Dissecting the neuronal circuit for olfactory learning in *Drosophila*

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Die vorliegende Arbeit wurde weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt. Zuvor habe ich keine akademischen Grade erworben oder versucht zu erwerben.

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Contribution of co-authors is described below.

1) Aso Y, Grübel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H. The mushroom body of adult Drosophila characterized by GAL4 drivers. *J Neurogenet.* 2009;23(1-2):156-72. Epub 2009 Jan 10.

   Y.A., H.T. and S.B. designed the experiments. Y.A., K.B., S.B., A.B.F. and I.S. performed the experiments and analyzed the data. AY and HT wrote the paper. In addition, B. Mühlbauer, M. Braun, S. Konrad, and S. Pünzeler supported the project.

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   Y.A. and H.T. designed the experiments. Y.A., I.S. and L.B. performed the experiments and analyzed the data. Y.A., K.I., T.K. and H.T. wrote the paper. Also, A.B. Friedrich, K. Grübel, A. Gruschka, and B. Mühlbauer supported the project.

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   Y.A. and H.T. designed the experiments. Y.A. performed the behavioral experiments and analyzed the data. I.S. dissected and stained brains. Y.A. and HT wrote the manuscript.

Contents

1. Zusammenfassung/Summary

Diese Dissertation umfasst drei Kapitel. Das erste Kapitel handelt von der anatomischen Charakterisierung des Pilzkörpers in adulten *Drosophila melanogaster.* Der Pilzkörper ist das Zentrum für olfaktorisches Lernen und viele andere Funktionen im Insektengehirn. Diese wurden mit Hilfe des GAL4/UAS Genexpressionssystems untersucht. Die vorliegende Arbeit charakterisiert die Expressionsmuster der gewöhnlich verwendeten GAL4 Treiberlinien für die Pilzkörperintrinsischen Neurone, den Kenyonzellen. Dabei zeigten ich die zahlenmäßige Zusammensetzung der unterschiedlichen Kenyonzelltypen und fanden einen Kenyonzellsubtyp, welcher bisher noch nicht beschrieben wurde. Das zweite und dritte Kapitel zeigen, dass verschiedene Typen dopaminerger Neurone aversive Verstärkungssignale (Unkonditionierte Stimuli) zum Pilzkörper übermitteln. Sie induzieren parallele Gedächtnisspuren, welche den unterschiedlichen zeitlichen Komponenten von aversivem Duftgedächtnis zugrunde liegen. Vor diesen Kapiteln enthält der Abschnitt „General introduction and discussion" einen Überblick und eine Diskussion über das derzeitige Verständnis des neuronalen Netzwerks, welches olfaktorischem Lernen in *Drosophila* zugrunde liegt.

This thesis consists of three major chapters, each of which has been separately published or under the process for publication. The first chapter is about anatomical characterization of the mushroom body of adult *Drosophila melanogaster*. The mushroom body is the center for olfactory learning and many other functions in the insect brains. The functions of the mushroom body have been studied by utilizing the GAL4/UAS gene expression system. The present study characterized the expression patterns of the commonly used GAL4 drivers for the mushroom body intrinsic neurons, Kenyon cells. Thereby, we revealed the numerical composition of the different types of Kenyon cells and found one subtype of the Kenyon cells that have not been described. The second and third

chapters together demonstrate that the multiple types of dopaminergic neurons mediate the aversive reinforcement signals to the mushroom body. They induce the parallel memory traces that constitute the different temporal domains of the aversive odor memory. In prior to these chapters, “General introduction and discussion” section reviews and discuss about the current understanding of neuronal circuit for olfactory learning in *Drosophila*.

2. General Introduction and Discussion

Many theories of learning and memory have been derived from the studies on classical conditioning using various animals (Menzel 2001; Medina, Repa et al. 2002; Heisenberg 2003; Kandel 2004; McGuire, Deshazer et al. 2005), in which presentation of a conditioned stimulus (CS) along with a biologically significant unconditioned stimulus (US) change the subsequent behavioral response to the CS. To elucidate how an ensemble of neurons processes such an associative memory, it is essential to identify elements of the circuit: neurons that mediate signals of the CS and US, neurons on which the CS and US signals converge to induce synaptic plasticity or a memory trace, and neurons that mediate the conditioned response (CR). Thanks to the relatively small number of neuronal cells, research on invertebrates system has a great advantage for such a cellular identification. Especially, researches on the olfactory conditioning of *Drosophila melanogaster* have been supported by elaborate genetic tools for manipulating gene expression and physiology of targeted cells.

Olfactory conditioning in Drosophila

Discriminatory olfactory learning in *Drosophila* is an extensively studied paradigm (Quinn, Harris et al. 1974; Tully and Quinn 1985; Heisenberg 2003; Gerber, Tanimoto et al. 2004; McGuire, Deshazer et al. 2005; Keene and Waddell 2007), in which flies are

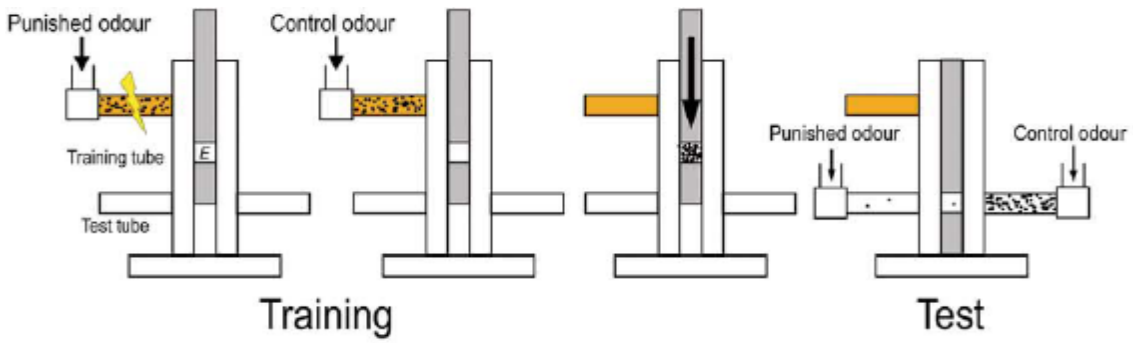

Fig.1: *Drosophila* olfactory learning: A group of flies are put into the training tube covered by a copper grid for electrification. For the delivery of odors, pump forms constant air flow from the distal end of tube to the proximal end. During training, flies receive one odor together with electric shock, and the control odor without shock. Subsequently, flies are transferred into the compartment and shuttled to a choice point where they can distribute between the previously punished odor and the control odor. (adapted from Gerber et al, 2004)

conditioned to avoid or approach a specific odor as a predictor of the reinforcing stimuli such as an electroshock punishment or sugar rearward (Fig. 1). The changed odor preference contributes to a learning index.

The formed memory can be viewed as a composite of memory phases or components characterized by various operational definitions (Fig.2) (Dubnau and Tully 1998; Margulies, Tully et al. 2005; McGuire, Deshazer et al. 2005): retention time, underlying molecular pathways, sensitivity to the cold anesthesia and protein synthesis inhibitor. Short-term memory (STM) is a memory component observed immediately after conditioning. Middle-term memory (MTM) exists until a few hours after conditioning and is sensitive to cold anaesthesia. MTM is preferentially impaired in the *amnesiac* mutant (Quinn, Sziber et al. 1979; Tully and Quinn 1985). Anesthesia-resistant memory (ARM) appears as memory is consolidated overtime (Quinn and Dudai 1976), and last longer than MTM. ARM is preferentially impaired in *radish* mutant (Tully and Quinn 1985; Folkers, Drain et al. 1993). Repeated training without interval (massed conditioning) preferentially induce 24 hour memory that is sensitive to the *radish* mutation (Tully, Preat et al. 1994). In contrast, protein synthesis dependent long-term memory (LTM) is formed after a spaced conditioning and insensitive to *radish* mutation (Tully, Preat et al. 1994).

