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The Mushroom Body of Adult *Drosophila* Characterized by GAL4 Drivers

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Abstract: The mushroom body is required for a variety of behaviors of *Drosophila melanogaster*. Different types of intrinsic and extrinsic mushroom body neurons might underlie its functional diversity. There have been many GAL4 driver lines identified that prominently label the mushroom body intrinsic neurons, which are known as “Kenyon cells.” Under one constant experimental condition, we analyzed and compared the expression patterns of 25 GAL4 drivers labeling the mushroom body. As an internet resource, we established a digital catalog indexing representative confocal data of them. Further more, we counted the number of GAL4-positive Kenyon cells in each line. We found that approximately 2,000 Kenyon cells can be genetically labeled in total. Three major Kenyon cell subtypes, the γ , α'/β' , and α/β neurons, respectively, contribute to 33, 18, and 49% of 2,000 Kenyon cells. Taken together, this study lays groundwork for functional dissection of the mushroom body.

Keywords: insect brain, morphology, GAL4/UAS system, expression database, Kenyon cells

INTRODUCTION

“For approaching the enormous complexity of the insect brain one may choose first to study the sensory and motor periphery in the hope to finally work one’s way up to the central processing stages of the brain or, alternatively, one may parachute in the midst of the jungle, experimentally altering the brain and try to understand the concomitant changes in behavior”

(Heisenberg, 1980).

The mushroom body (MB) is a major landmark in the “jungle” of the *Drosophila* brain (Fahrbach, 2006; Heisenberg, 2003), consisting of thousands of intrinsic and extrinsic neurons. Kenyon cells, the second-order olfactory interneurons in *Drosophila*, constitute the majority of the intrinsic neurons. Their cell bodies form a pair of quadruple clusters at the dorsal posterior cortex. Their extensive dendritic arborizations contribute to the globular structure beneath the cell bodies, called the calyx, in which the collaterals of the olfactory projection neurons terminate (Stocker et al., 1990). The axon bundle of the Kenyon cells further project anteriorly through the pedunculus to the lobes. The lobes of the MB are

considered as the main output site of Kenyon cells, but also receive many inputs from extrinsic neurons (Ito et al., 1998; Tanaka et al., 2008; Johard et al., 2008).

Various behavioral and physiological functions that the MB is known to support range from olfactory learning to decision making under uncertain conditions. Its structural heterogeneity may anatomically reflect the organization of circuits that are required to achieve an array of distinct behavioral functions in one brain structure.

Drosophila Kenyon cells are roughly classified into three subtypes by their projections in the lobes: the γ , α'/β' , and α/β lobe neurons in order of birth (Crittenden et al., 1998; Jefferis et al., 2002). The γ -neurons project only to the medial lobe, while the α'/β' and α/β neurons bifurcate at the anterior end of the pedunculus to project to the medial and vertical lobes (Crittenden et al., 1998; Ito et al., 1998). Recent studies revealed that α'/β' and α/β lobes harbor further subdivisions (Strausfeld et al., 2003; Tanaka et al., 2008; Yang et al., 1995). In addition to their morphological distinction, these subtypes are differentiated with respect to their gene expression, neurotransmitter systems, connectivity to extrinsic neurons, and behavioral functions (Keene & Waddell, 2007; Strausfeld et al., 2003; Tanaka et al., 2008).

Compared to the other insect species with large numbers of Kenyon cells (Leitch & Laurent, 1996; Neder,

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1959; Witthöft, 1967), the advantages of studying the *Drosophila* MB are its smaller size with a conserved, layered cellular organization (Mobbs, 1984; Rybak & Menzel, 1993; Sjöholm et al., 2006; Strausfeld, 2002; Strausfeld et al., 2003) and its amenability to genetic manipulation (Venken & Bellen, 2005). This allows counting, rather than only estimating, the total number of Kenyon cells, and indeed, various studies have reported total numbers based on direct counting or extrapolation (Balling et al., 1987; Hinke, 1961; Ito & Hotta, 1992; Lee et al., 1999; Mader, 2004; Technau & Heisenberg, 1982; Technau, 1984). However, the average numbers in these studies range approximately from 1,000 to 2,500. Although the precise number is required to construct quantitative network models of the *Drosophila* MB and to assess the integrity of the genetic manipulation of Kenyon cells, no study has seriously addressed this discrepancy.

Laboratory members at the Biocenter in Würzburg have often witnessed Martin Heisenberg's extraordinary interest in determining the total number of Kenyon cells. His "favorite" number was 2,500, which was published in the early days of his laboratory (e.g., Technau & Heisenberg, 1982). Martin asserted that counting the fiber number in cross-sections of the posterior part of the pedunculus from electron micrographs resulted in the least errors, because 1) the fiber number represents the cell number, since Kenyon cell axons do not bifurcate at that level, and 2) the majority of thin and round fibers represent Kenyon cells.

We decided to reexamine the total number of Kenyon cells by using the GAL4/UAS system, since the cell type can be readily discerned. An array of GAL4 drivers have been identified with prominent expression in the entire or specific subsets of the MB (MB-GAL4; Figure 1). The specificity of GAL4 expression inside and outside the MB is critical, especially for the interpretation of functional manipulation when using MB-GAL4s (Ito et al., 2003). Here, we show the expression patterns of 25 different MB-GAL4s in the brain and the subesophageal ganglion under one constant experimental condition. We present a catalog that indexes the confocal micrographs of all analyzed drivers. By counting GAL4-positive Kenyon cells, we here endeavor to reveal the total number of Kenyon cells. Further, we demonstrate the numerical composition of the Kenyon cell subtypes.

MATERIALS AND METHODS

Fly Strains

Fly stocks were grown on standard *Drosophila* medium (cornmeal, agar, molasses, yeast, and nipagin) under a constant light-dark cycle (14/10 hours) at 25°C and 60%

relative humidity. Twenty-five GAL4 strains were selected from the literature (Figure 1). These drivers have been used for behavioral, developmental, physiological, and/or anatomical studies of Kenyon cells (Supplemental Figure 1). To visualize the GAL4 expressing cells, males of these GAL4 lines were crossed with females of a reporter strain carrying multiple copies of *UAS-mCD8::GFP* (G3). No specific labeling was detected in the absence of a GAL4 driver (data not shown). For the GAL4 drivers on the X chromosome (D52H, NP3208, NP65, and NP7175), females of each driver were crossed with G3 males to detect the expression in F1 males. For the double drivers, one driver strain was crossed with another driver, and then F1 males heterozygous for two GAL4 insertions were crossed with G3 females. The genotypes of F2 progenies were determined with characteristic expression of each GAL4 line.

Immunohistochemistry

We examined flies between 5 and 10 days after eclosion and analyzed at least 2 males and 3 females for each cross. The brains were dissected in Ringer's solution, fixed in phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.3% Triton X-100 (PBT; Sigma, St. Louis, Missouri, USA) for 1 hour at room temperature and subsequently rinsed with PBT three times (3 × 10 minutes). After being blocked with PBT containing 3% normal goat serum (Sigma) for 1 hour at room temperature, the brains were incubated with the primary antibodies in PBT at 4°C overnight. The GAL4-expressing cells and overall neuropils were stained with the rabbit polyclonal antibody against green fluorescent protein (GFP) (1:1000; Molecular Probes, Eugene, Oregon, USA; A6455) and the mouse monoclonal antibody against Synapsin, a presynaptic protein (3C11; 1:20) (Klagges et al., 1996), respectively. The brains were washed with PBT for 20 minutes three times and incubated with secondary antibodies in the blocking solution at 4°C overnight. The employed secondary antibodies were Alexa Fluor488-conjugated goat antirabbit (1:1000 or 1:500; Molecular Probes) and Cy3-conjugated (1:250) goat antimouse IgG. Finally, the brains were rinsed with PBT (3 × 20 minutes + 1 × 1 hour) and mounted in Vectashield (Vector, Burlingame, California, USA). For the quantitative analysis of the cell numbers, we stained the brains of different genotypes in the same tube. Their genotypes were *post-hoc* identified according to their expression patterns.

