-course analyses and visualization of neural activity by detection of the AmEgr transcript

Next, I analyzed the time-course of *AmEgr* expression in response to seizure induced by awakening from anesthesia. Quantitative RT-PCR revealed that *AmEgr* is transiently induced approximately 10-fold, peaking at 30 to 60 min after seizure induction (Fig. 13A). Further quantitative analysis using the primer set designed to amplify the intron sequence of

AmEgr revealed that the *AmEgr* pre-mRNA is transiently induced approximately 30-fold, peaking at 15 min after seizure induction (Fig. 13B and C). These results suggest that both *AmEgr* mRNA and pre-mRNA are applicable for detecting neural activation, while pre-mRNA detection is more suitable for detecting neural activity with higher temporal resolution and sensitivity.

To examine whether *AmEgr* pre-mRNA can actually be used as a neural activity marker in the honeybee brain, I performed *in situ* hybridization using DIG-labeled antisense riboprobe designed to correspond to a part of the intron against worker samples at 0 and 15 min after seizure induction by awakening from anesthesia. In the seizure-induced bees, a large number of dotted signals of *AmEgr* pre-mRNA was detected in the somata of neurons in several brain regions, including antennal lobes (ALs), MBs, optic lobes (OLs), and the area between dorsal lobes (DLs) and OLs (Fig. 14K-R). In contrast, only a few positive cells were observed in the brains of the bees at 0 min after seizure induction (Fig. 14C-J). Dot-like signals, which seemed to reflect nuclear localization of the *AmEgr* pre-mRNA, enabled the detection of individual *AmEgr*-positive neurons with high spatial resolution. Quantitative RT-PCR using RNAs extracted from MBs, OLs, and the other brain regions confirmed that *AmEgr* pre-mRNA were induced in all brain regions tested after seizure induction, while the highest increase in expression was detected in the MBs (Fig. 15A). *kakusei* also showed induced expression in each brain regions after seizure induction (Fig. 15B). No dot-like signals of *AmEgr* were observed in sections hybridized with sense probes (Fig. 14S-Z). Therefore, I concluded that *AmEgr* is applicable as an IEG to identify active brain regions in the honeybee.

Detection of neural activity in the forager brains

In the former experiments, I detected *AmEgr* upregulation in response to artificially induced neural activity (using drug or seizure). It is important for using *AmEgr* as a neural activity marker to show increased expression in response to naturally occurred neural excitation. My collaborators previously used *kakusei* to show that the neural activity of the small- and middle-type KCs (s/mKCs), one subtype of the Class I KCs, is increased in the brains of foragers, whereas MB neurons are not significantly activated in the bees engaged in nursing their brood (nurse bees) [Kiya et al., 2007; Kaneko et al., 2013]. Therefore, I intended to detect neural activity in the forager brain by *in situ* hybridization using *AmEgr*. In the MBs of foragers, many more dotted signals were detected in s/mKCs, whose somata are located in the inner core of the MB calyces, whereas fewer signals were detected in the large-type KCs (lKCs), the other subtype of the Class I KCs, whose somata are located at the both edges inside of the calyces (Fig. 16C and D). In contrast, only a few signals were detected in the MBs of the nurse bees (Fig. 16E and F). The number of *AmEgr* signals was approximately 4 to 6-fold higher in both lKCs and s/mKCs in the forager brains compared with those in nurse bee brains. Furthermore, in the forager brains the number of *AmEgr* signals in s/mKCs was approximately 2-fold higher than that in lKCs (Fig. 16G).

I also questioned whether *AmEgr* expression in forager brains is foraging behavior-dependent rather than age-dependent because worker honeybees have an age-dependent division of labor [Gould and Gould, 1995]: young bees work as nurse bees while older ones work as foragers. To address this question, I expected that if the increased *AmEgr* expression in the MBs of the foragers is actually foraging-relevant neural activity-dependent, the *AmEgr* expression level would decrease to basal levels, almost

equivalent to that in nurse bee brains, when foragers were maintained for 5 h in an insect cage, because the increased *AmEgr* pre-mRNA expression decreased to almost basal levels within 2 h after neural excitation (Fig. 13B). In this experiment, the *AmEgr* expression level was approximately 2-fold higher in forager brains than in nurse bee brains (Fig. 16H), consistent with the results of the preceding experiment (Fig. 16G). The expression level decreased within 5 h to almost the same level as that in the nurse bee brains. Finally, I found that upon seizure induction *AmEgr* upregulation occurred not only in the forager brains, but also in the nurse bee brains (Fig. 16I). My findings further support the previous notion that neural activity is more predominant in s/mKCs than in lKCs in the forager brains [Kiya et al., 2007], and demonstrate that *AmEgr* is also applicable for detecting behavior-induced neural activity in the honeybee brain.

Detection of temperature-selective response of the KCs using AmEgr

I verified the increased *AmEgr* expression in the brains of honeybees exposed to a high temperature. I performed quantitative RT-PCR using RNAs extracted from the MBs, the OLs, and the other brain regions dissected from bees exposed to 33°C or 46°C and compared the *AmEgr* and *kakusei* expression levels. High-temperature induced *kakusei* expression was observed in each region, although the increase in the expression in the OLs was lower than that in the MBs and the other brain regions (Fig. 17B). In contrast, *AmEgr* was upregulated only in the MBs of bees exposed to 46°C (Fig. 17A). *In situ* hybridization analyses revealed that the expression pattern of *AmEgr* in the MBs of bees exposed to 46°C also resembled that of *kakusei* (Fig. 17C-F). These findings confirm that some KCs are activated upon exposure to 46°C, although there might be some differences between the

MBs versus the OLs and the other brain regions in the physiological processes or molecular mechanisms operating in these neurons under high temperature.

Finally, I attempted to detect the neural activity of the high-temperature responsive KCs using *AmEgr*. Similar to the findings using *kakusei* (Fig. 8), the *AmEgr* expression level was significantly increased at temperatures between 44°C and 46°C (Fig. 17G). These results strongly support temperature-selective response of some KCs in the honeybees.

Discussion

In this chapter, to identify honeybee homologs of well-known IEGs in vertebrates, I focused on the honeybee homologs of *Egr-1* and *Arc*, and demonstrated that the *Egr-1* homolog (*AmEgr*) shared similar properties as a neural IEG with *Egr-1* in vertebrates.

In addition, *in situ* hybridization analysis for *AmEgr* indicated that the neural activity is more predominant in s/mKCs than in lKCs in forager brains (Fig. 16C-G), strongly suggesting that *AmEgr* expression was also increased in response to more natural and physiological neural activity. Quantitative RT-PCR further confirmed that increased *AmEgr* expression in the MBs of the foragers was behavior-dependent rather than age-dependent (Fig. 16H). Based on these findings, I conclude that *AmEgr* is a neural IEG and can be utilized as a neural activity marker in the honeybee.

Most importantly, *AmEgr* expression was dramatically increased over 46°C in the MBs (Fig. 17G) and the distribution pattern of *AmEgr* signal was similar to that of *kakusei* (Fig. 17C-F), providing strong support for the temperature selectivity of some KCs. In the brains of bees exposed to 46°C, increased *AmEgr* expression was detected only in the MBs, whereas *kakusei* expression was not restricted to the MBs (Fig. 17A and B). Interestingly, both genes showed induced expression in various regions (MBs, OLs, and the other brain regions) after seizure induction (Fig. 15A and B). It is well known that in vertebrates the expression pattern of each IEGs show a significant spatial and temporal specificity in response to distinct stimuli [Hughes and Dragunow, 1995]. Such divergence is mainly ascribed to differential mechanisms of transcriptional regulation of these IEGs after nerve cell responses [Loebrich and Nedivi, 2009]. The expression of *kakusei* and *AmEgr* might be

regulated via distinct molecular pathways after neural activation due to high-temperature (46°C) exposure.

As mentioned above, in the forager brains induced *AmEgr* expression was observed in the s/mKCs (Fig. 16C-G). This result was consistent with earlier findings of my collaborators using *kakusei* as an IEG [Kiya et al., 2007]. Using *AmEgr*, characteristic *kakusei* expression pattern was reproduced in the brain of 46°C exposed bees (Fig. 17C-F). Additionally, Lutz and Robinson (2013) recently reported the identification of *Egr* from European honeybee and its application as an IEG to detect neural activity in the brains of pre-foragers that exhibited orientation flights (this work was published during the review process of my study [Ugajin et al., 2013]), although they did not examine the detailed characteristics of *AmEgr* as a neural IEG. In that study, they demonstrated increased expression of *AmEgr* in the whole KCs in the brains of pre-foragers that exhibited orientation flights, which also coincides with the previous study using *kakusei* as an IEG [Kiya et al., 2007]. These results conversely supported the previous notion that the expression of *kakusei*, which encodes a non-coding nuclear RNA [Kiya et al., 2007], reflects neural activity and can also be utilized as a neural activity marker.

Finally, my findings suggest that *Egr*-family genes with conserved motif structures are widely conserved in various animal species (Fig. 12). In mammals, there are four members of the EGR-family and their coding genes commonly show induced expression in response to neural activity, although there are considerable differences in the kinetics and spatial patterns of expression among them [Beckmann and Wilce., 1997; O'Donovan et al., 1999]. Taken together, my findings suggest that the *Egr*-family genes, which exhibit an immediate early response upon neural excitation in the central nervous

system, are evolutionarily conserved among various animal species. I expect that the findings of the present study will broaden the framework for the application of *Egr* homologs for neural activity mapping studies from vertebrates to invertebrates as a universal technique in behavioral neuroscience.

General Discussion

In the present study, using two immediate early genes (IEGs), *kakusei* (*Acks* in Japanese honeybee) and newly identified *AmEgr* (the honeybee homolog of *Egr-1* in vertebrates), I demonstrated that honeybees are equipped with thermosensitive neurons responding to approximately ~46°C in the mushroom bodies (MBs), and these neurons were significantly activated during hot defensive bee ball formation by Japanese honeybees [Ugajin et al., 2012]. To my knowledge, this is the first report of thermoresponsive neurons (Kenyon cells, KCs) in the MBs, a higher-order center in insect brains.

Heinrich (1980) reported that European honeybees fixed to a polystyrene pad and heated on the head with a focused beam of light start to regurgitate nectar from their honey-crop, and that aortic pulsations occur in the head when the head temperature reaches 44°C to 46°C, and as a result, the head temperature is stabilized near 45°C, even under continuous heating conditions. He considered that these responses cause evaporative cooling and act as a thermoregulatory behavior, which prevents their body temperature from reaching a lethal level. Thus, there seems to be a threshold for the temperature-dependent honeybee behavior of ~45°C. Concomitantly, I also observed another temperature-dependent behavioral change in European honeybees in my preliminary experiments. When the heads of bees fixed to corkboards as used in the picrotoxin injection experiment were heated by a beam of light while monitoring head surface temperature by thermography, the bee began to flutter their wings immediately after the surface temperature approached ~46°C. On the other hand, when the output of a beam of light was set to keep the temperature at 42°C, the bees showed no signs of fluttering.