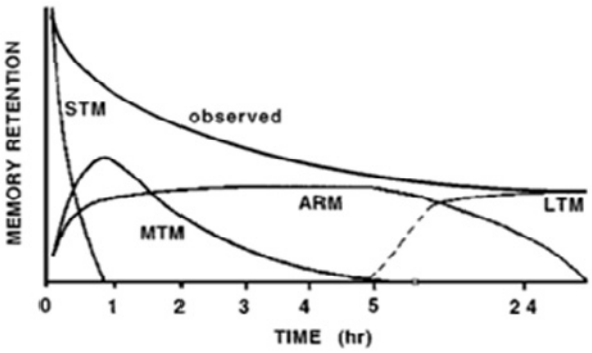

Fig.2: Memory phases of olfactory learning after conditioning Drosophila olfactory learning with electric shock:
From Dubnau and Tully 1998.

Overview: Circuit for olfactory learning

During conditioning, signals of odors and reinforcing stimuli converge on the mushroom body (MB) (Heisenberg 2003; Gerber, Tanimoto et al. 2004; McGuire, Deshazer et al. 2005; Keene and Waddell 2007). Structural mutants and chemical ablation of the MB lead to impaired memory (Heisenberg, Borst et al. 1985; de Belle and Heisenberg

1994). Also, blockade of major MB-intrinsic neurons, Kenyon cells, impairs memory (Dubnau, Grady et al. 2001; McGuire, Le et al. 2001; Schwaerzel, Heisenberg et al. 2002; Akalal, Wilson et al. 2006; Krashes, Keene et al. 2007). Furthermore, expression of many genes involved in learning and memory are highly enriched in the MB, and local expression of some of these genes in the MB can restore the learning deficits of the mutant (Keene and Waddell 2007) (McGuire, Deshazer et al. 2005).

The *Drosophila* MB is composed of three major parts: calyx, pedunculus and lobes. The signals of a given odor are detected in the antennae, and conveyed to the primary olfactory center, the antennal lobe. From there, the projection neurons transmit the processed olfactory information to the MB calyx and the lateral horn (Fig.3A; see (Stocker, Lienhard et al. 1990; Hallem and Carlson 2004; Masse, Turner et al. 2009) for the reviews on the odor processing in *Drosophila*).

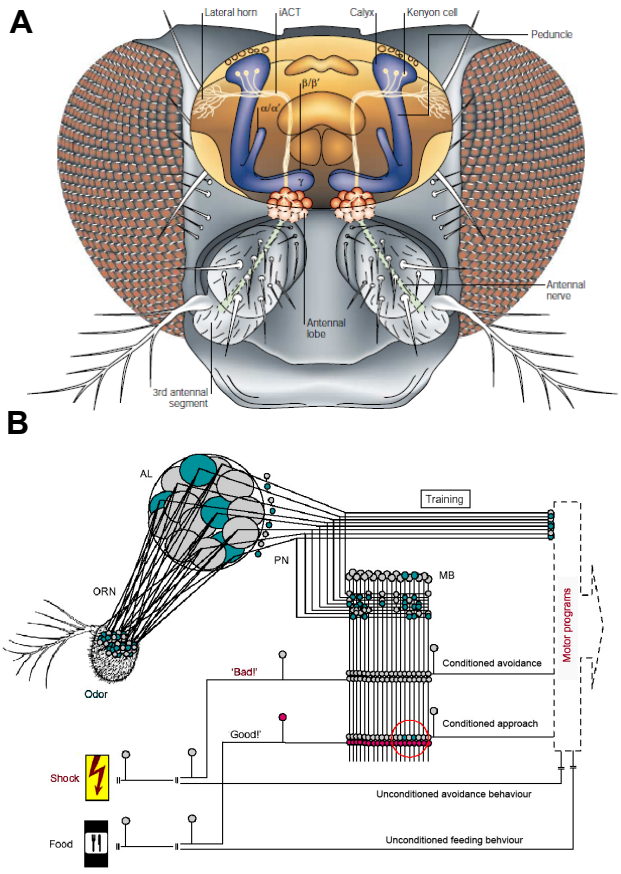

Fig. 3: Neural circuit for *Drosophila* olfactory learning: (a) Odor information is carried to the calyx of the mushroom body by ORN and PN. Mushroom body is composed of major intrinsic neurons, Kenyon cells: the α/ β neurons, the α'/β' neurons and the γ neurons. (b) Sugar reward and electroshock punishment are supposed to modulate olfactory information in the mushroom body (MB). ORN: Olfactory receptor neurons; PN: projection neurons.
(Adapted from (a) Heisenberg, 2003 and (b) Gerber et al. 2004)

In the MB, identity of odors is represented by the sparse activation pattern of its intrinsic neurons, Kenyon cells. On the other hand, reinforcing properties of aversive and appetitive stimuli are mediated to the MB by octopaminergic and dopaminergic neurons respectively (Schwaerzel, Monastirioti et al. 2003; Schroll, Riemensperger et al.

2006; Claridge-Chang, Roorda et al. 2009) (Fig.3B). In a model, these monoamines are hypothesized to modulate output synapses of Kenyon cells in the lobes of MB to facilitate the recruitment of MB-output neurons by the learned odor (Fig.3B). Taken together, MB has been considered as the site of memory formation and storage. However it is still unclear how individual Kenyon cells and MB-input/output neurons, collectively called as MB-extrinsic neurons, cooperatively function to support olfactory learning, mainly due to the limited anatomical and functional knowledge of individual MB-extrinsic neurons. Especially, little was known about where in the MB memory traces are formed and how MB-output neurons mediate the conditioned response based on these memory traces. Below, I will discuss approaches and recent progress on these subjects.