Confocal Microscopy

Frontal optical sections of whole-mount brains were taken with confocal microscopy (Leica SP1, Leica, Wetzlar,

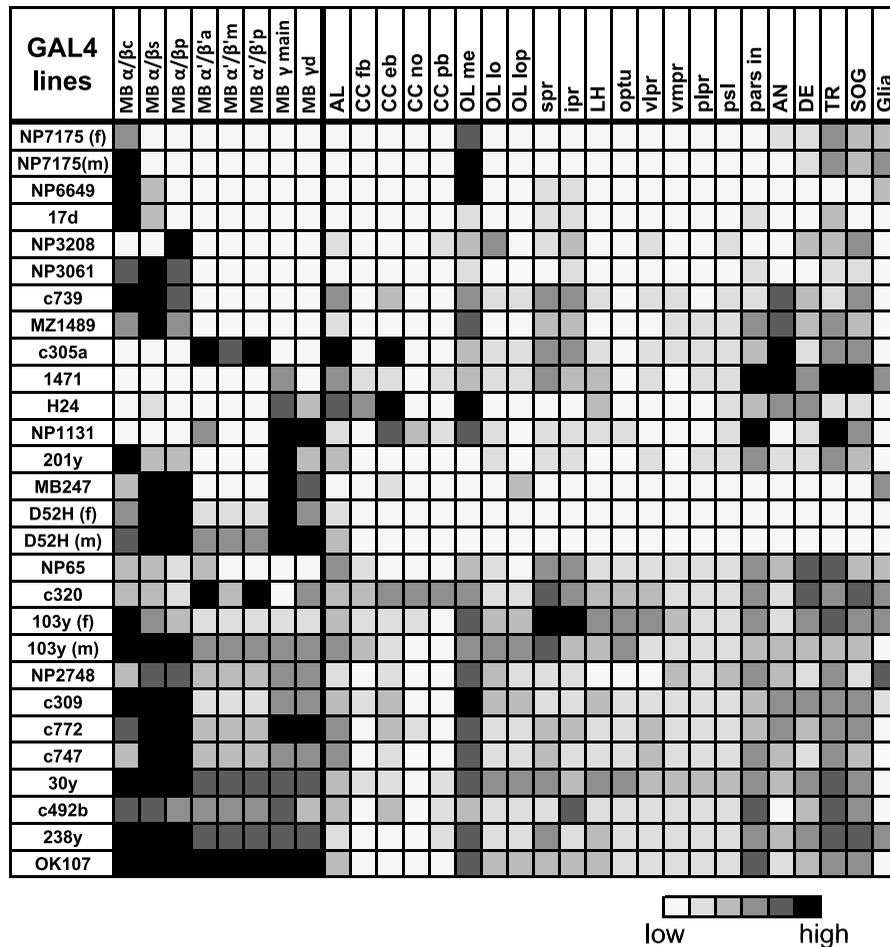


Figure 1. Expression pattern of 25 GAL4 lines. Summary of the expression levels of 25 MB-GAL4s in various brain areas defined by anti-Synapsin immunostaining. Gray scale indicates subjectively evaluated signal intensity. Note that a higher level of fluorescent signals in the certain brain area can result from larger population of GAL4 expressing cells and/or stronger GAL4 expression in each cell. MB, mushroom body; c, core subdivision; s, surface subdivision; p, posterior subdivision; a, anterior subdivision; m, middle subdivision; d, dorsal subdivision; AL, antennal lobe; CC, central complex; fb, fan-shaped body; eb, ellipsoid body; no, noduli; pb, protocerebral bridge; OL, optic lobe; me, medulla; lo, lobula; lop, lobula plate; spr, superior protocerebrum; ipr, inferior protocerebrum; LH, lateral horn; optu, optic tubercle; vlpr, ventrolateral protocerebrum; plpr, posteriorlateral protocerebrum; vmpr, ventromedial protocerebrum; psl, posterior slope; pars in, pars intercerebralis; AN, antennal nerve; DE, deutocerebrum; TR, tritocerebrum; SOG, subesophageal ganglion. References where these GAL4 drivers have been used are listed in Supplementary Figure 1.

Germany). Image stacks were collected at 1.5- μ m intervals with a 20X objective lens to cover an entire brain. For cell counting, we collected confocal stacks at 0.8- μ m intervals with a 40X objective lens. The posterior region of the MB was zoomed at such a magnification that all the GAL4-expressing cell bodies are in a frame. The posterior MBs in the left and right hemispheres were separately scanned and analyzed. For the quantitative analysis, brains were scanned with comparable intensity and offset. Images of the confocal stacks were analyzed with the open-source software (Image-J; National Institutes of Health, Bethesda, Maryland, USA). To avoid overlooking diffuse and/or weakly labeled structures, all pictures were repeatedly examined by different experimenters.

Cell Counting

Confocal stacks of the magnified cell-body region were first subjected to automatic marking of nuclei by a combination of ImageJ plug-ins: “Particle analyzer” and “Cell counter”. Particle analyzer, which was customized to recognize the circles (nuclei) by intensity thresholding at multiple different levels, automatically detected the nuclei of GAL4-expressing cells in each confocal slice. According to the coordinate information, the centers of the nuclei of the labeled cells were marked by “Cell counter.” Subsequently, all the stacks were reexamined manually to correct the errors in the automatic detection. Labeled, but not Kenyon, cells or unlabeled

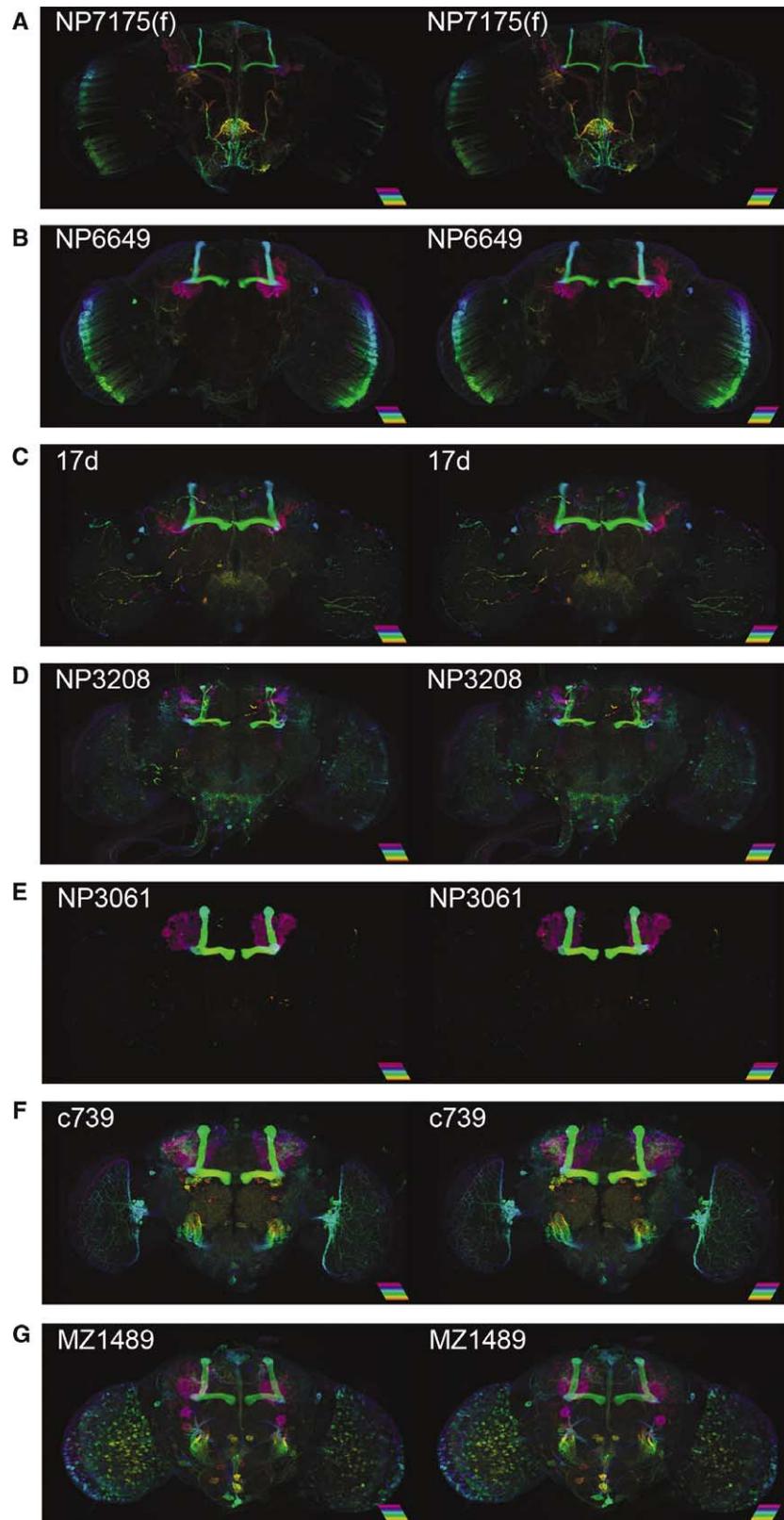


Figure 2 (Continued)

Kenyon cells surrounded by labeled cells were discriminated by their size, shape, location, fine neurites to the calyx, and reporter signals from the cytosol and membrane. Typically, one nucleus spanning different confocal sections was automatically marked twice or three times in the close vicinity. By manually checking duplicated markers in neighboring slices, we subtracted these redundant counts from the final number. Experimenters counted the numbers of Kenyon cells without information on genotypes. There was no statistical difference between experimenters, which was verified by counting the same samples (data not shown). The numbers of labeled Kenyon cells were statistically compared by using multiple pair-wise comparisons (*t*-test, followed by Bonferroni correction).

RESULTS

Collection of the Expression of MB-GAL4 Drivers

The interpretation of genetic manipulation using MB-GAL4 crucially depends on the expression pattern inside and outside of the MBs. We, thus, systematically reexamined the GAL4 expression of many published MB-GAL4 drivers under one constant experimental condition. We selected 25 MB-GAL4 drivers from the literature and evaluated their expression patterns with confocal microscopy (Figure 1; Supplemental Figure 1).

