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A new antigenic marker specifically labels a subpopulation of the class II Kenyon cells in the brain of the European honeybee *Apis mellifera*

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The mushroom bodies are the higher-order integration center in the insect brain and are involved in higher brain functions such as learning and memory. In the social hymenopteran insects such as honeybees, the mushroom bodies are the prominent brain structures. The mushroom bodies are composed of lobed neuropils formed by thousands of parallel-projecting axons of intrinsic neurons, and the lobes are divided into parallel subdivisions. In the present paper, we report a new antigenic marker to label a single layer in the vertical lobes of the European honeybee *Apis mellifera*. In the brain of *A. mellifera*, a monoclonal antibody (mAb) 15C3, which was originally developed against an insect ecdysone receptor (EcR) protein, immunolabels a single layer of the vertical lobes that correspond to the most dorsal layer of the γ-lobe. The 15C3 mAb recognizes a single ~200 kDa protein expressed in the adult honeybee brain. In addition, the 15C3 mAb immunoreactivity was also observed in the lobes of the developing pupal mushroom bodies. Since γ-lobe is well known to their extensive reorganization that occurs during metamorphosis in *Drosophila*, the novel antigenic marker for the honeybee γ-lobe allows us to investigate morphological changes of the mushroom bodies during metamorphosis.

Key words: *Apis mellifera*, mushroom body, antigenic marker, monoclonal antibody, vertical lobe

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The mushroom bodies, a pair of structures in the brain of insects and other arthropods, are the higher-order integration center of sensory information and are involved in higher brain functions such as learning and memory [1]. Each mushroom body is composed of parallel-projecting axons of a large population of intrinsic interneurons called Kenyon cells, the sensory input neuropiles called calyx, and the primary output neuropiles called lobes. The lobes are divided into parallel subdivisions which can be visualized by antigenic markers in some insects [2,3].

In the honeybee brain, the mushroom bodies are remarkably developed (occupy dorsal part of the brain), and receive visual, olfactory and other inputs. The Kenyon cells of the honeybee mushroom bodies are morphologically classified into three subpopulations (large-type, class I and class II small-type Kenyon cells), and the laminar organization of the lobes of the honeybee mushroom bodies was visualized using several histological techniques, such as immunohistochemistry using antisera against FMRFamide and gastrin cholecystokinin [4], and NADPH diaphorase histochemistry [5].

In the present paper, we report the antigenic marker, the 15C3 monoclonal antibody (mAb), to labal the neural fibers that might originate from a specific subpopulation of the Kenyon cells in the European honeybee *A. mellifera*. The 15C3 mAb was originally developed against an epitope in the common region of a *Manduca sexta* EcR [6]. We previously identified EcR isoforms in *A. mellifera* [7], and in this study we first attempted to examine their protein distribution in the honeybee brain. Against our expectations that the EcR isoforms would localize to nuclei as transcription factors, we

could not find any 15C3-immunoreactive nuclei in the adult honeybee brain, but found intensive 15C3 immunoreactivity in the mushroom body neurophiles. In the mushroom bodies of adult honeyebees, the neural fibers projecting to a single most dorsal layer of the γ-lobe were intensively labeled. Western blot analysis revealed that a single ~200 kDa protein in the brain of *A. mellifera* was recognized by the 15C3 mAb. Furthermore, the 15C3 mAb immunoreactivity was observed in the developing pupal mushroom bodies. The 15C3 mAb can be an useful tool to study structural feature of the mushroom bodies, as well as morphological changes of the γ-lobe neurons during metamorphosis in the honeybee.

Materials and Methods

Animals

Adult worker bees and pupae of the European honeybee (*Apis mellifera*) were collected from colonies purchased from Kumagaya Honeybee Farm (Saitama, Japan) and maintained on the roof of our laboratory building. Forager bees of Japanese honeybee (*Apis cerana japonica*) were collected in the Hongo campus of the University of Tokyo. Developmental stages of honeybee pupae were determined according to Ganeshina *et al*. (2000) [8].