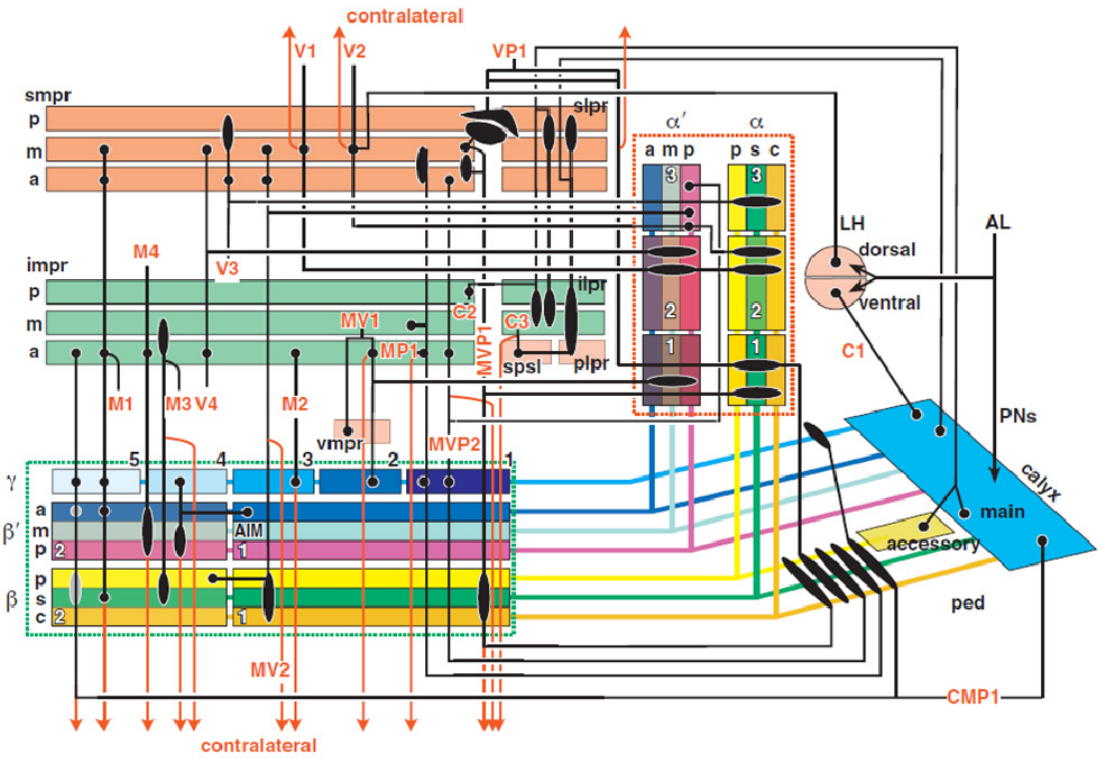

Fig. 4: Neural connection of the mushroom body of adult *Drosophila*

Circuit map of the MB (adapted from Tanaka et al. 2008). In addition to neurons depicted here, there are many other MB-extrinsic and intrinsic neurons: MB-DPM, GABAergic MB-APL, four types of octopaminergic neurons{Busch, 2009 #15082}, two types of dopaminergic neurons that respectively project to α'3 and α3 (Mao and Davis 2009), many types of dopaminergic neurons from PAM cluster and PPL2ab, contralaterally projecting serotonin-immunoreactive deutocerebral (CSD) interneurons (Roy, Singh et al. 2007), neurons immunoreactive to vesicular glutamate transporter{Daniels, 2008 #15092}. Also a population of neurons defined as single cell type by GAL4 drivers may contain multiple cell types that can be morphologically distinguished by visualizing individual cell.

Tools for anatomically indentify individual cell types

To identify individual cell types in the complex network of neurons in the brain, it is a key to visualize a small subset of neurons. In addition to the Kenyon cells, the MB comprises various MB-extrinsic neurons. In early studies, stochastic labeling such as the Golgi method was successfully to indentify various types of MB-extrinsic neurons in *Drosophila* (Ito, Suzuki et al. 1998). Later, GAL4/UAS gene expression system was developed for reproducibly expressing a given trangene in the restricted pattern for selectively visualizing and manipulating the neurons of interest (Brand and Perrimon 1993). Due to the power of GAL4/UAS system, therefore, identification of GAL4 drivers that label specific neurons projecting to the MB was the essential first step to draw a circuit map of the MB. Such a screening can be supported by collections of GAL4 enhancer trap lines that have been established by the collective efforts of many labs. Expression patterns of some driver collection are archived as a confocal image database for public use (flytrap; http://www.fly-trap.org/). In addition to GAL4 enhancer trap lines, there are ongoing efforts to generate a new collection of ca. 5,000 drivers (Pfeiffer, Jenett et al. 2008) . In contrast to the conventional enhancer trap lines, these lines were generated by inserting the specific promoter regions of genes into a defined genomic location. Thus GAL4 is interchangeable with any transgenes of interest.

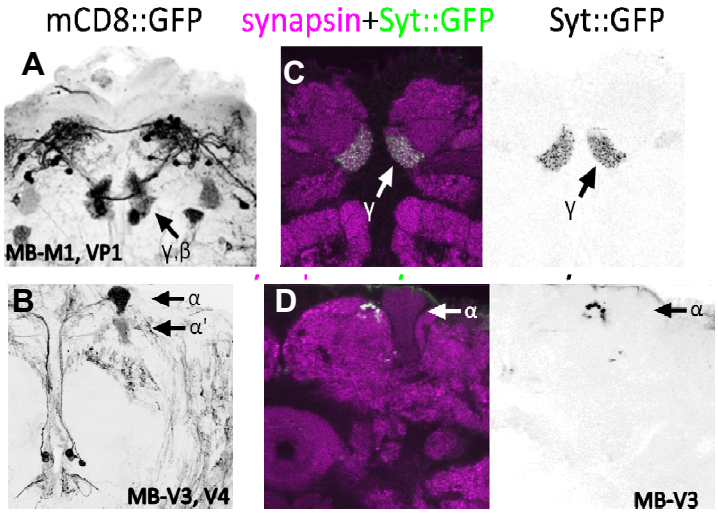

Fig. 5: Polarity of MB-extrinsic neurons

MB-extrinsic neurons are visualized with membrane targeted GFP (*UAS-mCD8::GFP;* black) or presynaptic marker synaptotagmin::GFP (Syt::GFP; green or black) by NP6014 (A and C) NP2150 (B and D). Magenta is counterstaining of neuropiles with anti-Synapsin.
(A) and (B) show confocal projections of the regions including the MB.
(C) and (D) show each a single confocal slice. mCD8::GFP and Syt::GFP both label terminals of MB-M1 in the distal tip of the γ lobe (γ_5), indicating these terminals are presynaptic. On the other hand, Terminals of MB-V3 in the tip of α lobe (α_3) is labeled by mCD8::GFP but not by Syt::GFP, indicating dendritic nature of these terminals.

To draw a circuit of the MB, Tanaka et al. systematically identified the GAL4 drivers for MB-intrinsic and -extrinsic neurons by examining expression patterns of ca. 4,000 GAL4 driver lines generated by the NP consortium (Hayashi, Ito et al. 2002; Tanaka, Tanimoto et al. 2008). This screening identified dozens of MB-intrinsic and -extrinsic neurons (Fig. 4). Detailed examination of these lines revealed that the MB has complex internal structures (Fig. 4). As in the MB of other insects (Rybak and Menzel 1993; Strausfeld, Hansen et al. 1998; Li and Strausfeld 1999), each MB-extrinsic neuron projects to a specific subdomain of the MB, forming a synaptic unit or compartment in which a specific set of MB-intrinsic and extrinsic neurons may interact.

The identified drivers can be used for further in depth anatomical characterization. By expressing a reporter that preferentially localizes to pre or post-synaptic terminals such as synaptotagmin::GFP and Rdl::HA, one can determine the polarity of neurons (Fig.5). Also the transmitter of neurons can be deciphered by co-labeling of neurons with antibody to the transmitter itself or an enzyme essential for the synthesis and release of a particular transmitter. For instance, an antibody to tyrosine hydroxylase revealed putative dopaminergic MB-extrinsic neurons (Fig.6).