The bumblebee, which is another eusocial bee that belongs to the genus *Bombus* (another genus in family *Apidae*), also has thermosensitive KCs, although the activation threshold was ~6°C lower than that of honeybees (Fig. 8F). Interestingly, there is also a ~6°C difference in the preferred temperature (temperature inside of their hives) between honeybees and bumblebees, 33°C and 27°C, respectively [Okada, 1975; Weidenmüller, 2004; Yoshida, 2005]. Thus, it is plausible that the high-temperature responsive KCs (especially Class II KCs) in the bee (*Apidae*) brains are activated when bees are confronted with the significant elevation of the body temperature over their preferred temperature (Fig. 18A). Japanese honeybees might have applied this neural mechanism for the maintenance of the temperature inside of a hot defensive bee ball around 46°C, just like a 'thermostat' (Fig. 18B).

Heinrich (1980) suggested that the temperature sensor that elicits these behavioral responses is located in the honeybee head, as thoracic heating did not elicit these behavioral responses. In my experiments, quantitative reverse transcription-polymerase chain reaction using workers whose appendages were ablated revealed that peripheral appendages, including the antennae and legs, did not mainly contribute to activate the Class II KCs under high temperature (Fig. 9). It might be that bees can sense the high temperature by some neurons in the brain.

In insect, the molecular mechanism of temperature sensing has been studied extensively in *Drosophila melanogaster*. For example, some transient receptor potential (TRP) channels [Tracey et al., 2003; Viswanath et al., 2003; Rosenzweig et al., 2005] are well characterized. *Drosophila* TRPA1 (DTRPA1) [Viswanath et al., 2003; Hamada et al., 2008; Rosenzweig et al., 2008; Tang et al., 2013], Pyrexia [Lee et al., 2005], and Painless

[Tracey et al., 2003; Xu et al., 2006; Sokabe et al., 2008], all of which are TRPA subfamily members, respond to “warm” or “hot” temperatures and are required for thermotaxis from warm temperature to preferred range (18-24°C) and avoidance of noxious heat (above 40°C) (The temperature thresholds are 24-29°C, 37.5-40°C, and 42-45°C, for DTRPA1, Pyrexia, and Painless, respectively). In honeybee, nest temperature is maintained near 33°C [Okada, 1975] and Hymenoptera specific TRPA (AmHsTRPA) is needed to warmth (above 36°C) avoidance (The temperature thresholds are 34°C for AmHsTRPA) [Kohno et al., 2010]. These thermosensitive TRPA channels are expressed in several body parts not only at the periphery but also in the brain [Xu et al., 2006; Hamada et al., 2008; Kohno et al., 2010; Kang et al., 2012]. In addition to the TRP channels, involvement of other temperature selective responsive ion channels in temperature preference behavior was reported in *Drosophila* [Gallio et al., 2011; Ni et al., 2013]. In honeybee, these temperature sensitive ion channels could express in some KCs or other brain neurons projected to the KCs and act as thermosensor when they are exposed to high temperature.

There are some differences between the Class I and Class II KCs other than the location of the somata [Strausfeld, 2002; Farris et al., 2004; Fahrbach, 2006]. In the honeybee, the calyces are partitioned into three concentric subdivisions: the lip, collar, and basal ring (Fig. 5C) [Gronenberg, 2001; Fahrbach, 2006]. Sensory interneurons providing axons to the calyces segregate according to their modalities: the lip receives olfactory input from the antennal lobes, whereas the collar receives visual input, and the basal ring receives olfactory and visual inputs, respectively [Gronenberg, 2001; Ehmer and Gronenberg, 2002; Fahrbach, 2006]. The basal ring might also receive gustatory and mechanosensory inputs from the proboscis [Schröter and Menzel, 2003]. Class I KCs typically restrict their

dendrites into these specific calycal subdivisions [Strausfeld, 2002; Fahrbach, 2006]. In contrast, the dendrites of Class II KCs represent all calyx regions [Strausfeld, 2002; Farris et al., 2004; Fahrbach, 2006]. Thus, it has been proposed that each subtype of the Class I KCs monitors the activity from a specific modality, whereas the role of the Class II KCs remains unclear [Gronenberg, 2001; Strausfeld, 2002; Fahrbach, 2006]. In addition, previous studies from our laboratory demonstrated that some genes involved in Ca^{2+}-signaling [Kamikouchi et al., 1998 and 2000; Takeuchi et al., 2002; Uno et al., 2013] or neural plasticity [Takeuchi et al., 2001; Kage et al., 2005; Hayashi et al., 2009] were preferentially expressed in the large-type KCs (lKCs), one subtype of the Class I KCs, suggesting that neural plasticity mediated by Ca^{2+}-signaling is enhanced in the lKCs. Furthermore, neural activity mapping using the IEGs in the present study as well as in the previous study revealed that the neural activity is enhanced in the small-type KCs and some middle-type KCs in the brains of foragers, suggesting that these neurons are related to processing of sensory information that the foragers perceive during their foraging flights [Kiya et al., 2007; Kaneko et al., 2013]. On the other hand, no gene has been reported to preferentially express in the Class II KCs. Thus, the present study first provides evidences for the putative function of the Class II KCs in the honeybee brain.

Insects are generally thought to be poikilothermic. Studies of their temperature-related behavior have been restricted to temperature preference and avoidance. The hot defensive bee ball formation in Japanese honeybees, in which the bees actively generate heat and maintain the appropriate temperature in the bee ball by sensing the temperature in the environment, seems to be a suitable model to study the mechanisms of thermoregulation in insects.

The other important achievement in my doctoral course studies is the first identification of *Egr* homologs as a potential universal neural IEG [Ugajin et al., 2013]. Yellowjackets (*Vespula germanica*), which is a social wasp species that belongs to another family in Hymenoptera (*Vespidae*), began to regurgitate when the head temperature reached ~42°C [Coelho and Ross, 1996]. In other social wasp species, nest temperature is maintained at ~30°C and they exhibit wing fanning to prevent overheating of the nest when the nest temperature is elevated [Kovac et al., 2009], like honeybees and bumblebees. It is possible that the thermoresponsive KCs are evolutionarily conserved in both *Apidae* and *Vespidae*. Future comparative studies using the *Egr* homologs as the neural activity marker in other insects, including the wasps, will help to elucidate the evolutionary origin of the thermoresponsive KCs.

There was no distinct *Acks*-signal in the brains of isoamyl acetate (IAA)-exposed workers (Fig. 6). Interestingly, my collaborators recently observed that IAA acts as one of releaser pheromones of forming a hot defensive bee ball, and that the amount of emitted IAA affect the size of a bee ball [Ono et al., unpublished], raising the possibility that Japanese honeybees receive and process the pheromonal stimulation to regulate their bee ball formation behaviors. In vertebrates, each IEGs have a significant spatial and temporal specificity of expression pattern in response to distinct stimuli [Hughes and Dragunow, 1995]. It might be that the *Acks* (and *kakusei*) expression was not sensitive enough to detect the neural activity that is evoked by olfactory input including the IAA stimulation. Application of *AmEgr* to map the active brain regions during IAA exposure and hot defensive bee ball formation might allow identifying neural activity relevant to IAA detection and regulation of bee ball size.

Insects are the most diverse group of animals on the earth, including more than a million described species and representing more than half of all known living organisms [Grimaldi and Engel, 2005]. They inhabit almost all environments and show greatly diverse adaptive behaviors [Matthews and Matthews, 2010]. Recently, Fujita et al. (2013) reported that *hr38*, a homolog of *NGFI-B* in vertebrates, also had IEG properties and was applicable for detecting active brain regions, at least in the *Bombyx mori* and *Drosophila*, and succeeded in identifying neurons relevant to the female pheromone response in male brains. I expect that application of the IEGs for neural activity mapping studies will facilitate our understanding of the neural mechanisms underlying unique behaviors in insects.

Figures

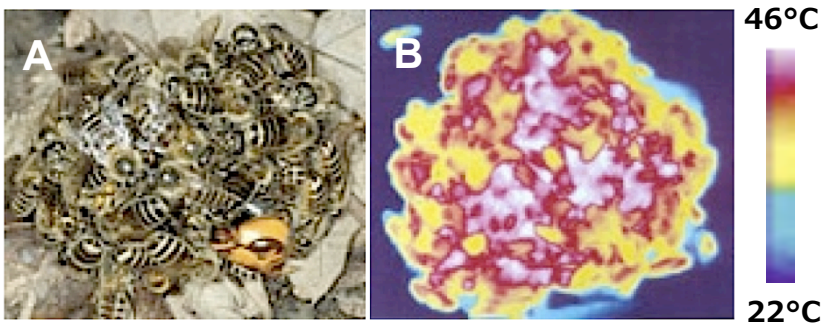

Figure 1. Hot defensive bee ball against a giant hornet.

(A) A lot of Japanese honeybee workers surround the giant hornet. (B) A thermography image of a hot defensive bee ball. These pictures were used with permission of Professor Masato Ono in Tamagawa University.

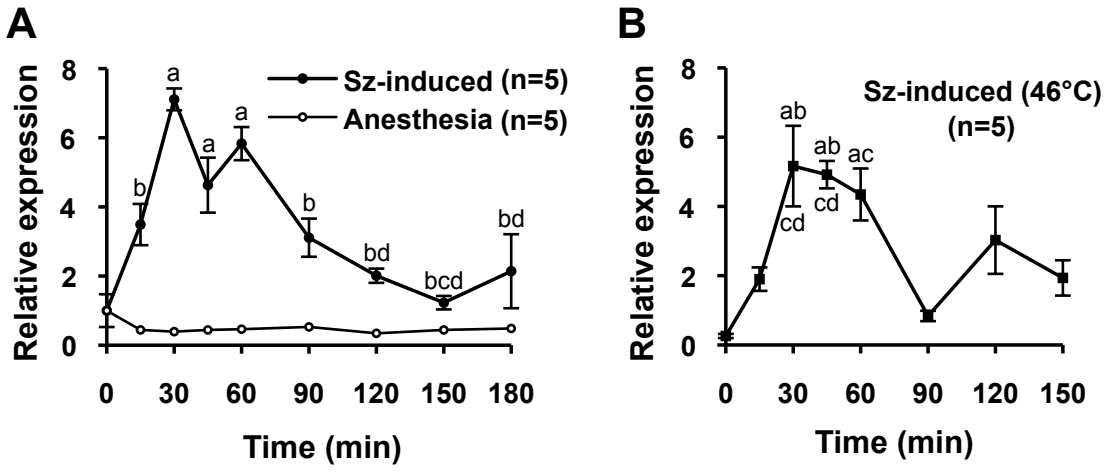

Figure 2. Time course of induced expression of *Acks* after seizure induction.