As a reporter gene, we chose multiple copies of *UAS-mCD8::GFP* inserted in different genomic loci (G3). Since CD8 is a membrane protein, GFP is targeted predominantly to the plasma membrane of GAL4-express-

ing cells. After immunostaining, we frequently noticed that these multiple copies of the reporter construct enabled visualization of weakly labeled cells that had been overlooked in previous reports. Moreover, G3 is advantageous for counting the number of labeled cells, since the nuclei of the GAL4-expressing cells are clearly outlined (e.g., Figure 8A).

A previous study showed that different reporter genes, different staining conditions, and the insertion site of a reporter gene may influence the results (Ito et al., 2003). G3 can minimize such insertion-specific biases, since there are multiple different insertions of *UAS-mCD8::GFP*.

The expression patterns of MB-GAL4s in the brain and subesophageal ganglion (SOG) are summarized in Figure 1 and Supplemental Figure 1. By relative signal intensity, we subjectively ranked the degree to which fibers were resolved in various neuropils. Therefore, these ranks are neither to indicate the absolute intensities nor to compare the expression levels between different GAL4 drivers, but are rather to compare the signal intensities within a sample. MB-GAL4s vary, to a great extent, in terms of the specificity within the MB and the expression outside the MB (Figure 1; supplemental Figure 1). In the following sections, we describe the expression pattern of these drivers. Based on the expression pattern within the MB of female samples, we categorized them into five groups: MB-GAL4s labeling the α/β lobes, α'/β' lobes, γ lobes, multiple lobes, and all of the lobes. Except for three lines (NP7175, D52H, and 103y), expression pattern was very similar in males and females.

As an Internet resource, we indexed representative confocal data of all analyzed driver strains (“der Pilz”;

Figure 2. Drivers labeling the α/β neurons. Stereopairs show reconstructions of MB-GAL4s preferentially labeling the α/β neurons. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). Because of the space limitation, labels of the neuropils have been omitted from all figures. See also original confocal stacks for detail (<http://mushroombody.net>). Expression pattern was indistinguishable between males and females, unless stated. (A) Female NP7175 exclusively innervated the α/β lobes in the MB. It also labeled the subesophageal ganglion, the cells in the medulla, and the processes in the tritocerebrum. See Figure 7A for the expression pattern in the male. (B) NP6649 strongly labeled the α/β , while the expression in the α/β s was weak. As in NP7175, the cells in the medulla were strongly labeled. It also has expression in the dorsal giant interneuron (DGI) (Ito et al., 1997). (C) As in NP6649, the α/β were strongly labeled in 17d, while the expression in the α/β s was weak. In addition, weak labeling was occasionally detected in the pars intercerebralis, cells located around the subesophageal ganglion, DGI, and large neurons located around the calyx and protocerebral bridge. One very large neuron located ventrally to the calyx projects anteriorly and terminates in the superior and inferior protocerebrum. Collaterals of this neuron may also project to the deutocerebrum. Similar neurons were labeled by NP3208, c739, MZ1489, NP65, and c492b. Especially in MZ1489, their huge cell bodies are clearly visible (see G). (D) NP3208 specifically innervated the α/β p. The accessory calyx is visible as a protrusion originating from the dorsal calyx. The paired giant neurons located ventral to the calyx (see also the legend of C) and processes in the subesophageal ganglion were labeled. Further faint signals were detected in many neuropils, including the optic lobes. (E) NP3061 labeled all over the α/β lobes. Occasionally, barely detectable signals were in the pars intercerebralis, DGI, tritocerebrum, and subesophageal ganglion. NP3061 contained the least expression outside the MB in all the MB-GAL4s analyzed here. (F) c739 strongly innervated the entire α/β lobes. Outside the MB, it labeled elements in a wide range of neuropils, including local interneurons in the antennal lobe, the antennal nerve, ellipsoid body, a cluster of neurons projecting from/to the optic lobes, and many other cells throughout the brain (see also Figure 1). (G) MZ1489 innervated the entire α/β lobe, though the signal in the α/β c was less intense. Outside of the MB, it labeled a wide range of neuropils, including the pars intercerebralis, many cells in the medulla, antennal nerve, paired giant neurons located ventral to the calyx (see also the legend of C), and many other cells throughout the brain (see also Figure 1).

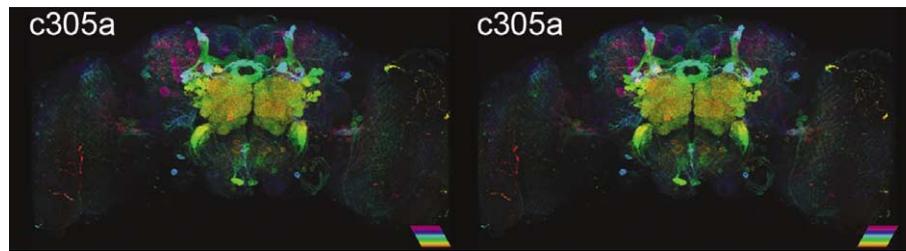


Figure 3. Driver labeling the α/β' neurons. Stereopair shows a reconstruction of c305a preferentially labeling the entire α/β' lobes. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). See also original confocal stacks for detail (<http://mushroombody.net>). Outside the MB, it labeled broad neuropils, including the local interneurons in the antennal lobe, antennal nerve, ellipsoid body, large paired neurons originating from the subesophageal ganglion, and many other cells throughout the brain (see also Figure 1).

<http://mushroombody.net>). This web-based database is publicly available. Thus, readers are encouraged to download and scrutinize the raw data by themselves, rather than solely relying on the subjectively ranked expression and brief description here.

Drivers Labeling the α/β Neurons

GAL4 expression in NP7175, NP6649, 17d, NP3208, NP3061, c739, and MZ1489 in the MB was restricted to the α/β neurons. NP7175, NP6649, 17d, and NP3208 labeled limited subdivisions (Figure 2A–2D). GAL4-positive Kenyon cells in NP7175, 17d, and NP6649 preferentially innervated the core of the α/β lobes (α/β c). Given the variable occupancy, the α/β c lobes are likely to consist of multiple populations with concentric organization (Figure 2; see also Tanaka et al., 2008). Within the MB, NP3208 has its expression specifically in the posterior division of the α/β lobes (α/β p), as previously described (Tanaka et al., 2008). The α/β p neurons have dendritic arbors specifically in the accessory calyx, where the projection neurons do not directly terminate (Tanaka et al., 2008). In NP7175, reporter signals inside and outside the MB differed between the

sexes (Compare Figure 2A and Figure 7A). With differences in expression levels among subdivisions, c739, MZ1489, and NP3061 seemed to drive GAL4 expression in most, if not all, subdivisions of the α/β lobes (Figure 2E–2G).

Driver Labeling the α'/β' Neurons

Among the MB-GAL4s we analyzed, only c305a had selective expression in the α'/β' neurons within the MB (Figures 1 and 3). The expression was detected in all subdivisions in the α'/β' lobes. NP65 and NP1131 labeled the specific subtype of the α'/β' lobe (i.e., the α'/β' a neurons), but had additional expressions in other subsets. Therefore, we categorized NP65 to the MB-GAL4s labeling multiple lobes, although the expression within the MB had been reported to be restricted in the α'/β' a neurons (Tanaka et al., 2008). Similarly, NP2748 has been described as a line specific for the α'/β' neurons (Tanaka et al., 2008), but we observed reporter signals also in the α/β and γ neurons. These differences in expression are presumably due to the employed reporter genes.

Figure 4. Drivers labeling the γ neurons. (A, B) Stereopairs show reconstructions of MB-GAL4s preferentially labeling the γ lobe. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). See also original confocal stacks for detail (<http://mushroombody.net>). (A) In the MB, expression of 1471 was restricted in the γ neurons. Expression in the γ d neurons was very weak, if any. It labeled a broad range of neuropils outside of the MB, including the pars intercerebralis, antennal lobe, antennal nerve, and sensory nerves. Large paired neurons located ventrally to the SOG and projecting to the deutocerebrum and/or tritocerebrum were also labeled. Similar neurons are also found in NP65, H24, and 201y. (B) H24 strongly labeled the γ neurons, with extremely weak additional expression in the α/β neurons. Outside the MB, local interneurons in the antennal lobe, the medulla, ellipsoid body, deutocerebrum, and large paired neurons located ventrally to the subesophageal ganglion showed the reporter signals. (C–G) Frontal views of the projections of three consecutive confocal slices, including the γ d lobe. In the left panels, the GAL4-positive processes (green) are shown with counterstaining of neuropils (anti-Synapsin; magenta). The right panels show only the reporter channel of the same stacks (black). In these sections, the γ lobe occupies the most dorsal part, while the β lobe occupies the most ventral part. The β' lobe typically lies in between them. Arrows indicate the medial end of the γ d subdivision. 1471 labeled the main part of the γ lobe, but not the γ d subdivision (C), whereas the expression both in the γ d subdivision and the main part of the γ lobe was seen in H24 and MB247 (D and E). (F) c320 labeled the γ d subdivision without labeling the main γ lobe. (G) c305a specifically labeled the α'/β' lobe, but not the γ d subdivision. Scale bar = 25 μ m for C–G.