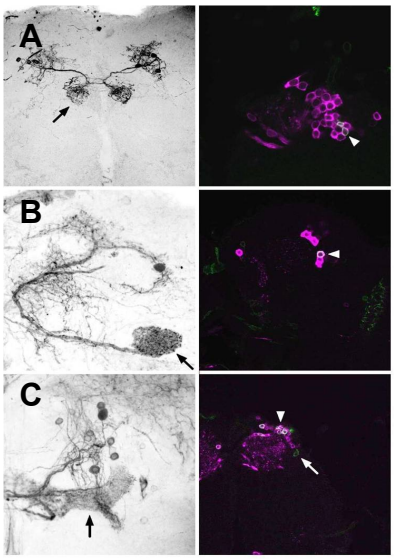

Fig. 6: Putatively dopaminergic MB-extrinsic neurons.
Left panels: Confocal projection of the regions including the MB (frontal view; dorsal is up). MB-extrinsic neurons are visualized by driving *UAS-mCD8::GFP* in MB-M3 with *NP5272* (A), MB-MP1 with *NP2758* (B) and MB/MVP1 with *NP6510* (C). Arrows indicate their terminals in the MB.
Right panels: Magnified view of the cell bodies in PAM (A and C) and PPL1 cluster (B). Green and magenta represent signal of mCD8::GFP and tyrosine hydroxylase (TH), respectively. Arrowheads indicate colocalization of signals. Arrow indicate a cell that is labeled by

In some cases, a neuron of interest has complicate branching patterns and it intermingled with processes of other GAL4 expressing neurons, making it difficult to precisely describe

projection patterns of individual neurons. In such a case, the Flp-out technique can be applied (Wong, Wang et al. 2002). Flp is a DNA recombinase in yeast and it binds to a specific target sequence (Flp recognition target; FRT). By varying the patterns and expression levels of Flp, it allows us to randomly label GAL4-expressing cells with the membrane-tethered GFP, where Flp is activated. Thus, the neuronal processes of individual neurons can be untangled (Fig.7).

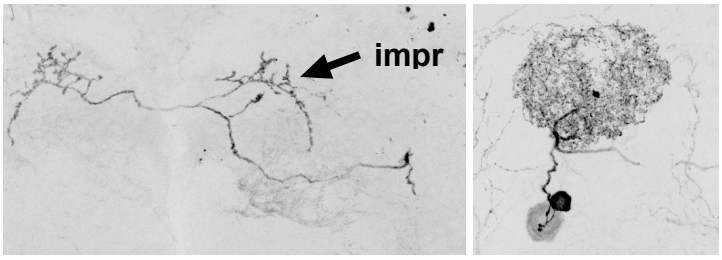

Fig.7 Single cell visualization of MB-CP1

Projection of confocal stacks showing individually labeled MB-CP1 projecting to the inferior medial protocerebrum (left panel) and calyx (right panel). NP2297 labels many other cells including Kenyon cells. By stochastic induction of DNA recombinase, only one MB-CP1 expresses mCD8::GFP in this brain.

Taken together, these analyses provide us working hypotheses on the functions of neurons in the circuit. For instance, dopaminergic neurons that terminate on the MB are the candidate neurons for mediating aversive reinforcement to the MB. Also, MB-output neurons are likely to be neurons that read out memory traces in the Kenyon cells. The design of the subsequent behavioral experiments can be based on these hypotheses.

Tools for functional characterization of individual cell types

Established genetic tools in *Drosophila* enable transient and non-invasive neuronal manipulation in intact behaving animals, being thus optimal for the systematic dissection of neural circuits. There are several choices for transgenes for manipulating the function of target neurons. *UAS-shi^{ts1}* is widely used to block the targeted neurons (Kitamoto 2001). *UAS-shi^{ts1}* encodes a temperature-sensitive allele of dynamin. Because dynamin is essential for recycling of synaptic vesicle, neuronal transmission from the neurons expressing *shi^{ts1}* is suppressed at restrictive temperature (>29°C). The effect is reversible; it recovers in a few minutes after the shift to permissive temperature (25°C). This is particularly useful for analyzing the circuit for olfactory learning because memory is a dynamic process in which

various neurons function sequentially for different processes of memory: formation, consolidation and retrieval. Transgenes for neuronal activation are also available. ChR2 and $P2X_2$ can depolarize neurons upon the illumination of light (Lima and Miesenbock 2005; Schroll, Riemensperger et al. 2006). *UAS-dTrpA1* encodes a *Drosophila* endogenous cation channel that opens by heat (>27°C), enabling thermo-activation of target neurons expressing this transgene (Hamada, Rosenzweig et al. 2008; Pulver, Pashkovski et al. 2009).

Selecting drivers for the circuit mapping

For a relatively time consuming behavioral paradigm, one may need to further select drivers for experiments from a large pool identified by screening of confocal databases. When GAL4 is mobilized with the P-element, insertion event does not take place at random, but tend to takes place at certain genomic loci. Therefore, some of enhancer trap lines are inserted into almost the same site. Because such drivers often have similar expression patterns, it is reasonable to select only one line for initial behavioral experiments. For the NP collection, information about their insertion sites is available from the data base: GETDB (http://flymap.lab.nig.ac.jp/~dclust/getdb.html).

The detailed quantitative assessment of expression pattern is a prerequisite to interpret the result of behavioral experiments, because it depends on several factors: 1) expression level of effector 2) whether the driver covers all cells of the target cell type 3) effect of manipulating off-target cells. When multiple drivers are available for the target cell type, one that has the strongest expression in the highest number of cells of the target cell type but least expression in other cells is a favorable option.

For quantifying GAL4 expression, the technical caveat is that both intensity and pattern of GAL4 can vary with type of reporter and the copy number (Pfeiffer, Ngo et al. 2010). Similarly, the expression level sufficient for the intended effect depends on the type of transgene. Furthermore, efficiency of transgenes such as dTrpA1 and shi^{ts1} highly depends on temperature (Schwaerzel, Heisenberg et al. 2002; Pulver, Pashkovski et al. 2009).

Cell bodies of a particular cell type are often clustered together with those of other cell types generated from the same neuroblasts, and they send axons using the same tract (Cardona, Saalfeld et al. 2010). Therefore, it is often difficult to count only the cells of interest when multiple cell types are labeled in the same cluster. In such cases, the precise counting requires knowledge about transmitter and morphology of target and other GAL4 expressing cells in the same cluster. By counting the number of labeled cells in individual drivers and combination of drivers, one can also test the identity of neurons. In case a GAL4 driver that covers all the cells of certain cluster is available, the numerical composition of cell types in the cluster can be unambiguously deciphered by twin-spot MARCM (Yu, Chen et al. 2009).

Given that a behavioral phenotype was observed with some driver, one needs to control the effect of manipulating other GAL4 expressing cells outside the target cells. One strategy is to test multiple drivers that have overlapping expression in the target cells but non-overlapping expression in other cells. If the phenotype is due to manipulation of target cells but not other GAL4 expressing cells, a consistent phenotype should be observed in theses drivers. In addition, GAL80, an inhibitor of GAL4, can be applied to refine the expression pattern of GAL4. Expression of GAL80 in the target cells should eliminate the behavioral phenotype, whereas that in other cells should not change the outcome. Compared to these conventional approaches, intersectional strategies have great advantage to manipulate minimum sets of neurons. For example, the split GAL4 approach restricts the expression of transgenes to the cells that express both DNA binding domain and transcription activating domain of the GAL4 (Luan, Lemon et al. 2006).

CS pathways: Representation of odors in the mushroom body

As an entry of the CS pathway, odor molecules are detected by olfactory receptor neurons (ORNs) on the antennae and the maxillary palps (Smith 2007; Vosshall and Stocker 2007; Masse, Turner et al. 2009) . Each ORN expresses a single type of olfactory receptor which defines its odor identity response (Hallem, Ho et al. 2004; Hallem and Carlson 2006). ORNs that express the same receptor converge to the same glomerulus of the antennal lobe

(Figure 2) (Vosshall, Amrein et al. 1999). In the antennal lobe, ORNs synapse with projection neurons (PNs). Many PNs innervate only one glomerulus (Marin, Jefferis et al. 2002). From the AL, odor information is further transmitted to the MB and the LH by the PNs via multiple tracts (Stocker, Lienhard et al. 1990; Tanaka, Tanimoto et al. 2008).