(A) Time course of *Acks* expression level investigated by quantitative RT-PCR after seizure induction under room temperature (25°C). Values are means ± SEM (a, different from 0 min $p<0.01$; b, different from 30 min $p<0.01$; c, different from 45 min $p<0.01$; d, different from 60 min $p<0.01$; Tukey-Kramer's test). Sz-induced, seizure-induced. (B) Time course of *Acks* expression level investigated by quantitative RT-PCR after seizure induction under the high temperature (46°C). Values are means ± SEM (a, different from 0 min $p<0.01$; b, different from 15 min $p<0.05$; c, different from 90 min $p<0.01$; d, different from 150 min $p<0.05$; Tukey-Kramer's test). Three worker honeybees were used per lot. The number of lots analyzed was shown in parentheses.

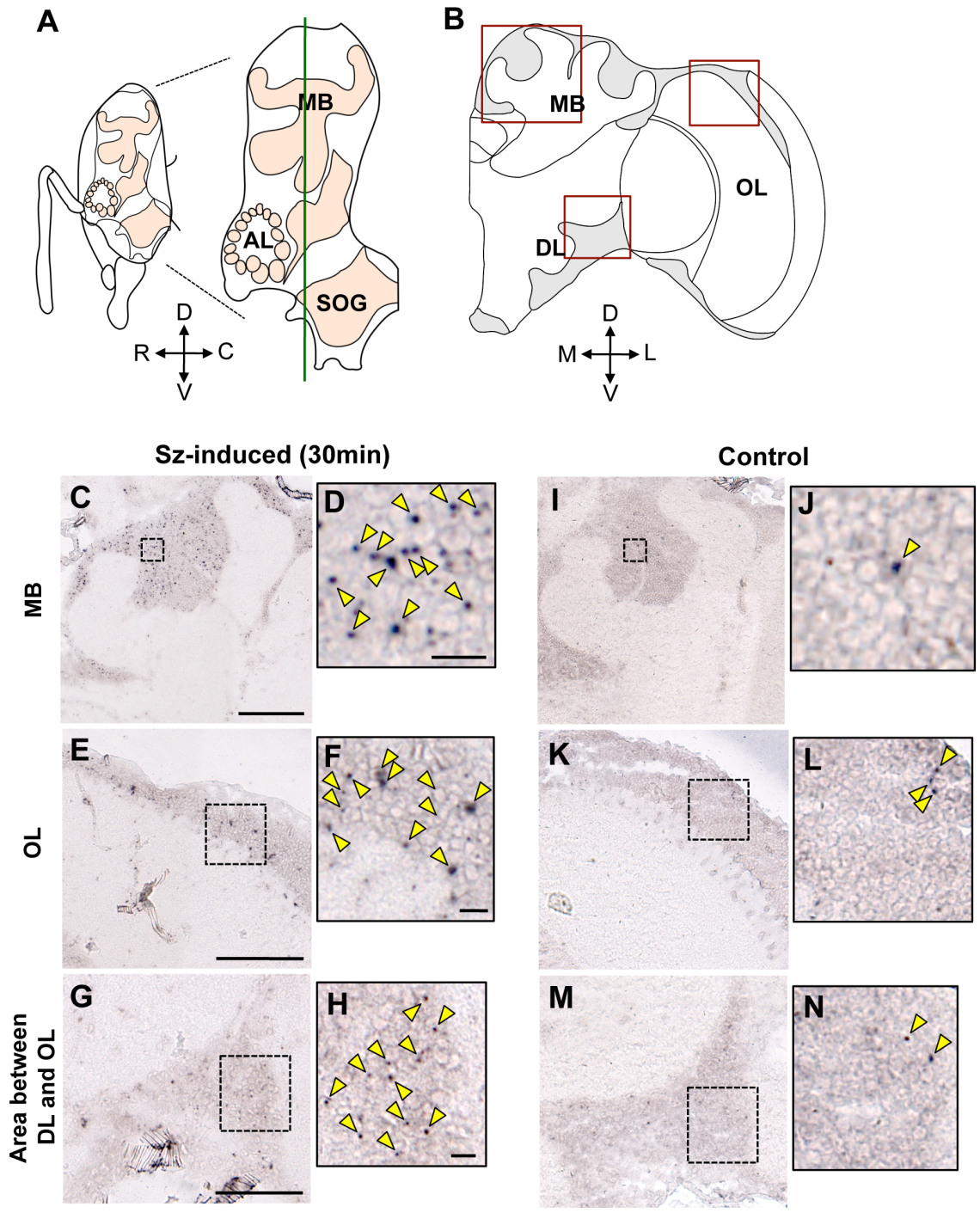
A
MB
AL
SOG
D
R
C
V
B
MB
OL
DL
D
M
L
V
Sz-induced (30min)
Control
MB
OL
Area between DL and OL
C
D
I
J
E
F
K
L
G
H
M
N

Figure 3. Seizure-induced neural activity in the brains of Japanese honeybee workers.

(A) Schematic diagram of the lateral view of a bee brain. Areas colored in light orange indicate neuropil regions. D, dorsal; V, ventral; R, rostral; C, caudal. MB, mushroom body; AL, antennal lobe; and SOG, subesophageal ganglion. The green line indicates the position of sections analyzed in this experiment. (B) A schematic diagram of a middle right brain hemisphere of Japanese worker honeybee. Areas colored in light grey indicate brain areas where the somata of neurons are located. Red squares correspond to brain areas whose *in situ* hybridization results are presented below. M, medial; L, lateral. OL, optic lobe; DL, dorsal lobe. (C-N) Expression analysis of *Acks* by *in situ* hybridization using coronal brain sections of seizure-induced (Sz-induced) (C-H) or control Japanese honeybee workers (I-N). The upper panels (C and I), middle panels (E and K), and lower panels (G and M) correspond to MB, OL, and area between the DL and OL, which are boxed in (B). Bars indicate 100 μm. (D, F, H, J, L, and N) Magnified views of the regions delineated by dotted lines in panels (C), (E), (G), (I), (K), and (M), respectively. Yellow arrowheads indicate *Acks* signals. Bars indicate 10 μm.

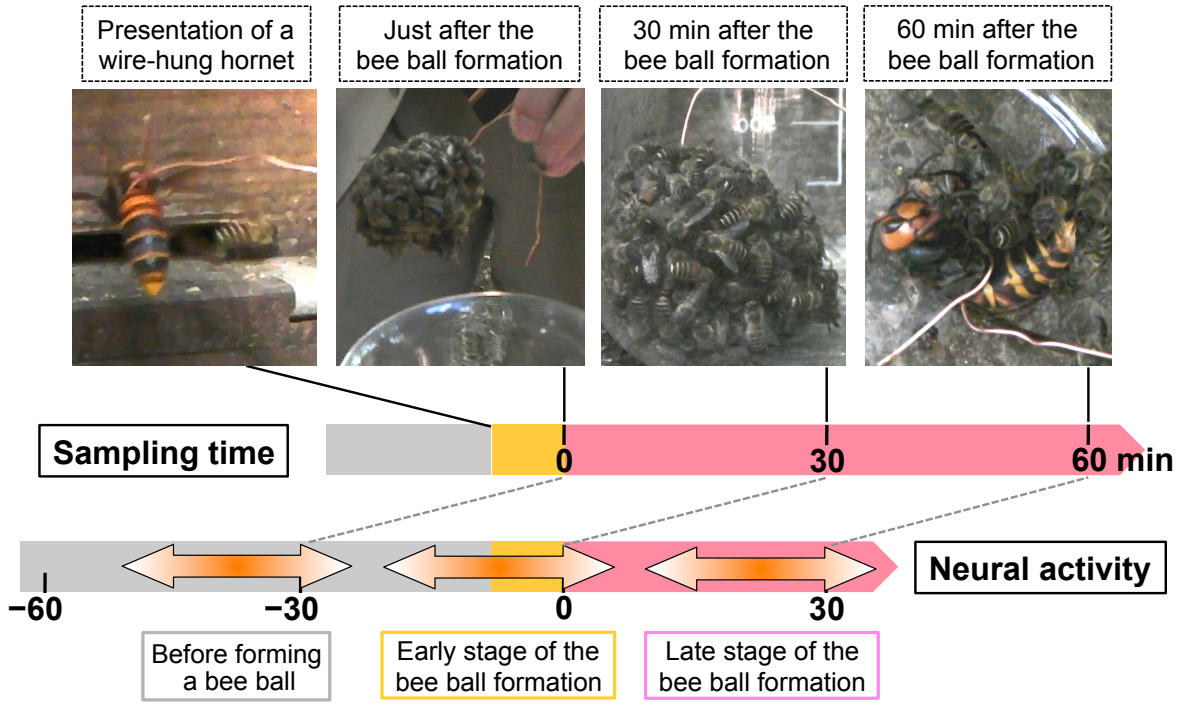

Figure 4. Sampling of workers from an artificially formed hot defensive bee ball.

The hot defensive bee ball is usually formed in the beehive. To collect only the workers involved in forming the bee ball, a giant hornet suspended on the tip of a wire was used as a decoy. The decoy hornet was inserted into a hive to allow the Japanese honeybees to form a bee ball around the hornet, and recovered the bee ball in a glass beaker. The decoy hornet inside the bee ball was dead 60 min after formation of the bee ball as usually occurs in nature. *Acks* expression can reflect neural activity that occurred 30 to 60 min before.