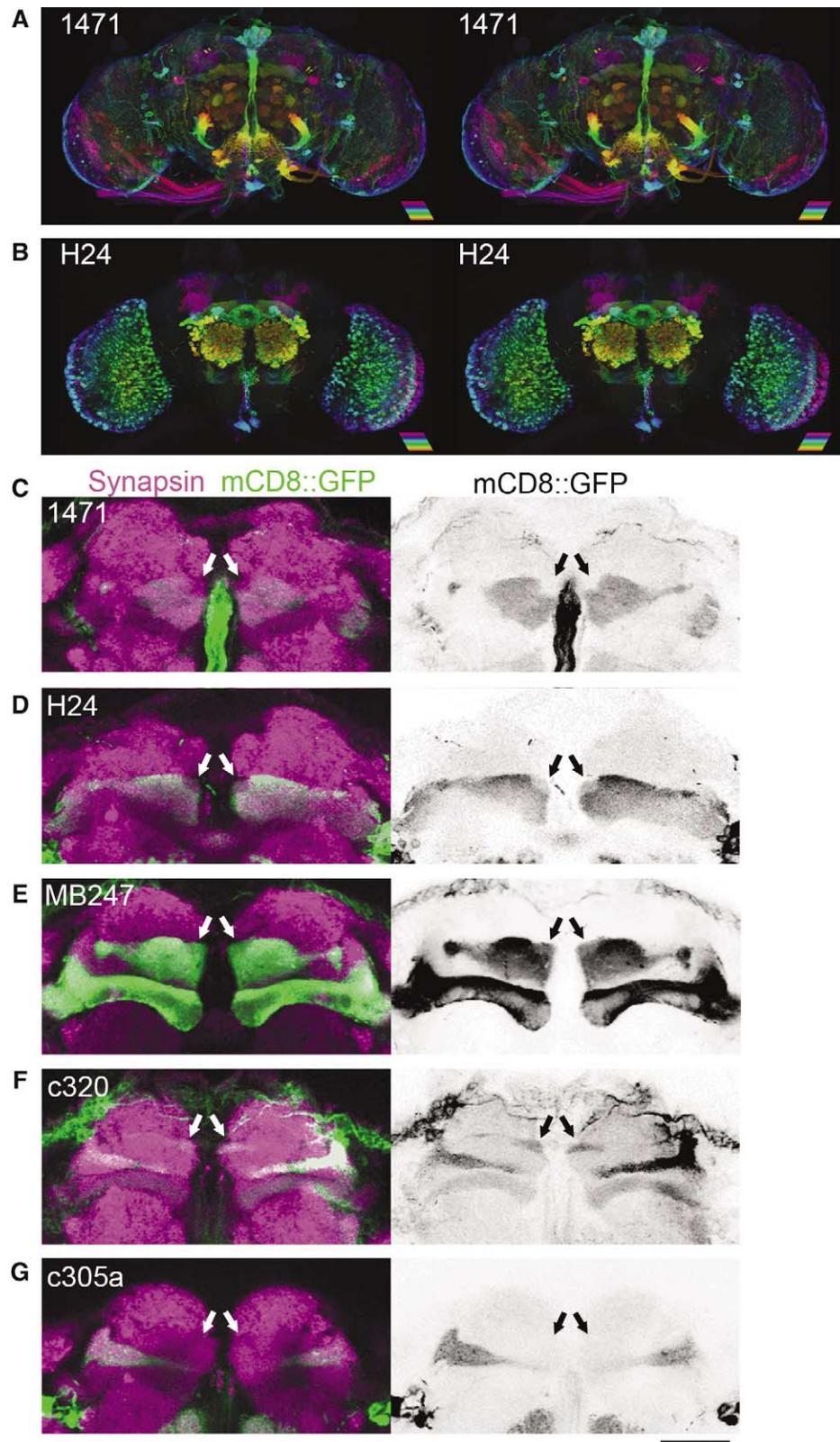


Figure 4 (Continued)

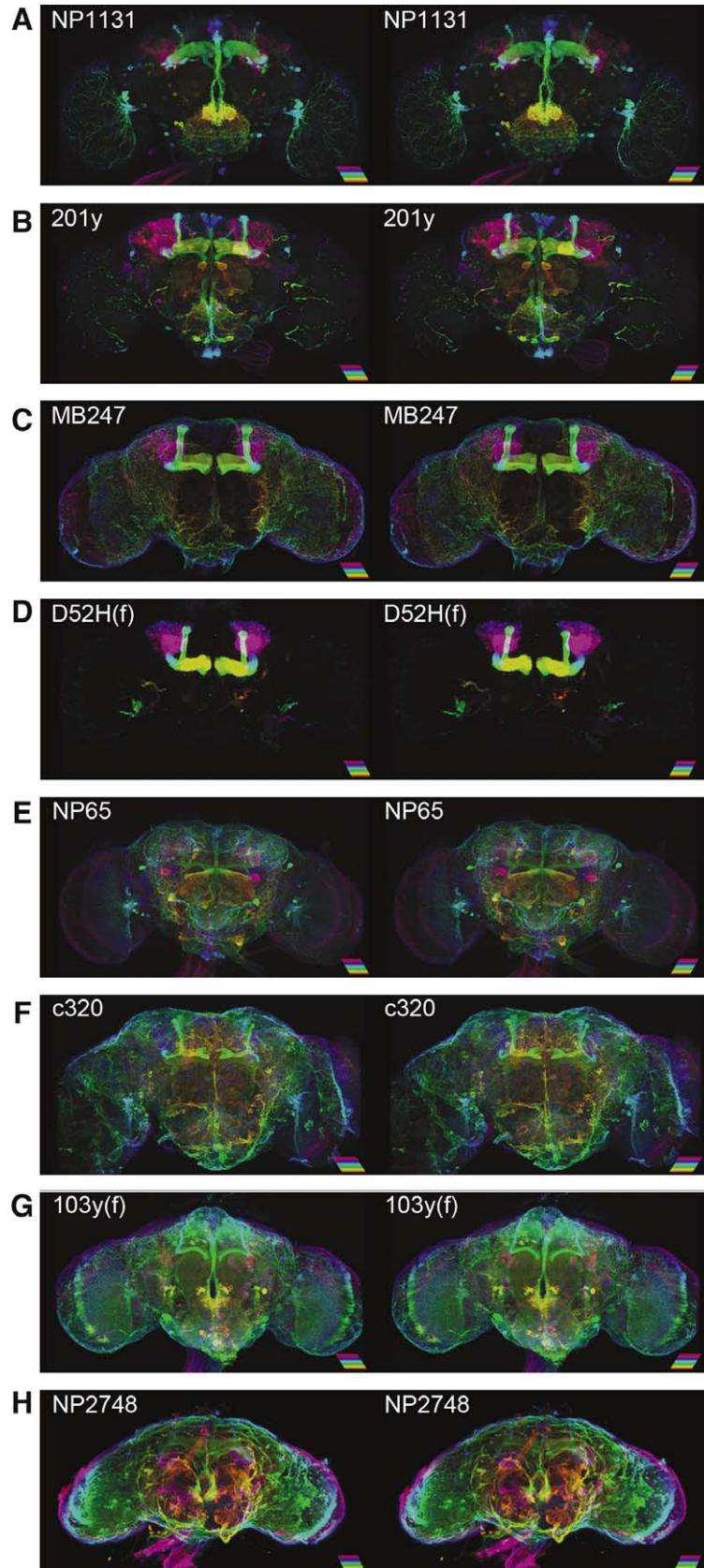


Figure 5 (Continued)

Drivers Labeling the γ -Neurons

H24 and 1471 preferentially marked the γ lobe within the MB (Figure 4). With G3, H24 additionally labeled the α/β neurons weakly. Also, 1471 had selective expression in the γ neurons of the MB, as originally reported (Isabel et al., 2004), although occasionally it seems to drive additional expression in the α/β neurons (Keleman et al., 2007). Further, 1471 seemed not to include all of the γ neurons, since the reporter expression in the dorsomedial tip of the γ lobe was conspicuously weak (Figure 4C and 4D). To distinguish this dorsal subdivision from the rest of the γ lobe (main part), we named it “ γd .” Does this subdivision belong to the γ neurons? We magnified the corresponding region of the γd in MB247, c320, and c305a (Figure 4E–G). MB247 labeled this part, but not the α'/β' lobes (Figure 4E). c320 had innervation there, but not in the rest of the γ lobe (Figure 4F). c305a labeled the entire α'/β' lobes, but not the γd (Figure 4G). These results indicate that the γd is supplied by a subpopulation of the γ neurons, but not the α'/β' neurons.

Drivers Labeling Multiple Types of Kenyon Cells

In NP1131, 201y, MB247, D52H (female), NP65, and c320, there was expression in many, but not all, the MB lobes (Figures 1 and 5). NP1131 selectively labeled the γ and α'/β' neurons in the MB (Figure 5A). Also, 201y had expression in the γ and $\alpha/\beta c$ neurons (Figure 5B). MB247 and D52H (female) strongly marked the α/β and γ neurons. MB247 and D52H (female) exhibited an unusually low level of background expression (Figure 5C and D). For both lines, there was very little, if any,

expression in α'/β' neurons. In D52H the expression showed a sexual difference in the MB. The males of D52H additionally strongly expressed the reporter in the α'/β' lobes (compare Figure 5D and Figure 7B). NP65 and c320 labeled subsets of the α/β and α'/β' neurons (Figure 5E and 5F). In NP2748 and female 103y, reporter signals were detectable in the all lobes, although very weak in some subdivisions (Figures 1 and 5).