Claw like dendritic structures extending from the KCs surround the presynaptic terminals of the PN, forming the microglomeruli in the calyx. Because Kenyon cells have higher firing threshold and its depolarization presumably requires coincident inputs from multiple PNs, odor signals are represented by sparse activity of Kenyon cells. There are three major cell types of KC which are distinguished by their projection pattern in the lobes: the α/β, α'/ β', and γ neurons (Crittenden, Skoulakis et al. 1998). They have distinct physiological properties; The α'/ β' neurons respond to the broader range of odors compared to the α/β and γ neurons (with statistical significance only for the γ neurons) (Turner, Bazhenov et al. 2008). The α'/β' neurons also shows higher spontaneous activity (Turner, Bazhenov et al. 2008). Given that KCs can be further categorized into subtypes based on their projection pattern in the lobe region (Yang, Armstrong et al. 1995; Strausfeld, Sinakevitch et al. 2003; Tanaka, Tanimoto et al. 2008; Aso, Grubel et al. 2009), it will be a future challenge to address how those subsystems represent odors in parallel.

US pathways: Mediating signals of aversive stimuli

In insects, dopamine mediates a reinforcing properties of aversive stimuli (Schwaerzel, Monastirioti et al. 2003; Unoki, Matsumoto et al. 2005; Schroll, Riemensperger et al. 2006; Vergoz, Roussel et al. 2007; Claridge-Chang, Roorda et al. 2009; Honjo and Furukubo-Tokunaga 2009; Selcho, Pauls et al. 2009; Aso, Siwanowicz et al. 2010). In *Drosophila*, dopaminergic neurons respond to electric shock (Riemensperger, Voller et al. 2005; Mao and Davis 2009), though the neuronal mechanism of electric shock sensation remains to be studies. Synaptic output of dopaminergic neurons is necessary during training (Schwaerzel, Monastirioti et al. 2003; Claridge-Chang, Roorda et al. 2009; Aso, Siwanowicz et al. 2010). Direct stimulation of the dopaminergic neurons by ChR2, $P2X_2$ and dTrpA1 can

substitute for the aversive US to induce odor memory in larvae and adult Drosophila (Schroll, Riemensperger et al. 2006; Claridge-Chang, Roorda et al. 2009; Aso, Siwanowicz et al. 2010). Also, the local expression of the D_1 dopamine receptor (dDA1) in the α/β and γ neurons is sufficient to rescue the defective memory of its mutant (Kim, Lee et al. 2007; Lebestky, Chang et al. 2009), suggesting that the dopamine signal to the MB is responsible for aversive reinforcement.

Immunolabeling of neurons that express tyrosine hydroxylase (TH), an enzyme required for dopamine biosynthesis, revealed a few hundred cells distributed to the different clusters in the protocerebrum (Nassel and Elekes 1992; Friggi-Grelin, Coulom et al. 2003; Mao and Davis 2009). Among them, subsets of the PAM, PPL1, and PPL2ab cluster neurons innervate the MB (Mao and Davis 2009). Importantly, one cluster contains distinct types of neurons that target different subdomains of the MB and show different physiological properties in response to odors and electric shock (Mao and Davis 2009). Because dopamine is involved in a variety of brain functions besides aversive reinforcement signaling (Friggi-Grelin, Coulom et al. 2003; Riemensperger, Voller et al. 2005; Zhang, Guo et al. 2007; Andretic, Kim et al. 2008; Seugnet, Suzuki et al. 2008; Zhang, Yin et al. 2008; Krashes, DasGupta et al. 2009; Lebestky, Chang et al. 2009; Liu, Dartevelle et al. 2009; Selcho, Pauls et al. 2009), it is obvious that individual dopaminergic neurons have different behavioral functions. Therefore, identification of individual dopaminergic neurons that mediate aversive reinforcement is an important step to understand the MB circuit underlying the formation of associative olfactory memory. Recent and present studies advance our understanding on this subject (Waddell 2010). In this thesis, I demonstrate that at least three types of dopaminergic neurons can induce aversive odor memory (Chapter II and III).

Sites of CS-US convergence: Induction of memory traces

A series of evidences support a model that synaptic plasticity underlying associative odor memory is formed in the output site of the MB (i.e. the presynaptic terminals of Kenyon cells) upon internal convergence of CS and US signals (Heisenberg 2003; Gerber, Tanimoto

et al. 2004; McGuire, Deshazer et al. 2005). The emerging view from the recent works is that CS-US convergence and induction of memory traces take place in the multiple compartments of the MB (discussed below and in Chapter II and III).

CS-US convergence might take place also in the antennal lobes because aversive conditioning changes the odor representation in the antennal lobes (Yu, Ponomarev et al. 2004; Ashraf, McLoon et al. 2006). For the appetitive memory, *rut* expression in the projection neurons restores the memory impairment of the mutant flies (Thum, Jenett et al. 2007). Also, in honey bee, microinjection of octopamine either in the antennal lobes or the calyx of the MB substitute for the sugar reward in olfactory conditioning of the proboscis extension reflex (Hammer and Menzel 1998).

Parallel memory traces and their interaction

Series of evidence indicate the existence of parallel memory traces in different types of Kenyon cells and their interaction in the maintenance of memory; 1) Expression of rut-encoded adenylate cyclase in the γ neurons restore a part of immediate memory (Zars, Fischer et al. 2000; Akalal, Wilson et al. 2006). On the other hand, restoring three hour memory requires *rut* expression both in the γ neurons and α/β (Blum and Dubnau 2010). 2) Selective blocking of the γ neurons, the α'/β' neurons or α/β neurons impairs a part of memory (Dubnau, Grady et al. 2001; McGuire, Le et al. 2001; Isabel, Pascual et al. 2004; Akalal, Wilson et al. 2006; Krashes, Keene et al. 2007). 3) Synergistic increase of cAMP after activation of Kenyon cells by acetylcholine and simultaneous application of dopamine was observed both in the α and the α' lobes (Tomchik and Davis 2009). 4) A small G protein Rac regulates memory decay. Expression of a dominant-negative form of Rac significantly slows down memory decay, but only when it is expressed both in the γ neurons and α/β neurons. 5) Similarly, mutation of a casein kinase Ig homolog, *gilgamesh*, further impairs a residual memory of *rut* mutants. Expression of *gilgamesh* in the γ neurons and the α/β neurons restores memory impairment, but expression either in the α/β neurons or the α'/β' neurons does not (Tan, Yu et al. 2010). 6) NMDA receptor accounts for a part of memory that is

independent of *rut* (Qin and Dubnau 2010). Knockdown of the glutamate NMDA receptor subunit, dsNR1, preferentially either in the γ neurons or the α/β neurons impairs part of three hour memory (Wu, Xia et al. 2007).

Identification of multiple types of dopaminergic neurons for aversive reinforcement in the present study (chapter II and III) also supports the existence of parallel memory traces in the MB. Although the terminals of individual dopaminergic neurons are restricted to the specific compartments of the MB, it is not restricted to a single type of Kenyon cells. Therefore, relation of parallel memory traces at the level of Kenyon cells and at the level of dopaminergic neurons remains to be investigated. Given that output of the α'/β' neurons and the dorsal paired medial neurons (DPM) is necessary for memory consolidation (Keene, Stratmann et al. 2004; Krashes, Keene et al. 2007), parallel memory traces might interact via these neurons (Keene and Waddell 2007). Future analysis of MB-output neurons may reveal not only the neurons for conditioned response but also novel types of neurons for memory consolidation.