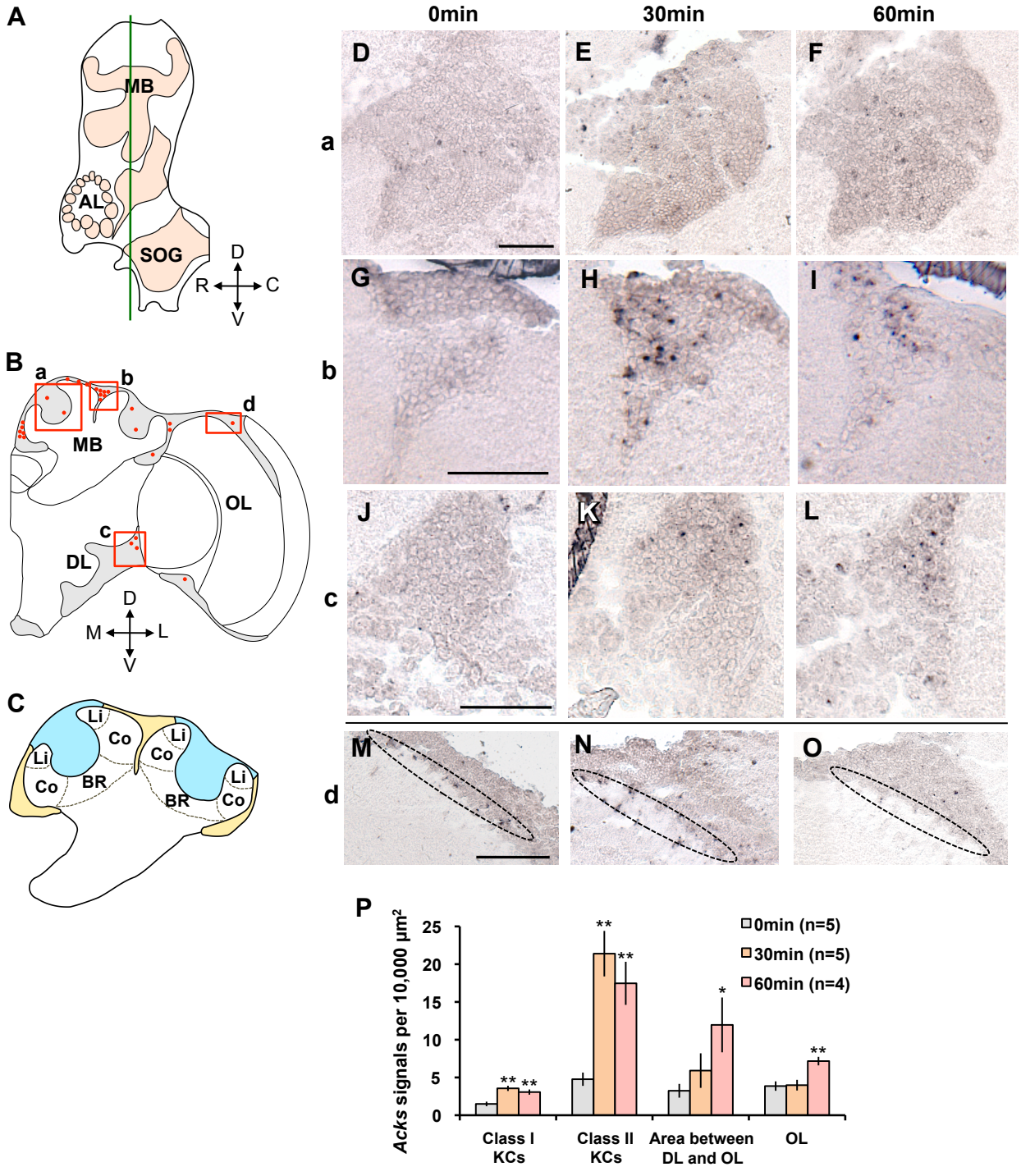
A
MB
AL
SOG
D
R
C
V
B
a
b
d
MB
OL
c
DL
D
M
L
V
C
Li
Co
BR
0min
30min
60min
D
E
F
G
H
I
J
K
L
M
N
O
a
b
c
d
P
Acks signals per 10,000 µm²
25
20
15
10
5
0
0min (n=5)
30min (n=5)
60min (n=4)
Class I KCs
Class II KCs
Area between DL and OL
OL

Figure 5. Neural activity in the brain during bee ball formation.

(A) Schematic diagram of the lateral view of a bee brain. The green line indicates the position of sections that correspond to a middle part of the brain used for this *in situ* hybridization experiment. (B) Schematic representation of the *Acks* signals detected in the right brain hemisphere of the workers that formed the bee ball. The red dots indicate induced *Acks* signals at 30 or 60 min after the bee ball formation. The boxed regions (a-d) correspond to Class I KCs whose somata are located inside the calyces (a), parts of Class II KCs whose somata are located outside of calyces (b), the restricted area located between the DL and lobula of the OL (c), and a part of the OL (d), whose *in situ* hybridization results are presented in the right panels (D-O). (C) Magnified schematic representation of the MB indicating the distribution of the somata of Class I (light blue) and Class II KCs (yellow), respectively, and calycal subdivision. Li, lip; Co, collar; and BR, basal ring. (D-O) *In situ* hybridization of *Acks* in each brain area shown in (B) in the brains of workers at 0 (D, G, J, and M), 30 (E, H, K, and N) and 60min (F, I, L, and O) after the bee ball formation. (D-F), (G-I), (J-L), and (M-O) correspond to the boxed brain regions (a), (b), (c), and (d), respectively. The dotted *Acks* signals were detected most densely in the Class II KCs (H and I), and less densely in the Class I KCs (E and F) at 30 and 60 min after the bee ball formation, respectively. Note that the *Acks* signals were detected moderately in the restricted region between the DLs and the lobula of the OLs (L), and less densely in the OLs (O) at 60 min after the bee ball formation. Staining observed in area surrounded by dotted ellipse (M-O) represents non-specific staining of trachea, which was also observed in sections hybridized with the sense probe (data not shown). Bars indicate 50 μm. (P) Quantification of

Acks-positive cells in various brain regions. Values are means ± SEM. Asterisks indicate significant difference compared to that at 0 min (*, $p<0.05$; **, $p<0.01$; Dunnett's test).

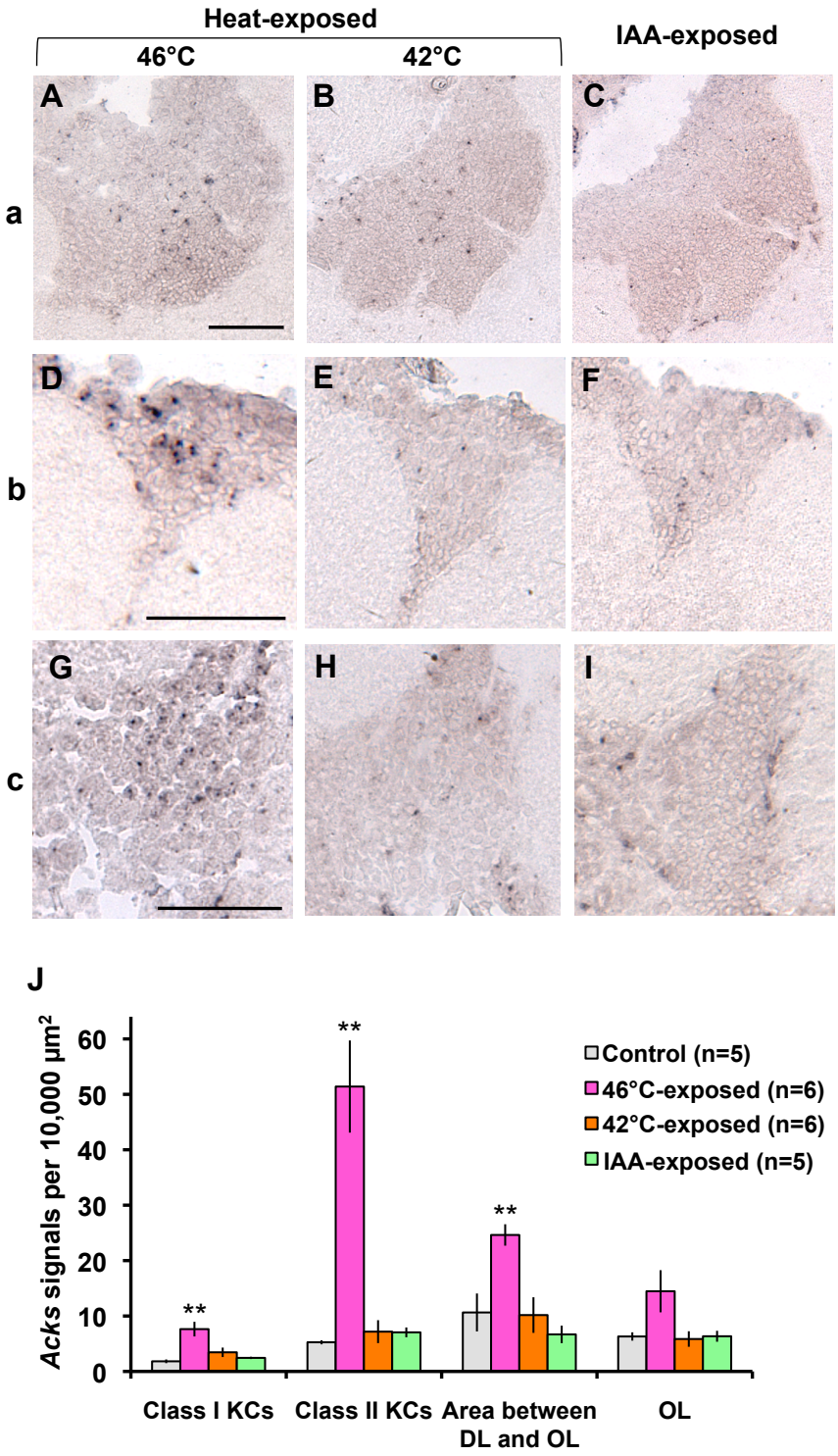
Heat-exposed
46°C
42°C
IAA-exposed
A
B
C
a
D
E
F
b
G
H
I
c
J
Acks signals per 10,000 µm²
60
50
40
30
20
10
0
**
**
**
Control (n=5)
46°C-exposed (n=6)
42°C-exposed (n=6)
IAA-exposed (n=5)
Class I KCs
Class II KCs
Area between DL and OL
OL

Figure 6. Neural activity in the brains of workers exposed to high temperature or IAA.

(A-I) *In situ* hybridization of *Acks* in each brain area shown as Figure 5B in the brains of workers exposed to 46°C (A, D, and G), 42°C (B, E, and H) and workers exposed to IAA (C, F, and I). (A-C), (D-F), and (G-I) correspond to the boxed brain regions shown as in Fig. 5B (a), (b), and (c), respectively. In the brains of 46°C-exposed bees, the dotted *Acks* signals were detected most densely in the Class II KCs (D) and moderately in the restricted area between the DLs and OLs (G), and much less densely in the Class I KCs (A). On the other hand, scarce or no significant signals were detected in these brain regions in 42°C or IAA-exposed workers (B, C, E, F, H, and I). Bars indicate 50 μm. (J) Quantification of *Acks*-positive cells in various brain regions. Values are means ± SEM. Asterisks indicate significant difference compared to control (33°C-exposed bees) (**, $p<0.01$; Dunnett's test).

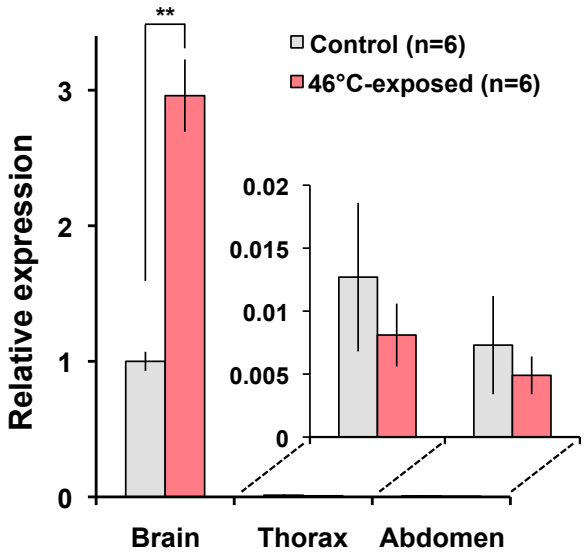

Figure 7. The results of quantitative RT-PCR of *Acks* in several body parts of 46°C-exposed bees.

The results of quantitative RT-PCR showing *Acks* expression in the brain, thorax, and abdomen of 46°C-exposed intact workers. Asterisks indicate a significant difference between heat-exposed and control workers within the same tissues (**, $p<0.01$; Student's *t*-test). The results for the thorax and abdomen are shown in the magnified graph because these values were extremely low. Values are means ± SEM. Two worker honeybees were used per lot. Each experimental group contained six lots of workers.