GAL4 Drivers Labeling All Lobe Systems

c309, c772, c747, 30y, c492b, 238y, and OK107 labeled the α/β , α'/β' , and γ lobes. OK107 covered all the subdivisions of all the lobes, whereas, in the other drivers, the expression in one or more subdivisions of a lobe system was weak or undetectable. For instance, the expression of c309 was strong in α/β and γ lobes, but weak in the α'/β' lobes (Figure 6A). All drivers in this group had significant GAL4 expression outside the MB (Figure 1 and 6). Therefore, functional manipulation of all lobe systems in the MB might require either a combination of selective GAL4 drivers with little additional expression or different MB-GAL4s covering all lobe systems with nonoverlapping expressions outside the MB. Based on the expression in the lobes, it is possible that some of these drivers (e.g., OK107) genetically label all Kenyon cells.

Three MB-GAL4s, NP7175, D52H, and 103y, showed clear sexual differences in their expression patterns (Figures 1 and 7). For NP7175 and D52H, a cause of the difference in reporter expression might be because the GAL4 insertions are on the X chromosome. Compared to the female, male 103y more strongly labeled the α'/β' and γ lobes (Figure 5G and 7C).

Figure 5. Drivers labeling multiple types of Kenyon cells. Stereopairs show reconstructions of MB-GAL4s preferentially labeling the multiple types of Kenyon cells. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). Some lines exhibited detectable expression in all types of Kenyon cells, but were categorized into this group, because expression in certain subdivisions was remarkably weak. See also original confocal stacks for detail (<http://mushroombody.net>). **(A)** NP1131 labeled the α'/β' and γ lobes. Additional expression was seen in the ellipsoid body, subesophageal ganglion, pars intercerebralis, large interneurons connecting the optic lobe and the central brain, and other cells distributed in the brain (see also Figure 1). **(B)** 201y labeled the $\alpha/\beta c$ and γ neurons. Outside the MB, additional expression was seen in the several glomeruli in the antenna lobe, pars intercerebralis, large paired neurons located ventral to the subesophageal ganglion, DGI, and other neurons. **(C)** MB247 strongly labeled the α/β and γ neurons with very low background expression. Expression in the $\alpha/\beta c$ was weaker than in the other α/β subdivisions. Additional expression was detected in the cells in the lobula plate and surface glia. **(D)** Female D52H strongly labeled the α/β and γ neurons with very low background expression. Expression in the $\alpha/\beta c$ was weaker than in the other α/β subdivisions. See Figure 7B for the expression pattern in the male. **(E)** NP65 labeled the $\alpha/\beta s$, $\alpha/\beta c$, and α'/β' neurons. We observed reporter signals also in the antennal lobe, deutocerebrum, tritocerebrum, DGI, large paired neurons located ventral to the calyx (see also the legend of Figure 2C), two large neurons projecting medially to the subesophageal ganglion, and many cells throughout the central brain. **(F)** In c320, the α/β , α'/β' , and γd lobes were labeled (Figure 3F). Expression in the α'/β' neurons was weaker than the rest of α'/β' subdivisions. Outside the MB, it labeled the majority of neuropils. Among them, relatively strong expressions were observed in surface glia, the protocerebral bridge, subesophageal ganglion, optic lobes, and superior protocerebrum (see also Figure 1). **(G)** Female 103y labeled the α/β neurons strongly. Outside the MB, it labeled the optic lobes, inferior and superior protocerebrum, cells on the subesophageal ganglion, surface glia, and many cells in the posterior cortex. See Figure 7C for expression pattern in the male. **(H)** In NP2748, weak reporter signals were observed in all of the lobes. It also labeled the medulla, pars intercerebralis, DGI, surface glia, and many other cells.

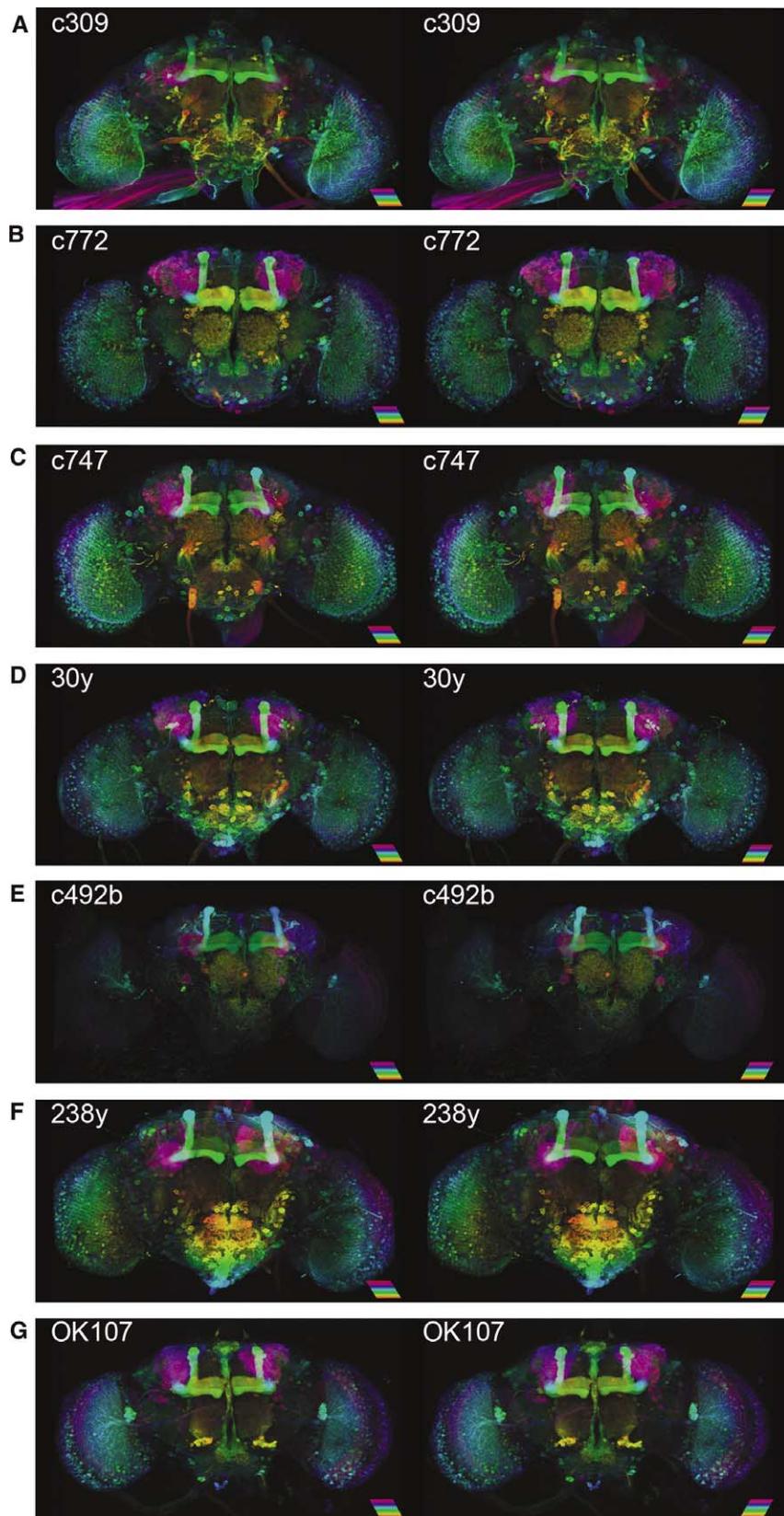


Figure 6 (Continued)

The Number of Genetically Labeled Kenyon Cells

The total number of Kenyon cells in the *Drosophila* MB was reported to range from 800 to 2,900 (Balling et al., 1987; Hinke, 1961; Ito & Hotta, 1992; Lee et al., 1999; Mader, 2004; Nikolai et al., 2003; Technau & Heisenberg, 1982; Technau, 1984). The variability mainly depends on the method of counting and estimation. MB-GAL4s with expression in all lobe systems (Figure 6) prompted us to address how many Kenyon cells of the *Drosophila* MB can be marked by the genetic method.

Consistent with their expression in the lobes, the largest numbers of Kenyon cells were stained in OK107 and 238y: on average, 1,917 and 1,898 cells, respectively (Figure 8B). These numbers were, however, smaller than those in some reports (Balling et al., 1987; Hinke, 1961; Technau & Heisenberg, 1982; Technau, 1984). To examine the highest possible number of genetically labeled Kenyon cells, we generated the flies carrying both OK107 and 238y (OK107/238y) and counted the labeled cells. If these single drivers incompletely labeled Kenyon cells with different patterns, the number of the GAL4-positive cells in OK107/238y could exceed that of each single driver. In OK107/238y, we counted 1,975 labeled Kenyon cells, on average, but not significantly higher than OK107 or 238y alone ($P > 0.05$; $n \geq 7$; Figure 8B).