CR pathways

How are memory traces in the MB translated into behavior? In the classical conditioning of the proboscis extension reflex of the honey bee, MB-output neurons from the pedunculus (PE1 neurons) reduce the response to the odor that had been paired with sugar (Okada, Rybak et al. 2007). Given that PE1 neurons terminate on the lateral horn, PE1 neurons are hypothesized to mediate the conditioned odor response by modulating odor processing via the lateral horn. In *Drosophila*, a large collection of MB-extrinsic neurons have been identified, but their neuronal polarity is largely not yet determined. Because there are multiple types of dopaminergic neurons for aversive reinforcement, corresponding MB-output neurons for the conditioned response may also be multiple. To understand a mechanism of memory retrieval and odor choice behavior, it will be essential to examine how individual MB-output neurons influence odor preference of flies. In depth analysis of

behaviors upon activation or suppression of individual MB-output neurons and anatomical identification of downstream neurons will provide significant insights on these issues. For instance, parameters such as general locomotion level and turning from the conditioned odor might be regulated by different MB-output neurons. Alternatively, activation of MB-output neurons may not induce any behavioral response unless an odor is presented. Interestingly, both appetitive and aversive olfactory memories require output of the Kenyon cells (Schwaerzel, Monastirioti et al. 2003; Krashes, Keene et al. 2007). Given that these memories induce opposite preference to the learned odor, it will be also essential to address whether these memories modulate different synapses of Kenyon cells or the same synapses of Kenyon cells in the opposite way.

References:

Akalal, D. B., C. F. Wilson, et al. (2006). "Roles for Drosophila mushroom body neurons in olfactory learning and memory." Learn Mem 13(5): 659-668.

Andretic, R., Y. C. Kim, et al. (2008). "Drosophila D1 dopamine receptor mediates caffeine-induced arousal." Proc Natl Acad Sci U S A 105(51): 20392-20397.

Ashraf, S. I., A. L. McLoon, et al. (2006). "Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila." Cell 124(1): 191-205.

Aso, Y., K. Grubel, et al. (2009). "The mushroom body of adult Drosophila characterized by GAL4 drivers." J Neurogenet 23(1-2): 156-172.

Aso, Y., I. Siwanowicz, et al. (2010). "Specific dopaminergic neurons for the formation of labile aversive memory." Curr Biol 20(16): 1445-1451.

Blum, A. and J. T. Dubnau (2010). "Parallel processing of olfactory memories in Drosophila." Fly (Austin) 4(2): 163-166.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development 118(2): 401-415.

Cardona, A., S. Saalfeld, et al. (2010). "Identifying neuronal lineages of Drosophila by sequence analysis of axon tracts." J Neurosci 30(22): 7538-7553.

Claridge-Chang, A., R. D. Roorda, et al. (2009). "Writing memories with light-addressable reinforcement circuitry." Cell 139(2): 405-415.

Crittenden, J. R., E. M. Skoulakis, et al. (1998). "Tripartite mushroom body architecture revealed by antigenic markers." Learn Mem 5(1-2): 38-51.

de Belle, J. S. and M. Heisenberg (1994). "Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies." Science 263(5147): 692-695.

Dubnau, J., L. Grady, et al. (2001). "Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory." Nature 411(6836): 476-480.

Dubnau, J. and T. Tully (1998). "Gene discovery in Drosophila: new insights for learning and memory." Annu Rev Neurosci 21: 407-444.

Folkers, E., P. Drain, et al. (1993). "Radish, a Drosophila mutant deficient in consolidated memory." Proc Natl Acad Sci U S A 90(17): 8123-8127.

Friggi-Grelin, F., H. Coulom, et al. (2003). "Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase." J Neurobiol 54(4): 618-627.

Gerber, B., H. Tanimoto, et al. (2004). "An engram found? Evaluating the evidence from fruit flies." Curr Opin Neurobiol 14(6): 737-744.

Hallem, E. A. and J. R. Carlson (2004). "The odor coding system of Drosophila." Trends Genet 20(9): 453-459.

Hallem, E. A. and J. R. Carlson (2006). "Coding of odors by a receptor repertoire." Cell 125(1): 143-160.

Hallem, E. A., M. G. Ho, et al. (2004). "The molecular basis of odor coding in the Drosophila antenna." Cell 117(7): 965-979.

Hamada, F. N., M. Rosenzweig, et al. (2008). "An internal thermal sensor controlling temperature preference in Drosophila." Nature 454(7201): 217-220.

Hammer, M. and R. Menzel (1998). "Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees." Learn Mem 5(1-2): 146-156.

Hayashi, S., K. Ito, et al. (2002). "GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps." Genesis 34(1-2): 58-61.

Heisenberg, M. (2003). "Mushroom body memoir: from maps to models." Nat Rev Neurosci 4(4): 266-275.

Heisenberg, M., A. Borst, et al. (1985). "Drosophila mushroom body mutants are deficient in olfactory learning." J Neurogenet 2(1): 1-30.

Honjo, K. and K. Furukubo-Tokunaga (2009). "Distinctive neuronal networks and biochemical pathways for appetitive and aversive memory in Drosophila larvae." J Neurosci 29(3): 852-862.

Isabel, G., A. Pascual, et al. (2004). "Exclusive consolidated memory phases in Drosophila." Science 304(5673): 1024-1027.

Ito, K., K. Suzuki, et al. (1998). "The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen." Learn Mem 5(1-2): 52-77.

Kandel, E. R. (2004). "The molecular biology of memory storage: a dialog between genes and synapses." Biosci Rep 24(4-5): 475-522.

Keene, A. C., M. Stratmann, et al. (2004). "Diverse odor-conditioned memories require uniquely timed dorsal paired medial neuron output." Neuron 44(3): 521-533.

Keene, A. C. and S. Waddell (2007). "Drosophila olfactory memory: single genes to complex neural circuits." Nat Rev Neurosci 8(5): 341-354.

Kim, Y. C., H. G. Lee, et al. (2007). "D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila." J Neurosci 27(29): 7640-7647.

Kitamoto, T. (2001). "Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons." J Neurobiol 47(2): 81-92.

Krashes, M. J., S. DasGupta, et al. (2009). "A neural circuit mechanism integrating motivational state with memory expression in Drosophila." Cell 139(2): 416-427.

Krashes, M. J., A. C. Keene, et al. (2007). "Sequential use of mushroom body neuron subsets during drosophila odor memory processing." Neuron 53(1): 103-115.

Lebestky, T., J. S. Chang, et al. (2009). "Two different forms of arousal in Drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits." Neuron 64(4): 522-536.

Li, Y. and N. J. Strausfeld (1999). "Multimodal efferent and recurrent neurons in the medial lobes of cockroach mushroom bodies." J Comp Neurol 409(4): 647-663.

Lima, S. Q. and G. Miesenbock (2005). "Remote control of behavior through genetically targeted photostimulation of neurons." Cell 121(1): 141-152.

Liu, T., L. Dartevelle, et al. (2009). "Reduction of dopamine level enhances the attractiveness of male Drosophila to other males." PLoS ONE 4(2): e4574.

Luan, H., W. C. Lemon, et al. (2006). "Functional dissection of a neuronal network required for cuticle tanning and wing expansion in Drosophila." J Neurosci 26(2): 573-584.

Mao, Z. and R. L. Davis (2009). "Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity." Front Neural Circuits 3: 5.

Margulies, C., T. Tully, et al. (2005). "Deconstructing memory in Drosophila." Curr Biol 15(17): R700-713.

Marin, E. C., G. S. Jefferis, et al. (2002). "Representation of the glomerular olfactory map in the Drosophila brain." Cell 109(2): 243-255.