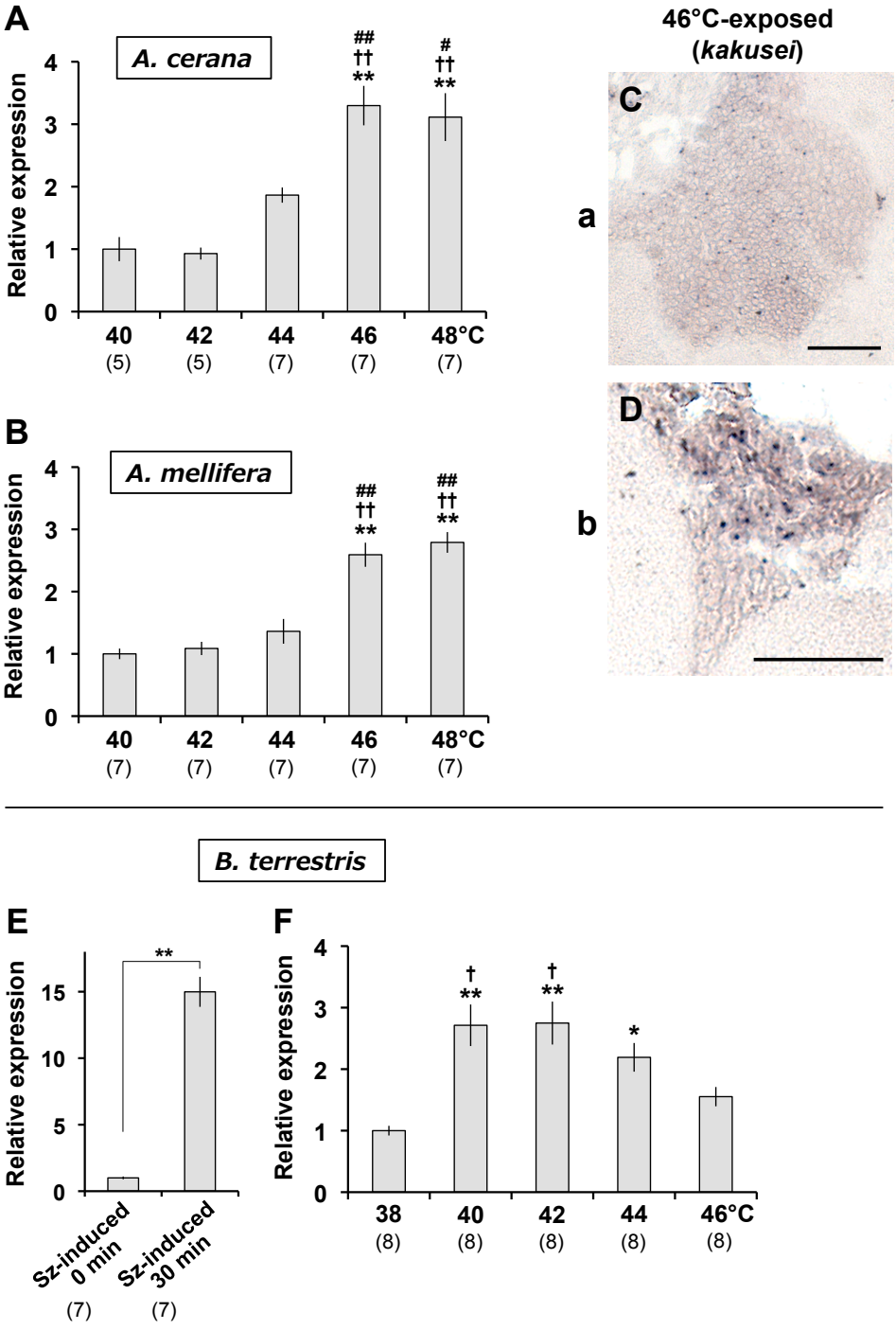
A
A. cerana
Relative expression
0
1
2
3
4
40
(5)
42
(5)
44
(7)
46
(7)
48°C
(7)
##
††
**
#
††
**
B
A. mellifera
Relative expression
0
1
2
3
4
40
(7)
42
(7)
44
(7)
46
(7)
48°C
(7)
##
††
**
##
††
**
46°C-exposed
(kakusei)
C
a
D
b
B. terrestris
E
Relative expression
0
5
10
15
**
Sz-induced
0 min
(7)
Sz-induced
30 min
(7)
F
Relative expression
0
1
2
3
4
38
(8)
40
(8)
42
(8)
44
(8)
46°C
(8)
†
**
†
**
*

Figure 8. Neural activity in the MBs of honeybees and bumblebees exposed to various high temperatures.

(A and B) The results of quantitative RT-PCR showing the *Acks* or *kakusei* expression in the MBs of high-temperature exposed (40°C-48°C) Japanese (A) or European (B) honeybees. Values are means ± SEM (**, different from 40°C $p<0.01$; ††, different from 42°C $p<0.01$; #, different from 44°C $p<0.05$; ##, different from 44°C $p<0.01$; Tukey-Kramer's test). Two worker honeybees were used per lot. The number of lots analyzed was shown in parentheses. (C and D) *In situ* hybridization of *kakusei* in the MBs of European honeybee workers exposed to 46°C. Each panels correspond to the boxed brain regions shown as in Fig. 5B (a) and (b), respectively. Bars indicate 50 μm. (E) The results of quantitative RT-PCR in the whole brains of bumblebees at 0 and 30 min after seizure induction (shown as Sz-induced 0 and 30 min, respectively). Student's *t*-test was used for analysis (**, $p<0.01$). All data are shown as the means ± SEM. A worker bumblebee was used per lot. The number of lots analyzed was shown in parentheses. (F) The results of quantitative RT-PCR showing the *kakusei* expression in the MBs of high-temperature exposed (38°C-46°C) buff-tailed bumblebees. Values are means ± SEM (*, different from 38°C $p<0.05$; **, different from38°C $p<0.01$; †, different from 46°C $p<0.05$; Tukey-Kramer's test). A worker bumblebee was used per lot. The number of lots analyzed was shown in parentheses.

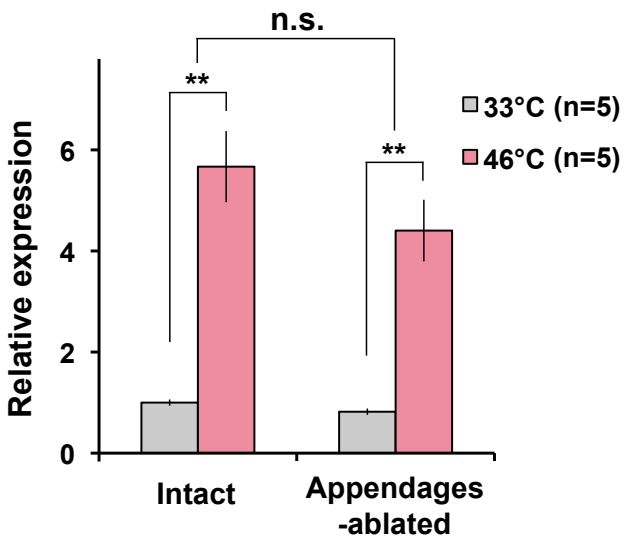

Figure 9. Increased neural activity in the MBs of appendage-ablated bees after 46°C exposure.

The results of quantitative RT-PCR showing the *kakusei* expression in the MBs of intact and appendages-ablated workers under usual (33°C) and high (46°C) temperature. Two worker honeybees were used per lot. Each experimental group contained five lots of workers. A two-way ANOVA revealed that there was no interaction between temperature and ablation of appendages (n.s.=non-significant, p=0.20), and then Student's t-test was conducted for intergroup comparison (**, p<0.01). Values are means ± SEM.

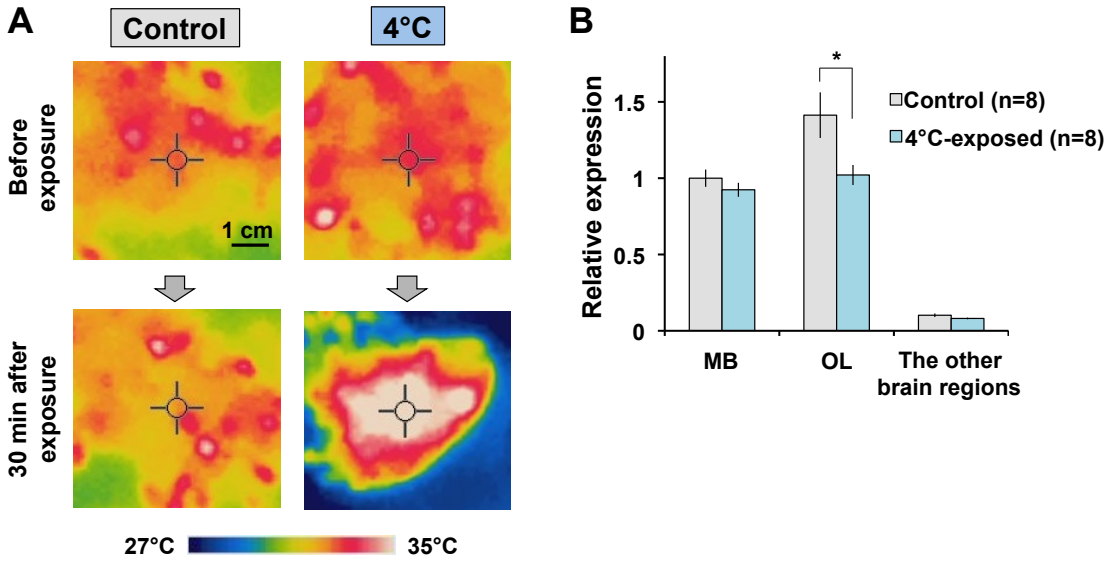

Figure 10. *kakusei* expression in the brains of European honeybee workers forming a bee ball under 4°C.

(A) Thermography images of the bees exposed to 4°C (right column) and those to 33°C as a control (left column). When the bees were exposed to 4°C, they formed an assemblage and avoided cooling their bodies. A bar indicates 1 cm. (B) Results of quantitative RT-PCR of *kakusei* in various brain regions 30 min after lower-temperature exposure. *kakusei* expression did not significantly increase in any brain region. Student's *t*-test was used for analysis (*, $p<0.05$). All data are shown as the means ± SEM. Three worker honeybees were used per lot. Each experimental group contained eight lots of workers.