Likewise, the numbers of GAL4-expressing Kenyon cells were counted in MB-GAL4s with expression in specific subdivisions of the lobes (Figure 8B). Interestingly, the expression pattern in the lobe does not always predict the number of the labeled neurons. For example, the expression patterns of NP3061 and c739 were similar in the α/β lobes (Figure 1, 2E, and 2F), whereas the number of

GAL4-expressing Kenyon cells of NP3061 was significantly less than that of c739 ($P < 0.001$; $n \geq 5$; Figure 7B). Similarly, the combination of MB247/NP6649 only slightly increased the number of labeled cells, compared to MB247 alone, indicating that weaker expression in the α/β of MB247 was sufficiently strong for cell counting. The MB-GAL4 that labels the smallest population in the analyzed lines was NP3208. The expression was detected in 76 cells, on average, and all of them projected to the α/β p (Tanaka et al., 2008) (Figure 1 and 2E).

Based on these results, we next examined the total number of genetically labeled Kenyon cells by summing up the numbers of MB-GAL4s labeling specific lobes. If the marked cell population in OK107/238y did not represent the entire Kenyon cells, the sum could exceed 1,975. Since we counted, on average, 1,002, 370, and 671 cells for c739 (α/β), c305a (α'/β'), and H24 (γ), respectively, the sum of these lines was 2,043 cells, which matches the number in OK107/238y with $\sim 3\%$ of a mean difference (Figure 7). The sum of MB247/NP6649 (α/β and γ ; 1,630 cells) and c305a (α'/β' ; 370 cells) also matched the number in OK107/238y (2,000 vs. 1,975 cells). Taken together, we conclude the average total number of Kenyon cells of the MB that can be genetically labeled is 2,000.

DISCUSSION

The Number of *Drosophila* Kenyon Cells

How many Kenyon cells does adult *Drosophila* have? With genetic labeling and confocal microscopy, we reached a number of 2,000 Kenyon cells per hemisphere (Figure 8B). This is roughly, on average, 500 cells fewer than previous

Figure 6. GAL4 strains labeling all of the lobes. Stereopairs show reconstructions of MB-GAL4s labeling all of the lobes. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). Since most of the lines in this category exhibited expression in many neuropils, we describe only strong signals here (see also Figure 1). See also original confocal stacks for detail (<http://mushroombody.net>). (A) In the MB, c309 strongly labeled the α/β and γ neurons. We also observed very weak expression in the α'/β' neurons. Outside the MB, the majority of the labeled neuropils included the antennal lobe, tritocerebrum, subesophageal ganglion, and optic lobes (see also Figure 1). Additionally, many different sensory nerves and the cervical connectives were stained. (B) c772 preferentially labeled the α/β p, α/β s, and γ neurons with weaker expression in the α/β c and α'/β' neurons. It also labeled the optic lobes, ventrolateral protocerebrum, local interneurons in the antennal lobe, and many cells on the subesophageal ganglion. (C) As in c772, c747 labeled the α/β p, α/β s, and γ neurons strongly and the α/β c and α'/β' neurons weakly. Outside the MB, we observed reporter signals in the optic lobes, antennal nerve, local interneurons in the antennal lobe, pars intercerebralis, and many cells on the subesophageal ganglion. (D) In 30y, all the MB subdivisions were innervated. It also labeled the optic lobes, antennal lobe, pars intercerebralis, and many cells surrounding the subesophageal ganglion. It also labeled a cluster of small neurons that are located dorsolateral to the optic tubercle and that project first ventrally and extend laterally toward the dorsolateral edge of the ventrolateral protocerebrum. Similar neurons were observed in 238y. (E) c492b labeled all types of Kenyon cells, although expression in the γ d neurons was relatively weak. Compared to other lines in this category, expressions outside the MB were less pronounced. We observed reporter signals in the pars intercerebralis, local interneurons in the antennal lobe, deutocerebrum, subesophageal ganglion, and large paired neurons located ventral to the calyx (see also the legend of Figure 2C). (F) All types of the Kenyon cells were strongly labeled by 238y. Outside the MB, this line labeled the optic lobe, superior protocerebrum, pars intercerebralis, and many neurons surrounding the entire subesophageal ganglion. (G) In OK107, all the subdivisions of the MB were strongly and uniformly labeled. Outside the MB, we observed strong reporter signals in the optic lobe, antennal lobe, pars intercerebralis, and cells on the subesophageal ganglion.

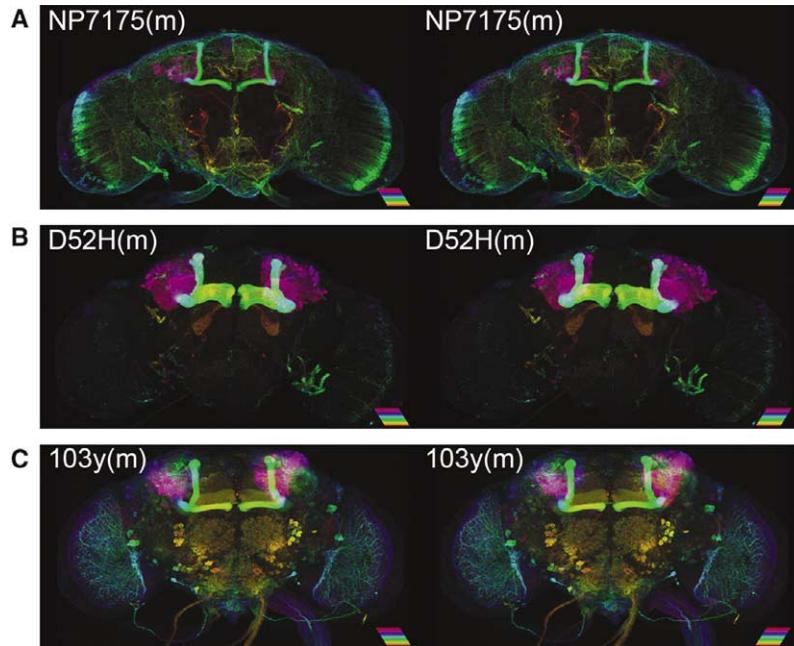


Figure 7. GAL4 strains with sex-dependent difference. Stereopairs show reconstructions of MB-GAL4s with sex-specific reporter expression. The applied color illustrates the depth (see scale bar [25 μ m] for the color code). See also original confocal stacks for detail (<http://mushroombody.net>). (A) Compared to the females, male NP7175 labeled slightly broader α/β (Figure 2 A). In addition to the background expression seen in the female, surface glia was strongly labeled. (B) In contrast to the female, male D52H additionally labeled the α/β' neurons. Moreover, the innervation of one glomerulus by the olfactory receptor neurons was more pronounced in the male. Otherwise, background expression was unusually low as in females. (C) Male 103y labeled all subtypes of Kenyon cells, whereas in the female, the expression in the α/β' and γ lobes was very faint (see Figure 5G). In addition, expression in surface glia and dense terminals in the superior medial protocerebrum were less pronounced in males. Outside the MB, it labeled the processes in the medulla and lobula, middle superior lateral protocerebrum, local interneurons in the antennal lobe, and many neurons supplying the subesophageal ganglion and tritocerebrum.

reports, where the numbers of cell bodies (2,380–2,640) and axon fibers (2,140–2,990) were estimated and counted, respectively (Balling et al., 1987; Hinke, 1961; Technau & Heisenberg, 1982; Technau, 1984).

We demonstrated that any combinations of MB-GAL4s with complementary or overlapping patterns covering the entire lobe systems failed to exceed 2,000 (Figure 8B). This is consistent with the number of cell bodies in one MB neuroblast clone, using *elav-GAL4* or *OK107* (i.e., \sim 500 cells) (Lee et al., 1999). It is still possible to assume that MB-GAL4s systematically fail to label 500 Kenyon cells. One parsimonious explanation for the discrepancy could be a retardation of Kenyon cell proliferation induced by overexpression of *mCD8::GFP*.

This possibility can be clarified by counting the number of Kenyon cell fibers at the posterior pedunculus. Indeed, we observed that the fiber number in *OK107/G3* was smaller than that in the wild-type *Canton-S* on electron micrographs (A.B.F. and Y.A., unpublished observation). In any event, this study suggests that approximately 2,000 Kenyon cells might be the limit that can be labeled by the *GAL4/UAS* system.

Alternatively, the lack of cell type-specific labeling in the previous studies could obscure the total numbers. Indeed, MB-APL, one of the non-Kenyon-cell intrinsic neurons, and GABA-ergic neuron(s) have thin parallel fibers projecting through the pedunculus as Kenyon cells do (Tanaka et al., 2008; Yasuyama et al., 2002). In our

Figure 8. The number of genetically labeled Kenyon cells. (A) Single confocal sections through the calyx and cell-body cluster of Kenyon cells as representative pictures of the counting procedure (*c739* and *NP7175*). Nuclei of Kenyon cells are marked with red, while non-Kenyon cells, unlabeled cells, or spaces between cells are marked with yellow. The arrow and arrowhead indicate microglomeruli innervated and not innervated by labeled Kenyon cells, respectively. Scale bar = 25 μ m. (B) The numbers of genetically labeled Kenyon cells in different MB-GAL4 drivers of the females. Labeled Kenyon cell subtypes are indicated below (see also Figure 1). The label in gray shows weak expression. See the legend of Figure 1 for abbreviations. $N = 5-8$. Error bars represent standard error of the mean. (C) Model of the numerical composition of the *Drosophila* MB. The γ , α'/β' , and α/β neurons, respectively, contribute to 33, 18, and 49% of \sim 2,000 Kenyon cells. One circle and line represents \sim 75 cells. Approximately 150 iACT projection neurons terminate on the Kenyon cells, except for the α/β neurons (rightmost line).