Immunohistochemistry

The concentrated culture supernatant of the 15C3 mAb was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Dissected honeybee brains were fixed with 4% paraformaldehyde in phosphate-buffered saline [PBS; 135 mM NaCl, 2.9 mM NaH_2PO_4, 9 mM Na_2HPO_4, pH 7.4] at 4°C overnight. After three 30-min rinses in PBS, the fixed brains were embedded in 4% agar in PBS, and sectioned at 100 μm with a vibrating blade microtome (Leica VT1000 S, Leica Microsystems, Wetzlar, Germany). Free-floating sections were rinsed three times in 0.2% Triton X-100 in PBS (PBS-Tx), and incubated with 2% goat serum in PBS-Tx at 4°C for 3 h. Then, the brains were incubated with the 15C3 mAb diluted to 1:100 with 2% goat serum in PBS-Tx at 4°C for 5 days. After five-time 30-min-rinses and an overnight rinse in PBS-Tx, the brains were incubated with Alexa Fluor 555-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) diluted to 1:500 with blocking solution at 4°C for 3 days. After antibody treatment, the sections were incubated with 0.1 μg/ml DAPI, and rinsed three times in PBS-Tx for 30 min. Then, they were cleared through 30–70% glycerol and mounted in Shandon Immu-Mount (Thermo Scientific, Waltham, MA, USA). Samples were viewed with a Zeiss Axio Imager Z1 Microscope with Apotome (Carl Zeiss MicroImaging, Jena, Germany) and a LSM 700 Confocal Laser Scanning Microscope (Carl Zeiss). Captured images were processed using Image J and Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

Western blot analysis

The brains of the European honeybee (*A. mellifera*) and the Japanese honeybee (*A. cerana japonica*) were dissected from worker bees in ice-cold PBS (pH 7.4). The whole-brain, the central brain (whole brain minus the optic lobes), and the optic lobes were homogenized in lysis buffer [50 mM Tris-HCl (pH 9.5), 5 mM EDTA, 1% SDS containing 0.1 mM phenylmethylsulphonyl fluoride], and protein concentration of lysates was measured using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples (50 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel. After separation by SDS-PAGE, the protein samples were electrotransferred to the Immobilon-P Transfer Membrane (Millipore, Billerica, MA, USA). After blocking with 2% goat serum in PBS-Tx, the membrane was incubated with the 15C3 mAb diluted to 1:300 with 2% goat serum in PBS-Tx at room temperature for 3 h. After three 5-min rinses with 0.05% Tween 20 in Tris-buffered saline [TBS; 150 mM NaCl, 20 mM Tris-HCl (pH 7.5)], the membrane was incubated with horseradish-peroxidase-labeled anti-mouse IgG (Amersham Biosciences, Piscataway, NJ, USA) diluted to 1:5000 with 5% skim milk and 0.05% Tween 20 in TBS at room temperature for 1 h. After three 10-min rinses with 0.05% Tween 20 in TBS and a 10-min rinse with TBS, the membrane was treated with LumiGlo Reagent (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 5 min. The luminescence of the specific spots was detected with Amersham Hyperfilm MP (GE Healthcare, Buckinghamshire, UK).

Results and Discussion

The 15C3 mAb specifically labels a subgroup of the class II Kenyon cells in the brain of *A. mellifera*

The 15C3 mAb, which originally developed against an insect EcR protein [5], showed cross-immunoreactivity with an intrinsic epitope(s) localized in the mushroom body of the European honeybee *A. mellifera*.

In the adult worker brain, a 15C3 mAb-immunoreactive layer was observed in the vertical lobe of the mushroom bodies (Fig. 1(A)). In the vertical lobe, the most dorsal layer of the γ-lobe (the glia-rich part of the vertical lobe revealed by DAPI staining) was intensively immunolabeled by the 15C3 mAb (Fig. 1(B), (C)). The descending fibers in the pedunculus were also labeled with the 15C3 mAb (arrowheads in Fig. 1(D)). In the calyces, the 15C3 mAb-immunoreactive fibers travel through the lip region (arrows in Fig. 1(G)). The γ-lobe is subdivided into three layers (γ1, γ2 and γ3), and the most dorsal layer (γ1) possibly receives projections from the class II Kenyon cells surrounding the lip of the calyces [9]. The 15C3 mAb-immunoreactive fibers, traveling from the lips to the dorsal layer of the γ-lobe, might correspond to the neural fibers of the class II Kenyon cells supplying the γ1 layer. Immunoreactivity of the 15C3 mAb