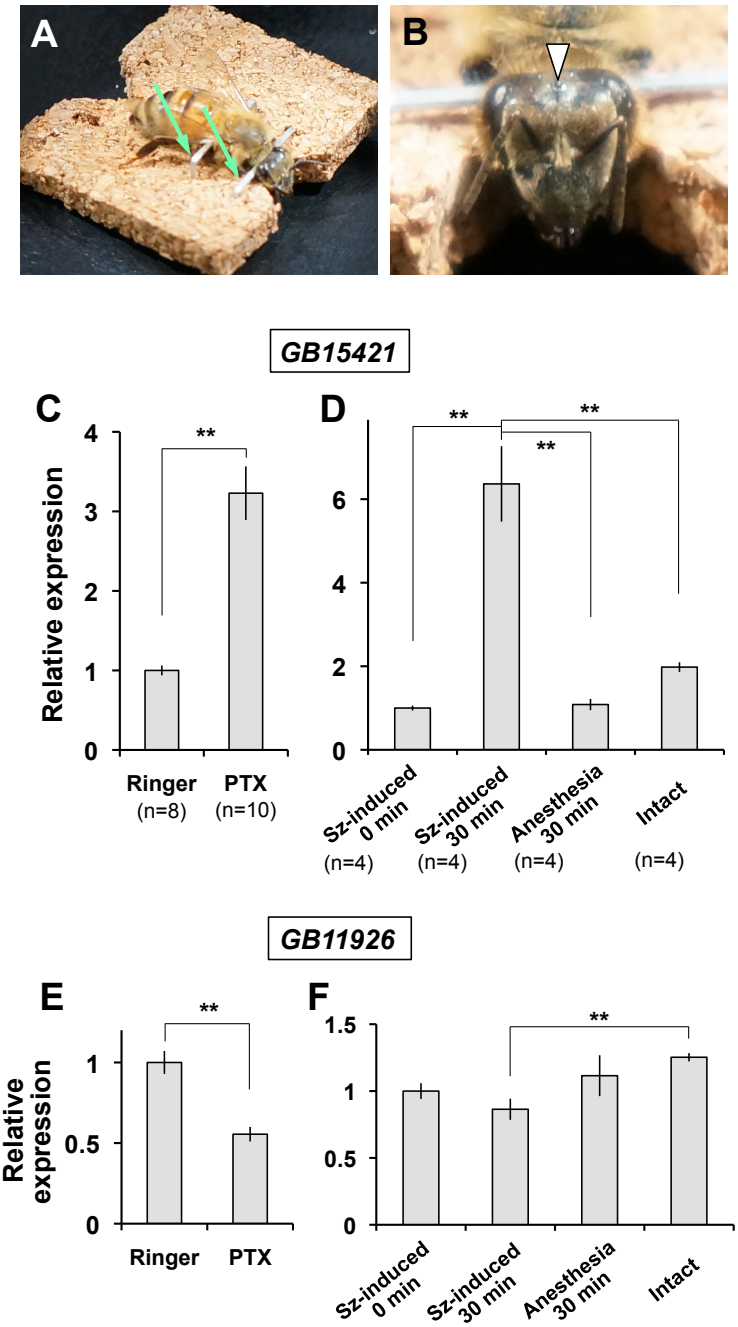
A
B
GB15421
C
D
Relative expression
Ringer
(n=8)
PTX
(n=10)
Sz-induced
0 min
(n=4)
Sz-induced
30 min
(n=4)
Anesthesia
30 min
(n=4)
Intact
(n=4)
GB11926
E
F
Relative
expression
Ringer
PTX
Sz-induced
0 min
Sz-induced
30 min
Anesthesia
30 min
Intact

Figure 11. Induced expression of GB15421 in response to PTX treatment or seizure induction.

(A) A worker honeybee in the experimental set-up. Two staples are fitted into the bee's neck and petiole (arrows). (B) Magnified view of the head of the cork board-fixed bee. White arrowhead indicates the drug injection site (median ocellus). (C-F) The results of quantitative RT-PCR showing GB15421 (C and D) or GB11926 (E and F) expression. (C and E) Using the MBs of Ringer injected and PTX-injected bees. A worker honeybee was used per lot. The number of lots analyzed was shown in parentheses. Student's *t*-test was used for analysis (**, $p<0.01$). All data are shown as the means ± SEM. (D and F) Using the whole brains of the bees at 0 and 30 min after seizure induction (shown as Sz-induced 0 and 30 min, respectively) and maintained under anesthesia continuously for 30 min (Anesthesia 30 min), and without anesthesia or seizure induction (Intact). Two worker honeybees were used per lot. Each experimental group contained four lots of workers. Multiple comparisons were conducted using Tukey-Kramer's test (**, $p<0.01$). All data are shown as the means ± SEM.

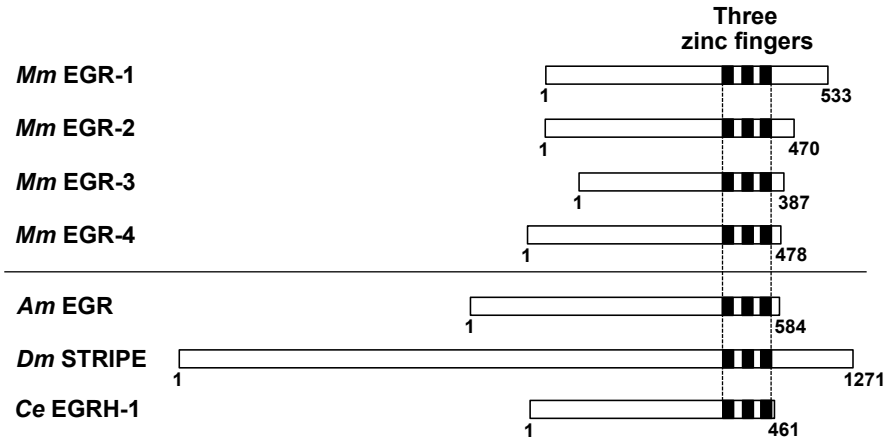

Figure 12. Comparison of the domain structure of *Am*EGR with that of other EGRs.

Schematic structures of members of the EGR-family from *Mus musculus* (*Mm*) and three invertebrate species: *Apis mellifera* (*Am*), *Drosophila melanogaster* (*Dm*), and *Caenorhabditis elegans* (*Ce*). The three conserved zinc fingers are represented by the black boxes. Numbers indicate the positions of amino acid residues.

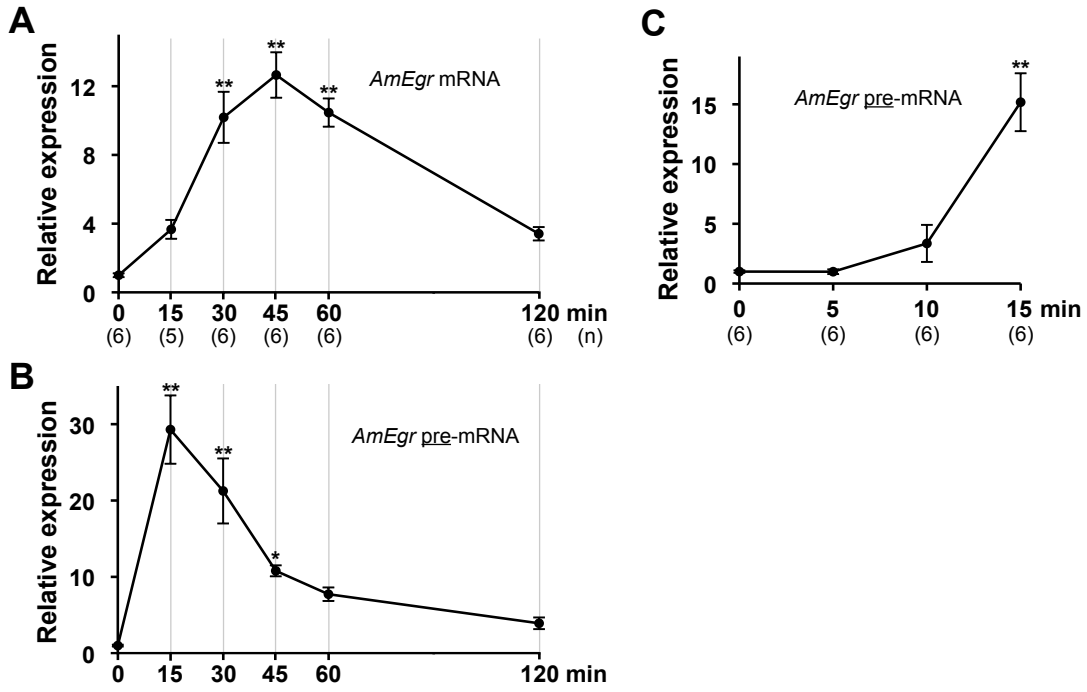

Figure 13. Time-course analyses of *AmEgr* transcript expression in response to seizure induction.

(A) Time-course of *AmEgr* mRNA expression investigated by quantitative RT-PCR after seizure induction. Asterisks indicate significant difference compared to the bees at 0 min (**, $p<0.01$; Dunnett's test). (B) Time-course of the *AmEgr* pre-mRNA expression investigated by quantitative RT-PCR after seizure induction. Asterisks indicate significant difference compared to the bees at 0 min (*, $p<0.05$; **, $p<0.01$; Dunnett's test). Quantitative RT-PCR was performed using the same sample set in both experiments. (C) Time-course of early phase of *AmEgr* pre-mRNA expression after seizure induction. Asterisks indicate significant difference compared to the bees at 0 min (**, $p<0.01$; Dunnett's test). Two worker honeybees were used per lot. The number of lots analyzed was shown in parentheses. All data are shown as the means ± SEM.