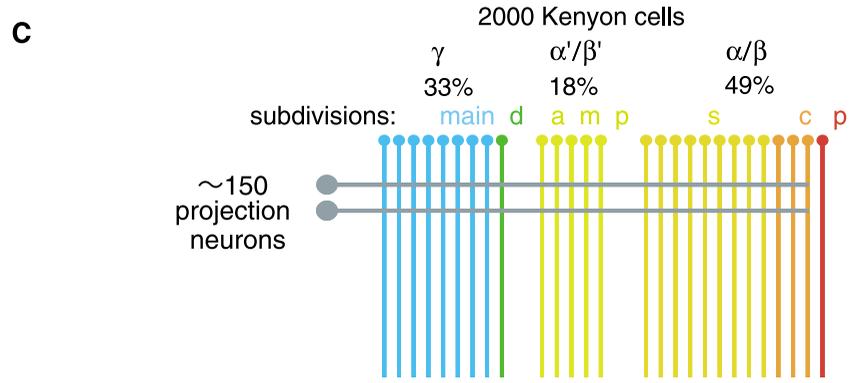
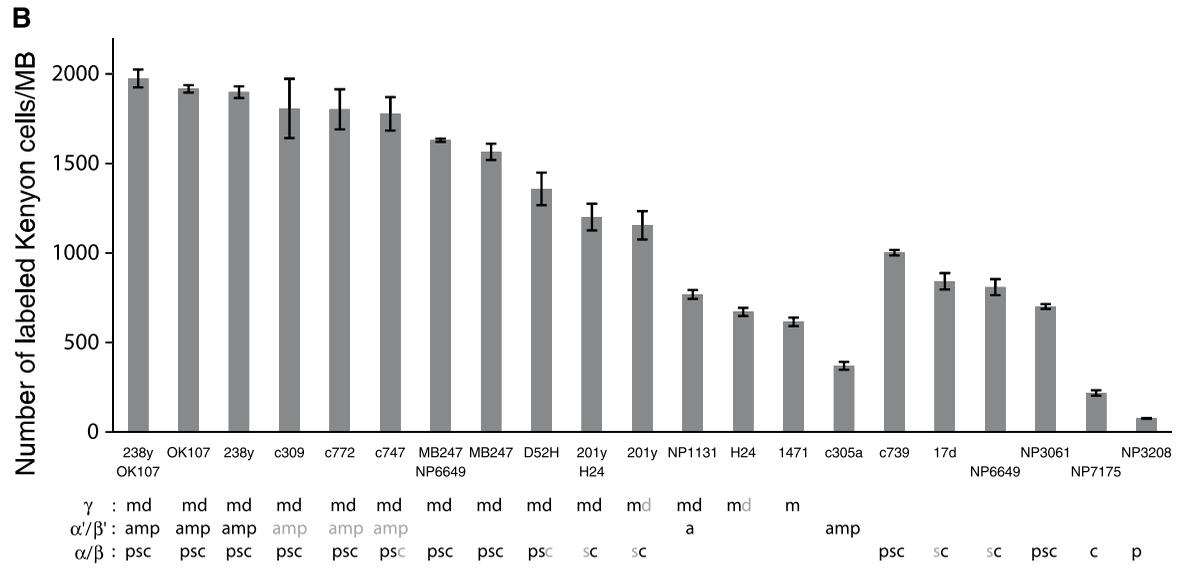
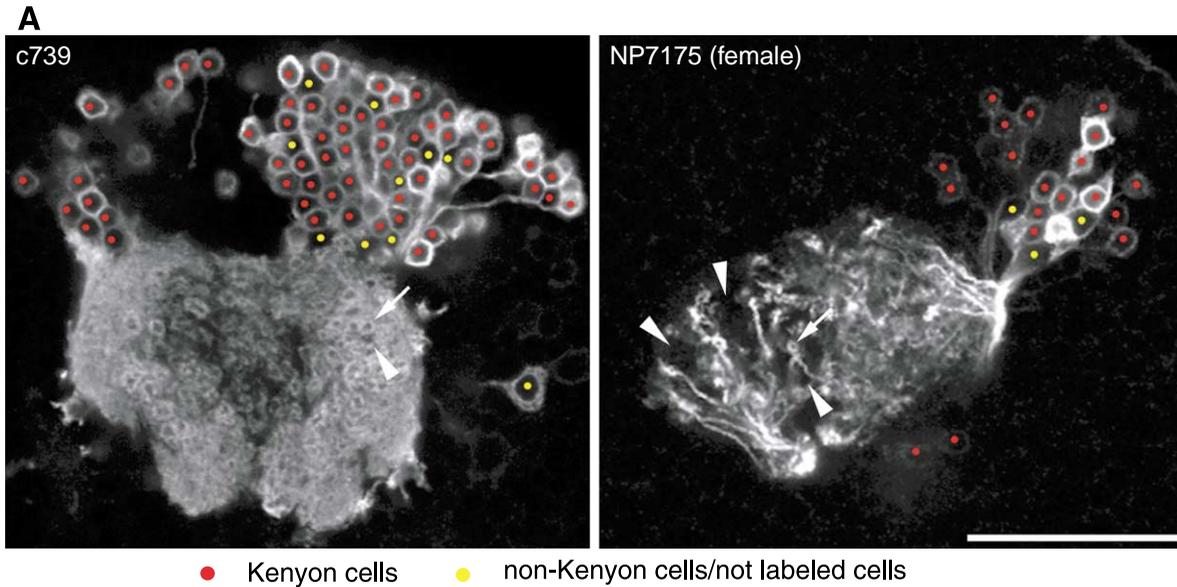


Figure 8 (Continued)

confocal micrographs, we counted at least 45 fibers at the caudal pedunculus derived from a single MB-APL (K.G. and Y.A., unpublished observation). Without specific

labeling, such thin fibers, but not of Kenyon cells, might potentially lead to an overestimation of the total fiber number. Lack of specific labeling might have obscured

previous cell-body counting (Hinke, 1961; Technau & Heisenberg, 1982). Although Kenyon cell nuclei look distinct from those of surrounding cells without cell type-specific labeling, such a border turned out to be not always reliable after genetic labeling of Kenyon cell nuclei (data not shown).

It is noteworthy that there also exist studies that have estimated significantly smaller numbers of Kenyon cells than this one (Ito & Hotta, 1992; Mader, 2004). Mader, in his diploma thesis, analyzed 19 different MB-GAL4s by expressing *nls-lacZ*. His tallies were systematically fewer than ours (e.g., 825 nuclei in MB247; Figure 8). This difference might be due to a mere technical pitfall. For instance, there could be a significant overlap of multiple Kenyon cell nuclei due to the usage of the 20X objective lens, leading to lower Z resolution in confocal microscopy. Based on the number of Kenyon cells that incorporated BrdU in defined time periods, another study estimated that MB neuroblasts give rise to 800–1,200 until adulthood (Ito & Hotta, 1992). Although we have no clear explanation, the deviation might, again, be technical, such as inefficient BrdU incorporation.

Considering the production of 2,000 neurons in the adult, using the typical cell-division scheme of the neuroblast, the cell cycle of MB neuroblasts must be unusually short—within an hour (2,000 cells during ~200 hours of MB neuroblast proliferation). Thus, one or more of the assumptions of the neuroblast division might not be applied to the Kenyon cell proliferation. For instance, the ganglion mother cells from the MB neuroblasts might exceptionally divide more than once, not as in many other neurons in insects.

Seminal papers from the Heisenberg group showed that the structure and the fiber number of the MB undergo significant changes with many developmental and environmental factors, such as different wild-type strains (Balling et al., 1987; Heisenberg et al., 1995; Technau, 1984). Thus, direct comparison of these different counting methods under a constant experimental condition could, at least partially, settle the dispute on the total number of *Drosophila* Kenyon cells.

The total Kenyon cell number may have important implications when it comes to devising a quantitative network model of the *Drosophila* MB. For example, it might be important to reconsider the connectivity of the 2,000 genetically labeled Kenyon cells with the projection neurons at the calyx. Approximately 150 iACT projection neurons are the major source of afferents to the calyx and were calculated to supply in the calyx ~1,000 presynaptic boutons, which comprises the cores of microglomeruli (Jefferis et al., 2007; Stocker et al., 1990; Turner et al., 2008). Based on the electron microscopic data (Yasuyama et al., 2002), Turner et al. estimated 30 active zones, on average, at a single bouton (Turner et al., 2008). Since each Kenyon cell has dendritic arbors on five micro-

glomeruli, on average (Zhu et al., 2003), each projection neuron bouton could be presynaptic to 10 different Kenyon cell claws (=2000 cells \times 5 terminals/1,000 microglomeruli). Given that several postsynaptic sites can be assigned to a single presynaptic release site (Prokop & Meinertzhagen, 2005; Yasuyama et al., 2002), each Kenyon cell claw, on average, may have more than six postsynaptic sites (=multiple postsynapses [≥ 2] \times 30 active zones in a projection neuron bouton/10 Kenyon cell claw). These numbers, however, should be experimentally verified, such as by the high-resolution analysis of Kenyon cell dendrites and the reconstruction of microglomeruli with electron microscopy.