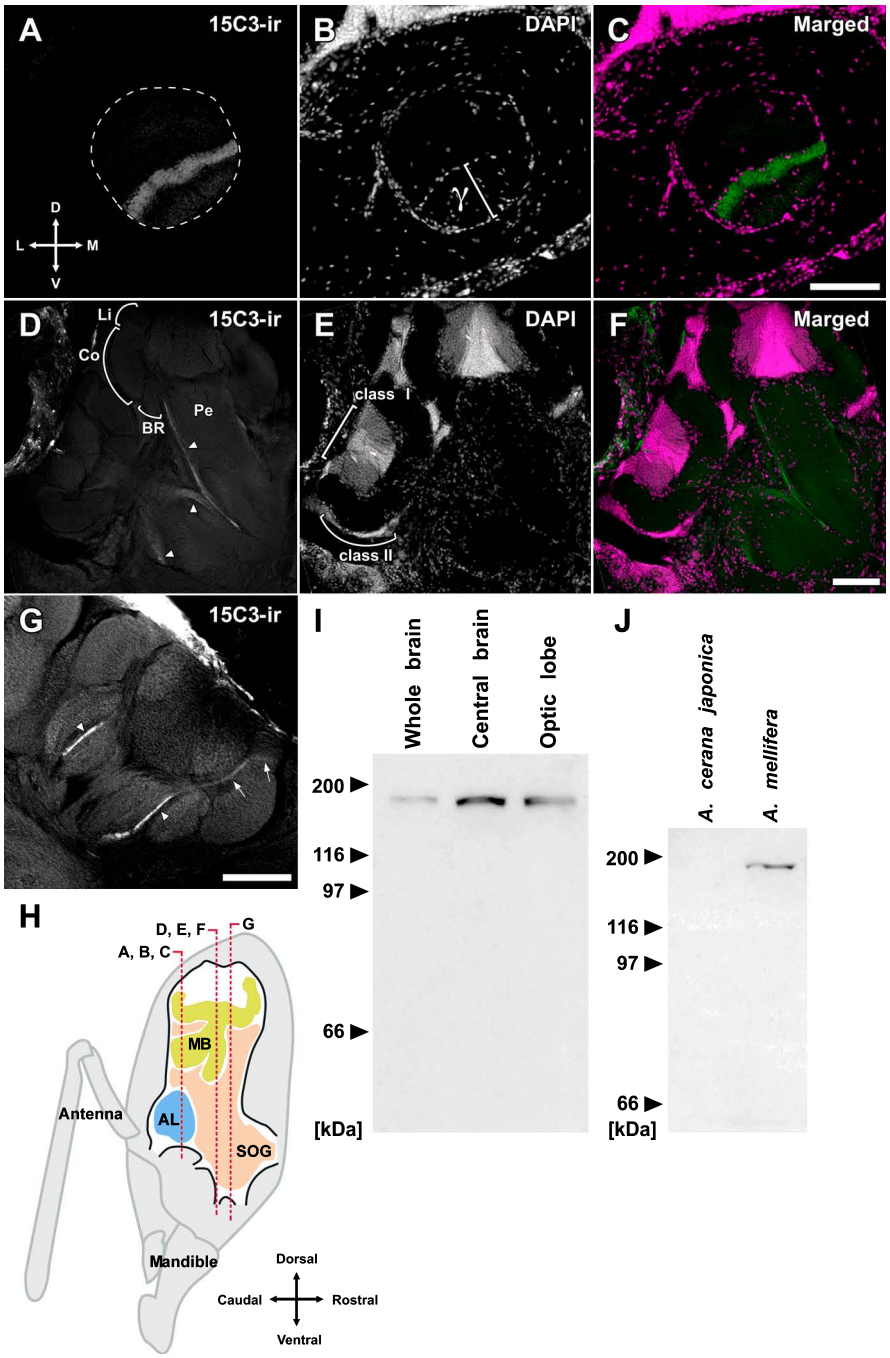

Figure 1 15C3 mAb-immunoreactivity in the honeybee brain. (A–G) Immunohistochemistry using the 15C3 mAb. An intensive immunoreactivity was observed in the mushroom body neuropiles. (A) A 15C3 mAb-immunoreactivity (15C3-ir) was observed in the vertical lobe of the mushroom body. White dotted line indicates the boundary of the vertical lobe. (B) DAPI staining reveals the glia-rich γ-lobe region (γ) in the vertical lobe of the mushroom body. (C) Merged image (green; 15C3-ir, magenta; DAPI). The 15C3 mAb strongly labeled the dorsal layer of the γ-lobe. (D) The 15C3 mAb-immunoreactive fibers observed in the pedunculus were indicated by arrowheads. (E) DAPI staining reveals that the clusters of the Kenyon cells (class I and class II). (F) Marged image (green; 15C3-ir, magenta; DAPI). (G) 15C3 mAb-immunoreactivity in the calyx. The 15C3 mAb-immunoreactive fibers in the pedunculus and the lip region were indicated by arrowheads and arrows, respectively. (H) A schematic diagram of the lateral view of the brain is shown. Positions of sections (A–G) are indicated by red dotted lines. Pe, pedunculus; Li, lip; Co, collar; BR, basal ring; AL, antennal lobe; MB, mushroom body; SOG, suboesophageal ganglion. Scale bars incdicate 100 µm. (I–J) Western blot analysis for 15C3 mAb-immunoreactive protein in the honeybee brain. (I) An intensely stained band of approximately ~200 kDa was detected in the lane of the central brain (whole brain minus the optic lobes), and weaker stained bands of the same size were detected in the lane of the whole brain and the optic lobes. (J) Protein samples of the worker brain of *A. cerana japonica* and *A. mellifera* were subjected to Western blot analysis. The ~200 kDa protein was detected in the lane of *A. mellifera* brain samples, but not in the lane of *A. cerana japonica*.

was also observed in the dorsal and ventral rim areas of the optic lobes and retina (Supplementary Fig. 1). In the mushroom bodies and optic lobes, the immunoreactivity of 15C3 mAb was localized in nerve fibers, but not in the somata and nuclei, suggesting cytosolic structural protein important for neurite structure (e.g. cytoskeletal protein or cytoskeleton interacting protein) or neurite-specific membrane protein is recognized by the 15C3 mAb in the brain of *A. mellifera*.