Masse, N. Y., G. C. Turner, et al. (2009). "Olfactory information processing in Drosophila." Curr Biol 19(16): R700-713.

McGuire, S. E., M. Deshazer, et al. (2005). "Thirty years of olfactory learning and memory research in Drosophila melanogaster." Prog Neurobiol 76(5): 328-347.

McGuire, S. E., P. T. Le, et al. (2001). "The role of Drosophila mushroom body signaling in olfactory memory." Science 293(5533): 1330-1333.

Medina, J. F., J. C. Repa, et al. (2002). "Parallels between cerebellum- and amygdala-dependent conditioning." Nat Rev Neurosci 3(2): 122-131.

Menzel, R. (2001). "Searching for the memory trace in a mini-brain, the honeybee." Learn Mem 8(2): 53-62.

Nassel, D. R. and K. Elekes (1992). "Aminergic neurons in the brain of blowflies and Drosophila: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons." Cell Tissue Res 267(1): 147-167.

Okada, R., J. Rybak, et al. (2007). "Learning-related plasticity in PE1 and other mushroom body-extrinsic neurons in the honeybee brain." J Neurosci 27(43): 11736-11747.

Pfeiffer, B. D., A. Jenett, et al. (2008). "Tools for neuroanatomy and neurogenetics in Drosophila." Proc Natl Acad Sci U S A 105(28): 9715-9720.

Pfeiffer, B. D., T. T. Ngo, et al. (2010). "Refinement of Tools for Targeted Gene Expression in Drosophila." Genetics.

Pulver, S. R., S. L. Pashkovski, et al. (2009). "Temporal dynamics of neuronal activation by Channelrhodopsin-2 and TRPA1 determine behavioral output in Drosophila larvae." J Neurophysiol 101(6): 3075-3088.

Qin, H. and J. Dubnau (2010). "Genetic disruptions of Drosophila Pavlovian learning leave extinction learning intact." Genes Brain Behav 9(2): 203-212.

Quinn, W. G. and Y. Dudai (1976). "Memory phases in Drosophila." Nature 262(5569): 576-577.

Quinn, W. G., W. A. Harris, et al. (1974). "Conditioned behavior in Drosophila melanogaster." Proc Natl Acad Sci U S A 71(3): 708-712.

Quinn, W. G., P. P. Sziber, et al. (1979). "The Drosophila memory mutant amnesiac." Nature 277(5693): 212-214.

Riemensperger, T., T. Voller, et al. (2005). "Punishment prediction by dopaminergic neurons in Drosophila." Curr Biol 15(21): 1953-1960.

Roy, B., A. P. Singh, et al. (2007). "Metamorphosis of an identified serotonergic neuron in the Drosophila olfactory system." Neural Dev 2: 20.

Rybak, J. and R. Menzel (1993). "Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe." J Comp Neurol 334(3): 444-465.

Schroll, C., T. Riemensperger, et al. (2006). "Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae." Curr Biol 16(17): 1741-1747.

Schwaerzel, M., M. Heisenberg, et al. (2002). "Extinction antagonizes olfactory memory at the subcellular level." Neuron 35(5): 951-960.

Schwaerzel, M., M. Monastirioti, et al. (2003). "Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila." J Neurosci 23(33): 10495-10502.

Selcho, M., D. Pauls, et al. (2009). "The role of dopamine in Drosophila larval classical olfactory conditioning." PLoS ONE 4(6): e5897.

Seugnet, L., Y. Suzuki, et al. (2008). "D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in Drosophila." Curr Biol 18(15): 1110-1117.

Smith, D. P. (2007). "Odor and pheromone detection in Drosophila melanogaster." Pflugers Arch 454(5): 749-758.

Stocker, R. F., M. C. Lienhard, et al. (1990). "Neuronal architecture of the antennal lobe in Drosophila melanogaster." Cell Tissue Res 262(1): 9-34.

Strausfeld, N. J., L. Hansen, et al. (1998). "Evolution, discovery, and interpretations of arthropod mushroom bodies." Learn Mem 5(1-2): 11-37.

Strausfeld, N. J., I. Sinakevitch, et al. (2003). "The mushroom bodies of Drosophila melanogaster: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes." Microsc Res Tech 62(2): 151-169.

Tan, Y., D. Yu, et al. (2010). "Gilgamesh is required for rutabaga-independent olfactory learning in Drosophila." Neuron 67(5): 810-820.

Tanaka, N. K., H. Tanimoto, et al. (2008). "Neuronal assemblies of the Drosophila mushroom body." J Comp Neurol 508(5): 711-755.

Thum, A. S., A. Jenett, et al. (2007). "Multiple memory traces for olfactory reward learning in Drosophila." J Neurosci 27(41): 11132-11138.

Tomchik, S. M. and R. L. Davis (2009). "Dynamics of learning-related cAMP signaling and stimulus integration in the Drosophila olfactory pathway." Neuron 64(4): 510-521.

Tully, T., T. Preat, et al. (1994). "Genetic dissection of consolidated memory in Drosophila." Cell 79(1): 35-47.

Tully, T. and W. G. Quinn (1985). "Classical conditioning and retention in normal and mutant Drosophila melanogaster." J Comp Physiol A 157(2): 263-277.

Tully, T. and W. G. Quinn (1985). "Classical conditioning and retention in normal and mutant Drosophila melanogaster." J Comp Physiol [A] 157(2): 263-277.

Turner, G. C., M. Bazhenov, et al. (2008). "Olfactory representations by Drosophila mushroom body neurons." J Neurophysiol 99(2): 734-746.

Unoki, S., Y. Matsumoto, et al. (2005). "Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study." Eur J Neurosci 22(6): 1409-1416.

Vergoz, V., E. Roussel, et al. (2007). "Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex." PLoS ONE 2(3): e288.

Vosshall, L. B., H. Amrein, et al. (1999). "A spatial map of olfactory receptor expression in the Drosophila antenna." Cell 96(5): 725-736.

Vosshall, L. B. and R. F. Stocker (2007). "Molecular architecture of smell and taste in Drosophila." Annu Rev Neurosci 30: 505-533.

Waddell, S. (2010). "Dopamine reveals neural circuit mechanisms of fly memory." Trends Neurosci.

Wong, A. M., J. W. Wang, et al. (2002). "Spatial representation of the glomerular map in the Drosophila protocerebrum." Cell 109(2): 229-241.

Wu, C. L., S. Xia, et al. (2007). "Specific requirement of NMDA receptors for long-term memory consolidation in Drosophila ellipsoid body." Nat Neurosci 10(12): 1578-1586.

Yang, M. Y., J. D. Armstrong, et al. (1995). "Subdivision of the Drosophila mushroom bodies by enhancer-trap expression patterns." Neuron 15(1): 45-54.

Yu, D., A. Ponomarev, et al. (2004). "Altered representation of the spatial code for odors after olfactory classical conditioning; memory trace formation by synaptic recruitment." Neuron 42(3): 437-449.

Yu, H. H., C. H. Chen, et al. (2009). "Twin-spot MARCM to reveal the developmental origin and identity of neurons." Nat Neurosci 12(7): 947-953.

Zars, T., M. Fischer, et al. (2000). "Localization of a short-term memory in Drosophila." Science 288(5466): 672-675.

Zhang, K., J. Z. Guo, et al. (2007). "Dopamine-mushroom body circuit regulates saliency-based decision-making in Drosophila." Science 316(5833): 1901-1904.

Zhang, S., Y. Yin, et al. (2008). "Increased dopaminergic signaling impairs aversive olfactory memory retention in Drosophila." Biochem Biophys Res Commun 370(1): 82-86.