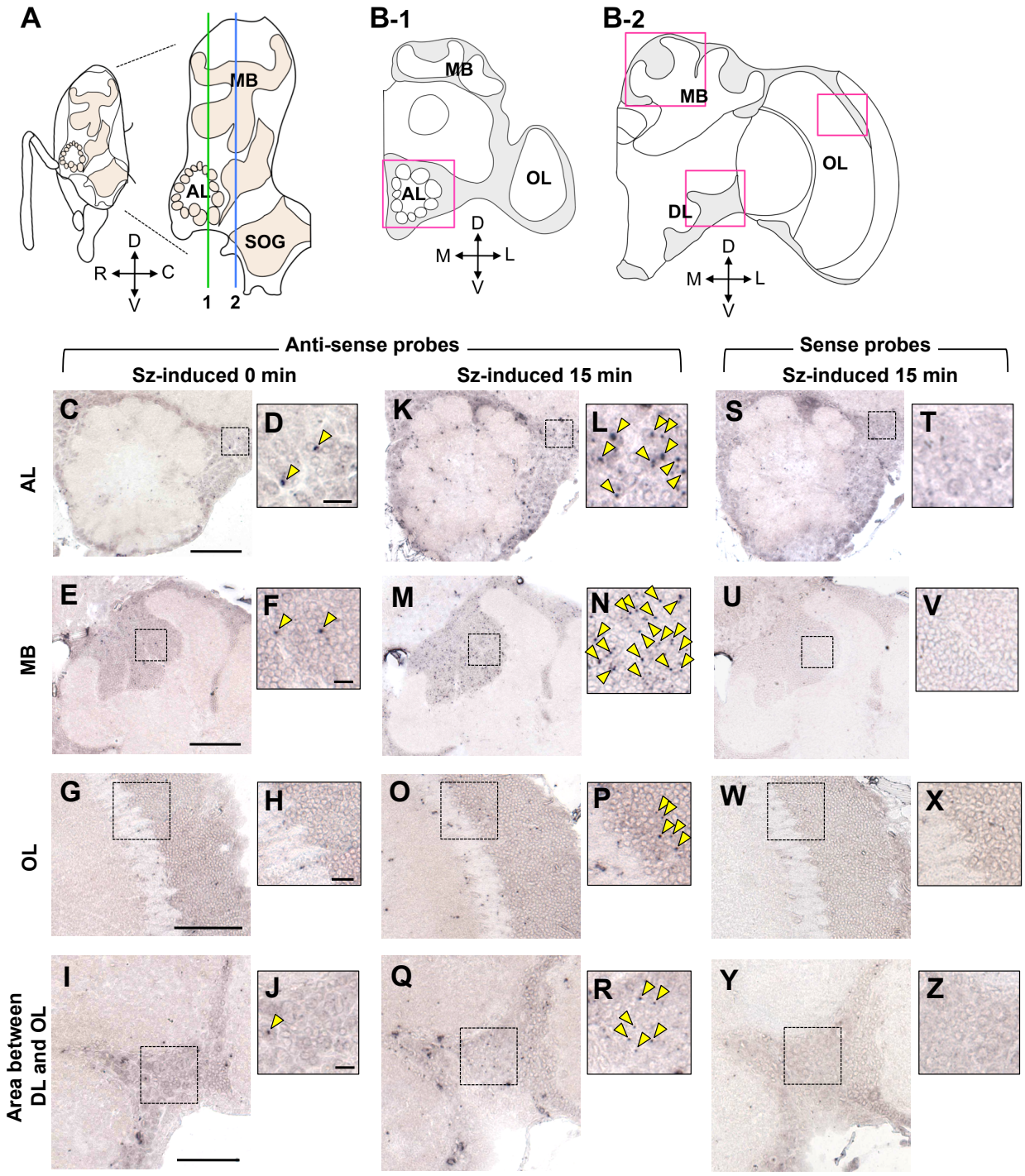
A
MB
AL
SOG
D
R
C
V
1
2
B-1
MB
AL
OL
D
M
L
V
B-2
MB
OL
DL
D
M
L
V
Anti-sense probes
Sz-induced 0 min
Sz-induced 15 min
Sense probes
Sz-induced 15 min
AL
MB
OL
Area between DL and OL
C
D
K
L
S
T
E
F
M
N
U
V
G
H
O
P
W
X
I
J
Q
R
Y
Z

Figure 14. Detection of *AmEgr* pre-mRNA expression in the worker brain by *in situ* hybridization.

(A) Schematic diagram of the lateral view of a worker brain. Areas colored in light orange indicate neuropil regions. D, dorsal; V, ventral; R, rostral; C, caudal. MB, mushroom body; AL, antennal lobe; and SOG, subesophageal ganglion. Green and blue lines indicate the position of sections analyzed in this experiment. (B) Schematic diagram of a rostral (B-1) or middle (B-2) right brain hemisphere of the worker honeybee. Areas colored in light grey indicate brain areas where the somata of neurons are located. Magenta squares correspond to brain areas whose *in situ* hybridization results are presented in the panels below. M, medial; L, lateral; OL, optic lobe; DL, dorsal lobe. (C-Z) Expression analysis of *AmEgr* pre-mRNA by *in situ* hybridization using coronal brain sections of workers at 0 (C-J) or 15 min (K-R) after seizure induction hybridized with anti-sense probes, or 15 min after seizure induction hybridized with sense probes (S-Z). The upper panels (C, K, and S), upper-middle panels (E, M, and U), lower-middle panels (G, O, and W), and lower panels (I, Q, and Y) correspond to AL, MB, OL, and the area between the DL and OL, which are boxed in (B). Bars indicate 100 μm. (D, F, H, J, L, N, P, R, T, V, X, and Z) Magnified views of the regions delineated by dotted lines in panels (C), (E), (G), (I), (K), (M), (O), (Q), (S), (U), (W), and (Y), respectively. Yellow arrowheads indicate *AmEgr* pre-mRNA signals. Bars indicate 10 μm.

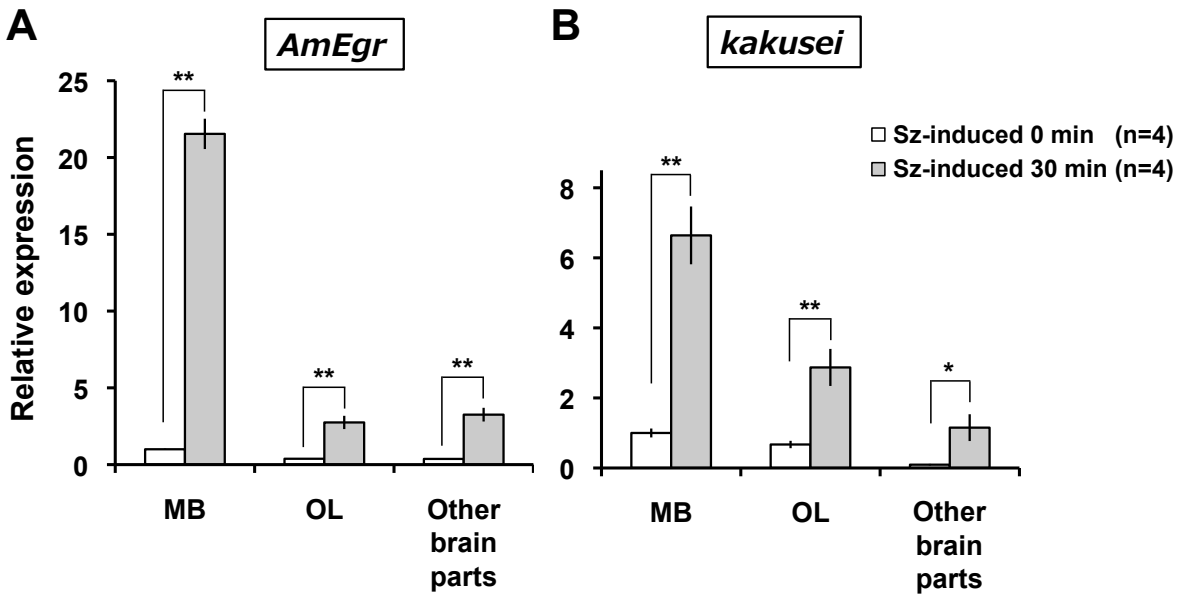

Figure 15. Expression analyses of *AmEgr* and *kakusei* after seizure induction.

The results of quantitative RT-PCR showing the *AmEgr* (A) or *kakusei* (B) expression in the MBs 30 min after seizure induction. Values are means ± SEM (*, $p<0.05$; **, $p<0.01$; Student's *t*-test). Quantitative RT-PCR was performed using the same sample set in both experiments. Two worker honeybees were used per lot. Each experimental group contained four lots of workers.

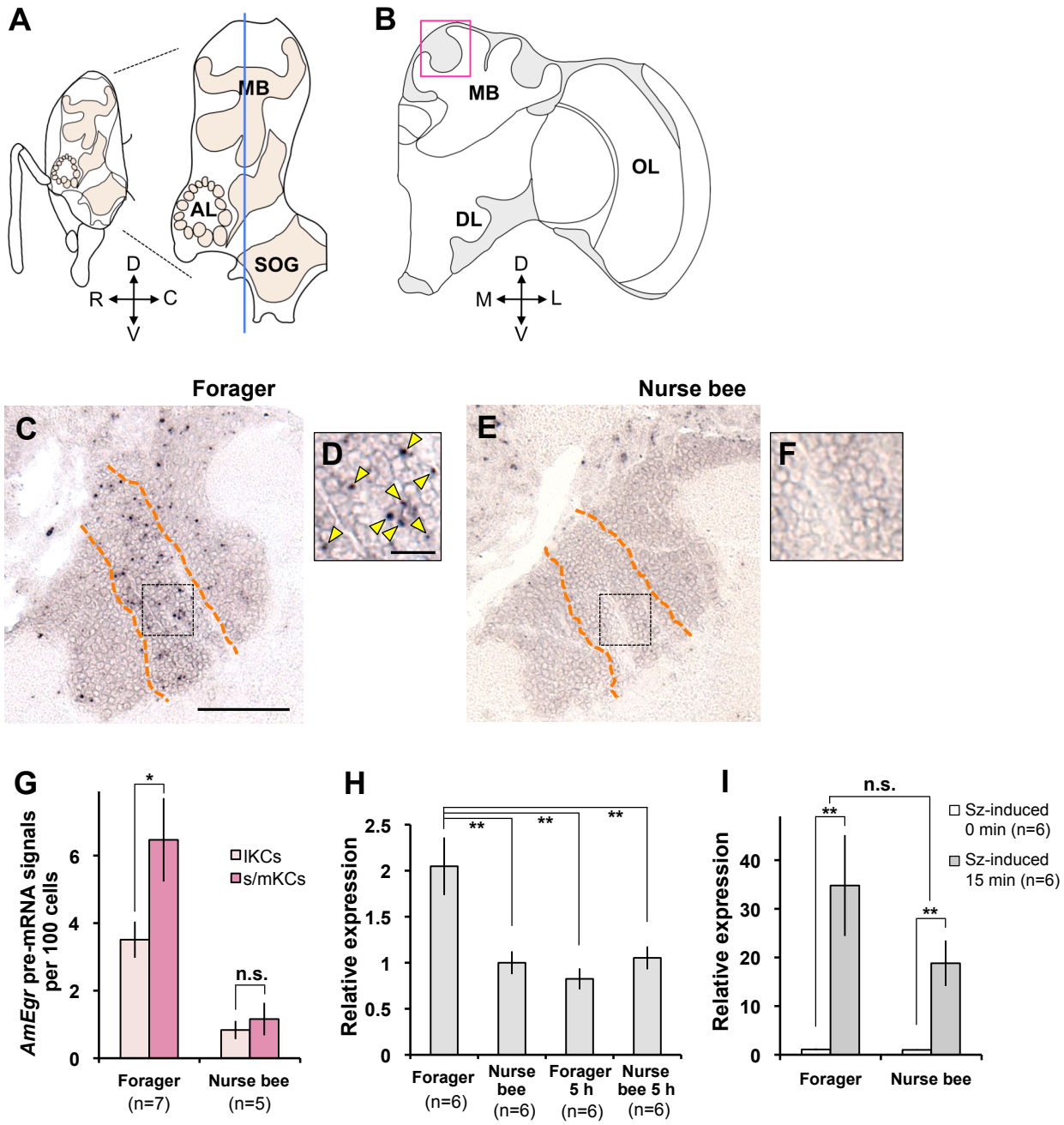
A
MB
AL
SOG
D
R
C
V
B
MB
OL
DL
D
M
L
V
Forager
Nurse bee
C
D
E
F
G
AmEgr pre-mRNA signals per 100 cells
IKCs
s/mKCs
*
n.s.
Forager (n=7)
Nurse bee (n=5)
H
Relative expression
**
**
**
Forager (n=6)
Nurse bee (n=6)
Forager 5 h (n=6)
Nurse bee 5 h (n=6)
I
n.s.
**
**
Sz-induced 0 min (n=6)
Sz-induced 15 min (n=6)
Relative expression
Forager
Nurse bee

Figure 16. Neural activity in the MBs of forager and nurse bee.