We also examined the 15C3 mAb-immunoreactivity in the brain of the Japanese honeybee *A. cerana japonica*, the sibling species of *A. mellifera*. Unexpectedly, the 15C3 mAb-immunoreactivity was not observed in the brain of *A. cerana japonica* (data not shown).

The 15C3 mAb recognizes a ~200 kDa protein expressed in the adult worker brain of the European honeybee

To determine the size of protein(s) that cross-immunoreacts with the 15C3 mAb, we performed Western blot analysis of honyebee brain lysate with the 15C3 mAb. First, we tried Western blot analysis with standard buffers/blocking condition (i.e. Tris-buffered saline instead of PBS, 5% skim milk instead of 2% goat serum), we could not obtain any positive signals. Therefore, we adjusted antibody reaction condition to that of our immunohistochemical procedure, and obtained single immunoreactive bands of approximately 200 kDa in the samples from *A. mellifera* (Fig. 1(I)). We detected ~200 kDa protein in the crude lysates of the whole brain, central brain (including mushroom bodies) and the optic lobes, suggesting that the ~200 kDa protein is a single source of 15C3 mAb-immunoreactivity in the brain of *A. mellifera*.

The 15C3 mAb was reported to recognize EcR protein in various arthropod species such as fruit fly, assassin bug and crayfish [10–13]. The calculated molecular mass of *A. mellifera* EcR isoforms are 60–70 kDa [A isoform, 66 kDa; B1 isoform, 60 kDa (GenBank accession numbers are BAH56300 and BAH56333, respectively)] [6]. Therefore, the 15C3 mAb did not immunoreact with intrinsic EcR protein(s), otherwise the EcR protein might be expressed under detectable level in the adult honeybee brain.

Next, we examined whether the 15C3 mAb recognizes the ~200 kDa protein in the brain of the Japanese honeybee *A. cerana japonica.* As consistent with the result of immunohistochemistry, the 15C3 mAb did not cross-immunoreact with any protein in the brain of *A. cerana japonica* (Fig. 1(J)). As described above, Western blot analysis with strong blocking condition (i.e. 5% skim milk) did not work well with the 15C3 mAb, suggesting weak interaction between 15C3 mAb and the ~200 kDa protein in the brain of *A. mellifera*. It is possible that there are species-specific variations in the 15C3-mAb recognizing epitope(s) of the ~200 kDa proteins between two honeybee species, which completely diminishes interaction between the 15C3 mAb and the ~200 kDa protein of *A. cerana japonica*.