Composition of the MB

In addition to the total number of Kenyon cells, this study reveals, for the first time, the proportion of genetically labeled Kenyon cells contributing to subdivisions. This opens new questions that are of direct functional relevance. What is the minimal set of MB subdivisions to which reproducible genetic manipulation can be applied? Can all lobe systems similarly receive odor information represented in the calyx? More generally, how is the *Drosophila* MB composed of different Kenyon cell subtypes?

According to the numbers of Kenyon cells contributing to subdivisions, we estimate that the γ neurons, α'/β' neurons, and α/β neurons occupy 33 (671 cells), 18 (370 cells), and 49% (1,002 cells) of the total labeled Kenyon cells (Figure 8). This composition fits well with the volumetric ratio of the cell bodies of MB neuroblast clones: 35, 23, and 42%, respectively (Lee et al., 1999). The lack of the γ neurons born before the induction of neuroblast clones might be compensated by the larger cell bodies of early-born Kenyon cells (Maurange et al., 2008). Interestingly, the cell number of each subtype does not seem to reflect the volume of each lobe, implying heterogeneous synaptic terminals of the subtypes.

Subtype-specific MB-GAL4s allowed us to estimate the smallest population of Kenyon cells; the α/β c and α/β p neurons in the α/β lobes have at least 203 (10%; NP7175) and 76 (4%; NP3208) cells, respectively. Given that the α/β p neurons exceptionally arborize in the accessory calyx, the α/β c lobes might contain the least Kenyon cells that project to the main calyx. For the γ d and α'/β' neurons, we could not calculate the number of each subtype due to the lack of a specific driver line. Yet, we estimated the numbers of the γ d and α'/β' a neurons to be approximately 55 and 98, respectively (H24 – 1471 and NP1131 – H24 for the γ d and α'/β' a neurons, respectively).

It may be important to consider whether each subdivision can represent all odors by contacting all the terminals of the projection neurons in the calyx. To completely cover 1,000 presynaptic boutons with five

dendritic terminals of each Kenyon cell, at least 200 Kenyon cells are theoretically required (=1,000 microglomeruli/5 terminals). Therefore, 203 of the α/β neurons could receive odor information from all microglomeruli, but at least two of the three subtypes in the α'/β' neurons (370 cells in total) could not. If we assume that the projection neurons innervating the same antennal lobe glomerulus convey identical information, only 10 Kenyon cells, in principle, could sufficiently receive the entire odor repertoire, given ~ 50 glomeruli in the antennal lobe (50 different terminals = 5 claws \times 10 Kenyon cells). However, in practice, it seems unlikely that even 200 cells cover all microglomeruli in the calyx, since each subtype has spatial preference of dendritic terminals (Jefferis et al., 2007; Lin et al., 2007; Tanaka et al., 2004). Consistently, we also found microglomeruli not innervated in MB-GAL4s that label 1,000 Kenyon cells (e.g., arrows and arrowheads in the calyx of *c739* in Figure 8A). Therefore, we suppose that each subtype of Kenyon cells can have its unique, yet overlapping, odor space.

Application of MB-GAL4s

According to the expression pattern in the lobes, *c739* and NP3061 have very similar, if not identical, patterns in the lobes (Figure 2). However, the numbers of labeled Kenyon cells are significantly different (ca. 300 cells more in *c739*; Figure 8B). This indicates that expression pattern in the lobes does not always predict the number of genetically labeled neurons; genetic subpopulations of Kenyon cells do not always correlate the morphological subdivisions of the MB. Since only a few Kenyon cells ($\sim 6\%$) represent each odor (Turner et al., 2008; Wang et al., 2004), the small difference in the labeled population may have a significant functional consequence. Indeed, Akalal et al. (2006) found the differential phenotypes of 17d and *c739* on the memories of different odor combinations (Akalal et al., 2006), although both label the α/β neurons (Figure 2). Therefore, outcome of genetic manipulation may vary with subtle difference in the population of labeled cells in the target lobes.

Our comprehensive analysis revealed that most MB-GAL4s have significant expression outside the MB. If the spatial specificity is absolutely required, combinatorial methods, such as “split GAL4” (Luan et al., 2006) or the GAL80 enhancer-trap system (Suster et al., 2004), should be applied to confine the effector-reporter expression to the MB.

In addition to the spatial specificity, the expression of MB-GAL4s analyzed here changes during development. For instance, *c739* is expressed broadly in the γ neurons during the larval stages (Kurusu et al., 2002). Therefore, additional temporal control is favorable to circumvent such a “developmental effect” and to restrict the

transgene expression to the adult (e.g., GeneSwitch or the GAL80[ts] system) (McGuire et al., 2003; Osterwalder et al., 2001; Roman et al., 2001).

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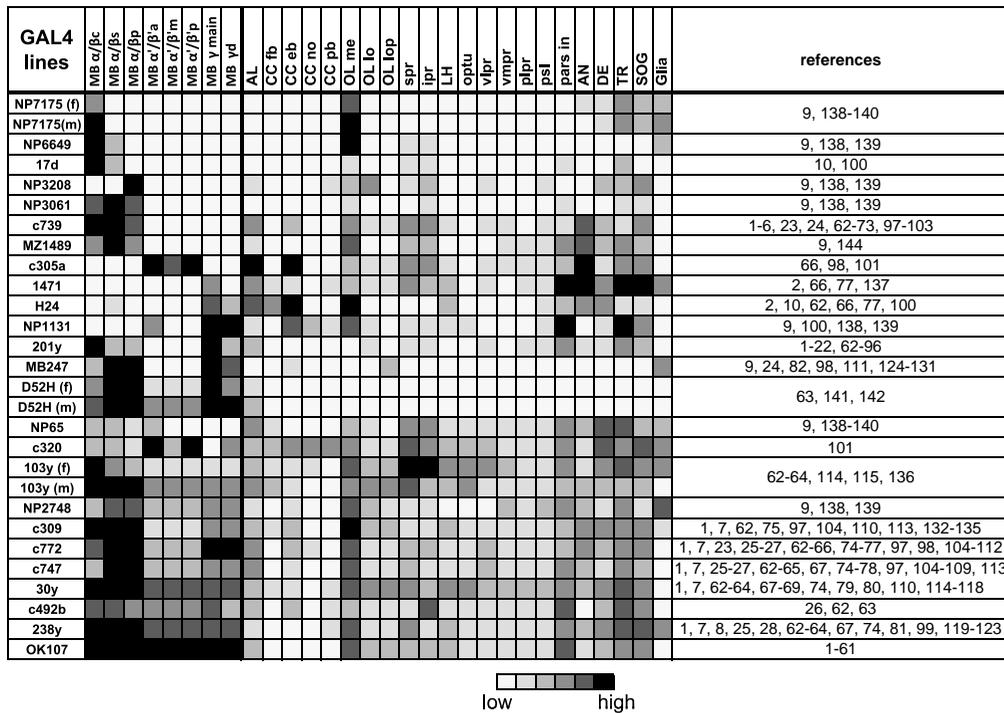
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Supplemental Figure 1.

SUPPL. FIG. 1. EXPRESSION PATTERN OF 25 GAL4 LINES AND REFERENCE LIST

Summary of the expression levels of 25 MB-GAL4s in various brain areas defined by anti-Synapsin immunostaining. Gray scale indicates subjectively evaluated signal intensity. Note that higher level of fluorescent signals in the certain brain area can result from larger population of GAL4 expressing cells and/or stronger GAL4 expression in each cell. *Abbreviations*: MB: mushroom body; c: core subdivision; s: surface subdivision; p: posterior subdivision; a: anterior subdivision; m: middle subdivision; p: posterior subdivision; d: dorsal subdivision; AL: antennal lobe; CC: central complex; fb: fan-shaped body; eb: ellipsoid body; no: noduli; pb: protocerebral bridge; OL: optic lobe; me: medulla; lo: lobula; lop: lobula plate; spr: superior protocerebrum; ipr: inferior protocerebrum; LH: lateral horn; optu: optic tubercle; vlpr: ventrolateral protocerebrum; plpr: posteriorlateral protocerebrum; vmpr: ventromedial protocerebrum; psl: posterior slope; pars in: pars intercerebralis; AN: antennal nerve; DE: deutocerebrum; TR: tritocerebrum; SOG: subesophageal ganglion. *References*: Original articles were sought using Flybase (<http://flybase.org>), and we manually tried to complement recent articles in addition. 1: (McGuire, Le, & Davis, 2001); 2: (Peng, Xi, Zhang, Zhang, & Guo, 2007); 3: (Ramaekers et al., 2005); 4: (Masuda-Nakagawa, Tanaka, & O’Kane, 2005); 5: (Wu et al., 2007); 6: (Fushima & Tsujimura, 2007); 7: (Connolly et al., 1996); 8: (Kurusu et al., 2002); 9: (Tanaka, Tanimoto, & Ito,

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