(A) Schematic diagram of the lateral view of a bee brain. Blue line indicates the position of sections analyzed in this experiment. (B) Schematic diagram of a middle right brain hemisphere of the worker honeybee. Two cup-shaped neuropils in the MB are calyces. Magenta square corresponds to brain area whose *in situ* hybridization results are presented in the panels below. (C-F) Expression analysis of *AmEgr* pre-mRNA in the MBs by *in situ* hybridization using coronal brain sections of the foragers or the nurse bees. (C and E) Pairs of dotted orange lines indicate boundary between the lKCs and the s/mKCs, and the somata of the s/mKCs are located in the sandwiched area between the pairs of the lines. Bars indicate 100 μm. (D and F) Magnified views of the regions delineated by dotted lines in panels (C) and (E), respectively. Bars indicate 10 μm. (G) Quantification of *AmEgr*-positive cells in the lKCs or the s/mKCs in foragers or nurse bees. Student's *t*-test was used for analysis (*, $p<0.05$; n.s.=non-significant). All data are shown as the means ± SEM. (H) The results of quantitative RT-PCR showing *AmEgr* pre-mRNA expression in the MBs of foragers, nurse bees, foragers kept in dark for 5 h, and nurse bees kept in the dark for 5 h. A worker honeybee was used per lot. Each experimental group contained six lots of workers. Multiple comparisons were conducted using Tukey-Kramer's test (**, $p<0.01$). All data are shown as the means ± SEM. (I) The results of quantitative RT-PCR showing increased *AmEgr* pre-mRNA expression 0 or 15 min after seizure induction in the MBs of foragers or nurse bees. A worker honeybee was used per lot. Each experimental group contained six lots of workers. A two-way ANOVA revealed that there was no interaction between seizure induction and division of labor (n.s.=non-significant, $p=0.178$), and then Student's *t*-test was

conducted for intergroup comparison (**, $p<0.01$). All data are shown as the means ± SEM.

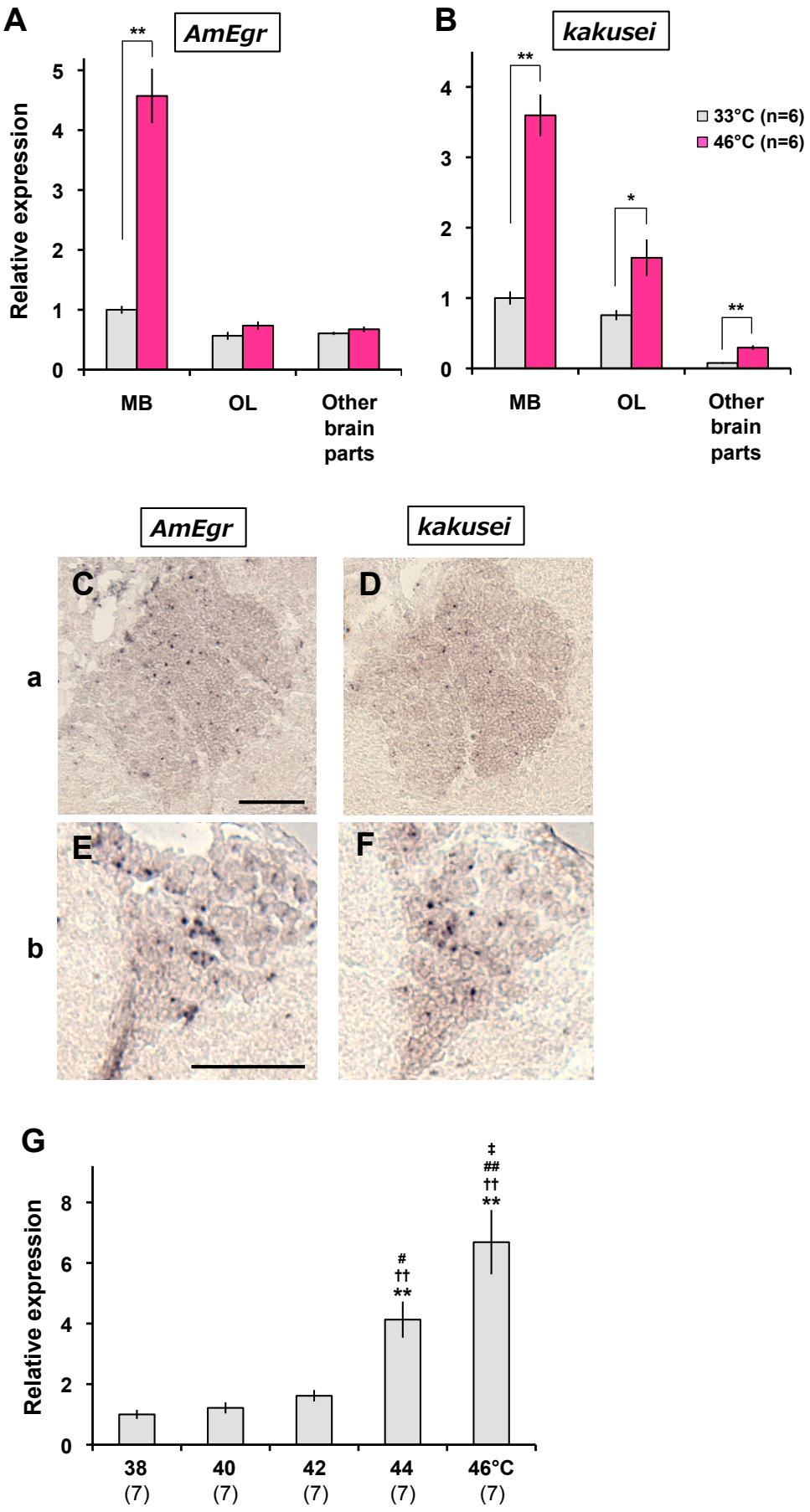
A
AmEgr
Relative expression
**
MB
OL
Other brain parts
B
kakusei
33°C (n=6)
46°C (n=6)
**
*
**
MB
OL
Other brain parts
AmEgr
kakusei
C
D
a
E
F
b
G
Relative expression
#
††
**
‡
##
††
**
38 (7)
40 (7)
42 (7)
44 (7)
46°C (7)

Figure 17. Neural activity in the brains of high-temperature exposed European honeybees detected by *AmEgr*.

(A and B) The results of quantitative RT-PCR showing the *AmEgr* (A) or *kakusei* (B) expression in the MBs 30 min after 46°C exposure. Values are means ± SEM (*, $p<0.05$; **, $p<0.01$; Student's *t*-test). Note that after 46°C exposure, *AmEgr* expression was increased only in the MBs. Two worker honeybees were used per lot. Each experimental group contained six lots of workers. (C-F) Expression analysis of *AmEgr* (C and E) or *kakusei* (D and F) in the MBs by *in situ* hybridization using coronal brain sections of the 46°C-exposed bees. (C and D) and (E and F) correspond to the boxed brain regions shown as in Fig. 5B (a) and (b), respectively. Bars indicate 50 μm. (G) The results of quantitative RT-PCR showing the *AmEgr* expression in the MBs of high-temperature exposed (40°C-48°C) honeybees. Values are means ± SEM (**, different from 40°C $p<0.01$; ††, different from 42°C $p<0.01$; #, different from 44°C $p<0.05$; ##, different from 44°C $p<0.01$; ‡, different from 46°C $p<0.05$; Tukey-Kramer's test). Two worker honeybees were used per lot. The number of lots analyzed was shown in parentheses.

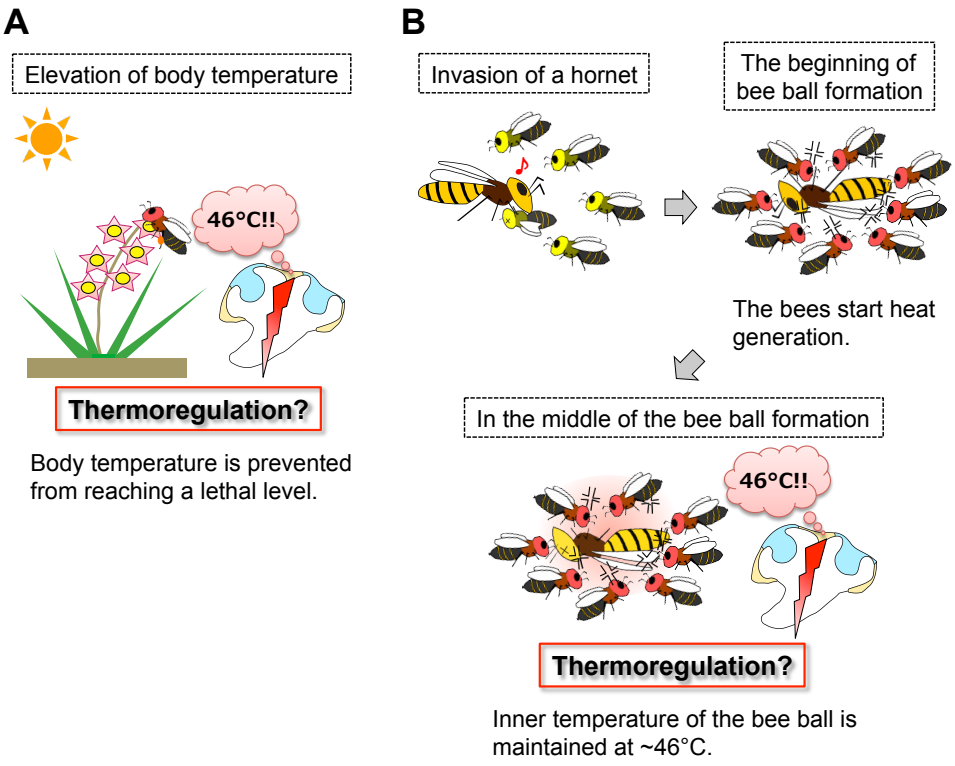

Figure 18. Possible role of the thermoresponsive KCs and application to temperature regulation inside the hot defensive bee ball.

(A) Model for the original role of thermoresponsive KCs in honeybees and bumblebees. When the bees undergo a significant elevation in body temperature (in this scheme, dedicated foraging flight under a sunny sky is shown as an example), the thermoresponsive KCs directly or indirectly perceive the thermal input and are activated to elicit thermoregulatory behavior. The body temperature is prevented from reaching a lethal level. (B) Model for the role of the thermoresponsive KCs of the Japanese honeybees during the formation of a hot defensive bee ball. After the inner temperature of the bee ball reaches ~46°C, the thermoresponsive KCs are activated to elicit thermoregulatory behavior to prevent further temperature elevation. During the bee ball formation, bees seem to continue to generate a considerable amount of heat. As a result, the inner temperature of the bee ball can be maintained at ~46°C.

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