15C3 mAb immunoreactivity in the developing mushroom bodies

The γ neurons of the mushroom bodies undergo axon

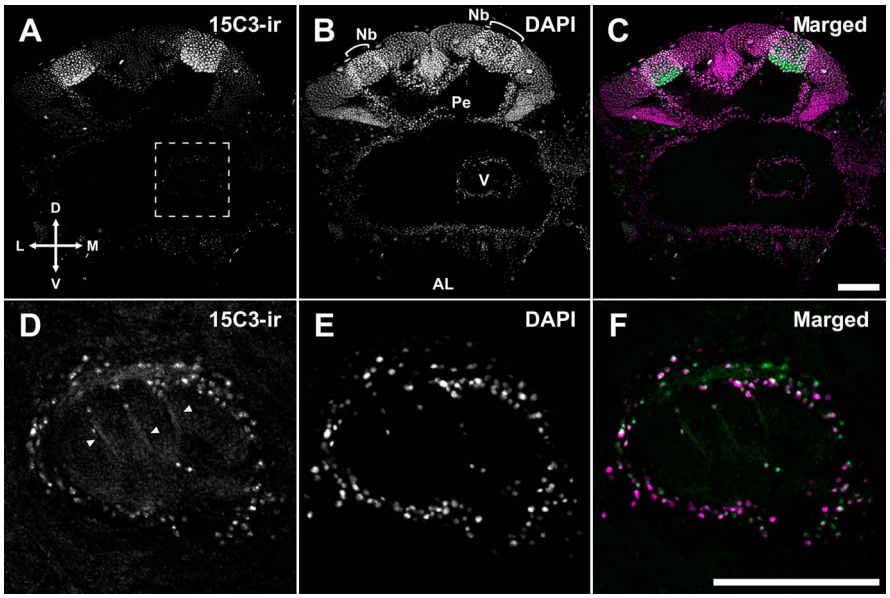

Figure 2 15C3 mAb-immunoreactivity in the developing brain of *A. mellifera*. 15C3 mAb-immunoreactivity in the worker brain of white-eyed pupa (P0/1 stage). (A) A intensive 15C3-ir was observed in the neuroblast claster of the mushroom body. (B) DAPI staining showing total nuclei. (C) Marged image (green; 15C3-ir, magenta; DAPI). (D) Magnified view of the vertical lobe indicated by the dotted square in A. 15C3-ir fibers indicated by arrowheads. (E) DAPI staining showing total nuclei. (F) Marged image (green; 15C3-ir, magenta; DAPI). Pe=pedunculus; Nb=neuroblast cluster; V=vertical lobe; AL=antennal lobe. Scale bars=100 μm.

pruning during metamorphosis in the *Drosophila* and honeybee [14,15]. To test whether 15C3 mAb can be a useful tool to analyze developmental remodeling of the axons of the Kenyon cells, the pupal worker brains were stained with the 15C3 mAb (Fig. 2).

Like the adult honeybee brain, the neural fibers in the vertical lobe of the mushroom bodies were immunostained in the pupal honeybee (arrowheads in Fig. 2(A)). Moreover, in contrast of the adult honeybee brains, the strong 15C3 mAb-immunoreactivity was observed in the nuclei. In *Drosophila*, EcR protein is widely distributed within the central nurvous system during early pupal stages [16], and is involved in metamorphic changes in neural circuits. Nuclear localization of 15C3 mAb-immunoreactivity in the pupal brain suggests that the 15C3 mAb recognizes the EcR proteins as well as the ~200 kDa protein expressed in the adult honeybee brain.

Conclusions

In conclusion, we newly developed a histochemical technique to label neural fibers innervating a single layer of the vertical lobe in the mushroom bodies of the adult European honeybee *A. mellifera*. Using the 15C3 mAb, a single most dorsal layer of the γ-lobe were labeled in the adult honeybee brain. Our new techenique allows us to examine the fine anatomical feuture of the mushroom bodies of adult honeybees, as well as the developmental morphological changes of the mushroom bodies during metamorphosis.

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Conflict of Interest

All the authors declare that they have no conflict of interest.

Author Contributions

TW carried out all experiments and drafted the manuscript. TK revised the manuscript.

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