3. Chapter I

The mushroom body of adult *Drosophila* characterized by GAL4 drivers

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Running head: Genetic dissection of the *Drosophila* mushroom body

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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 compared and cataloged 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. Furthermore, we counted the number of GAL4-positive Kenyon cells in each line. We found that approximately 2000 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 2000 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 (Heisenberg 2003; Fahrbach 2006). It consists 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, Lienhard 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, Tanimoto, & Ito, 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, Skoulakis et al. 1998; Jefferis, Marin 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, Skoulakis et al. 1998; Ito, Suzuki et al. 1998). Recent studies revealed that α'/β' and α/β lobes harbor further subdivisions (Yang, Armstrong et al. 1995; Strausfeld, Sinakevitch et al. 2003; Tanaka, Tanimoto et al. 2008). 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 (Strausfeld, Sinakevitch et al. 2003; Keene and Waddell 2007; Tanaka, Tanimoto et al. 2008).

Compared to the other insect species with large numbers of Kenyon cells (Neder 1959; Witthöft 1967; Leitch and Laurent 1996), the advantages of studying the *Drosophila* MB are its smaller size with a conserved layered cellular organization (Mobbs 1984; Rybak and Menzel 1993; Strausfeld 2002; Strausfeld, Sinakevitch et al. 2003; Sjoholm, Sinakevitch et al. 2006) and its amenability to genetic manipulation (Venken and 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 (Hinke 1961; Technau and Heisenberg 1982; Technau 1984; Balling, Technau et al. 1987; Ito and Hotta 1992; Lee, Lee et al. 1999; Mader 2004). However, the average numbers in these studies range approximately from 1000 to 2500. 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 2500, 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 pedunculus from electron micrographs resulted in least errors, because (1) the fiber number represents the cell number since Kenyon cell axons do not bifurcate at that level; (2) the majority of thin and round fibers represent Kenyon cells.

We decided to reexamine the total number of Kenyon cells 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; Fig. 1). The specificity of GAL4 expression inside and outside the MB is critical especially for interpretation of functional manipulation using MB-GAL4s (Ito, Okada 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. Furthermore, we demonstrate the numerical composition of the Kenyon cell subtypes.

Material and Methods

Fly strains

Fly stocks were grown on standard *Drosophila* medium (cornmeal, agar, molasses, yeast, and nipagin) under a constant light and dark cycle (14/10 hours) at 25°C and 60% relative humidity. Twenty five GAL4 strains were selected from the literature (Fig. 1). These drivers have been used for behavioral, developmental, physiological, and/or anatomical studies of Kenyon cells (Supplemental Fig. 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 two males and three females for each cross. The brains were dissected in Ringer's solution, fixed in phosphate-buffered saline containing 2% formaldehyde and 0.3% Triton X-100 (PBT; Sigma, St. Louis, MO) for 1 hour at room temperature, and subsequently rinsed with PBT three times (3x 10min). After 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 GFP (1:1000; Molecular Probes, Eugene, OR; A6455) and the mouse monoclonal antibody against Synapsin, a presynaptic protein (3C11; 1:20) (Klagges, Heimbeck et al. 1996), respectively. The brains were washed with PBT for 20min three times and incubated with secondary antibodies in the blocking solution at 4°C overnight. The employed secondary antibodies were Alexa Fluor488-conjugated goat anti-rabbit (1:1000 or

1:500 Molecular Probes) and Cy3-conjugated (1:250) goat anti-mouse IgG. Finally, the brains were rinsed with PBT (3x 20 min + 1x 1h) and mounted in Vectashield (Vector, Burlingame, CA). 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 a confocal microscopy, Leica SP1. Image stacks were collected at 1.5 μm intervals with a 20X objective lens to cover an entire brain. For cell counting, we collected confocal stacks at 0.8 μm 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 (Fig. 8A). 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 (NIH). 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 labeling 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 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 Kenyon cells surrounded by labeled cells were discriminated by their size, shape, location, fine neurites to the calyx, 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 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 (Fig. 1 and supplemental Fig. 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-expressing 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. Fig. 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, Okada 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 Fig. 1 and supplemental Fig. 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 (Fig. 1; supplemental Fig. 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 5 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 male and female.

As an internet resource, we indexed representative confocal data of all analyzed driver strains ("der Pilz"; 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 c739, MZ1489, NP3061, 17d, NP3208, NP7175 and NP6649 in the MB was restricted to the α/β neurons. 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 (Fig. 2E-G). The others labeled limited subdivisions (Fig. 2A-D). GAL4-positive Kenyon cells in NP7175, 17d and NP6649 preferentially innervated the core of the α/β lobes (α/βc). Given their variable occupancy of the α/βc lobes, it is likely to consist of multiple populations with concentric organization (Fig. 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/βp) as previously described (Tanaka, Tanimoto et al. 2008). The α/βp neurons have dendritic arbors specifically in the accessory calyx, where the projection neurons do not directly terminate (Tanaka, Tanimoto et al. 2008). The expression of NP7175 inside and outside the MB differed between the genders (Fig. 2A and 7A).

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 (Fig. 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 (Fig. 5A and E). 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, Tanimoto 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.

Drivers labeling the γ neurons

H24 and 1471 preferentially marked the γ lobe within the MB (Fig. 4). With G3, H24 additionally labeled the α/β neurons weakly. 1471 had selective expression in the γ neurons in the MB as originally reported (Isabel, Pascual et al. 2004), although occasionally it seems to drive additional expression in the α/β neurons (Keleman, Kruttner et al. 2007). 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 (Fig. 4C and 4D). To distinguish this dorsal subdivision from of 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 (Fig. 4E-G). MB247 labeled this part, but not the α'/β' lobes (Fig. 4E). c320 had innervation there, but not in the rest of the γ lobe (Fig. 4F). c305a labeled the entire α'/β' lobes, but not the γd (Fig. 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 (Fig. 1 and 5). NP1131 selectively labeled the γ and α'/β'a neurons in the MB (Fig. 5A). 201y had expression in the γ and α/βc neurons (Fig. 5B). c309, MB247 and D52H (female) strongly marked the α/β and γ neurons. MB247 and D52H (female) exhibited unusually low level of background expression (Fig. 5C and D). For both lines, there were very little, if any, expression in α'/β' neurons. D52H showed a gender difference in the MB. The males of D52H additionally strongly expressed the reporter in the α'/β' lobes (Fig. 7B). NP65 and c320 labeled subsets of the α/β and α'/β' neurons (Fig.5E and

F). In NP2748 and female 103y, reporter signals were detectable in the all lobes, although very weak in some subdivisions (Fig.1 and 5).

GAL4 drivers labeling all lobe systems

c309, c772, c747, 30y, 492b, 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 (Fig. 6A). All drivers in this group had significant GAL4 expression outside the MB (Fig. 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 non-overlapping 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 gender differences in their expression patterns (Fig. 1 and Fig. 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 (Fig.5G and 7C).

The number of genetically labeled Kenyon cells

The total number of Kenyon cells in the *Drosophila* MB was reported to range from 800 to 2900 (Hinke 1961; Technau and Heisenberg 1982; Technau 1984; Balling, Technau et al. 1987; Ito and Hotta 1992; Lee, Lee et al. 1999; Mader 2004). The variability mainly depends on the method of counting and estimation. MB-GAL4s with expression in all lobe systems (Fig. 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 1917 and 1898 cells, respectively (Fig. 8B). These numbers were however smaller than some reports (Hinke 1961; Technau and Heisenberg 1982; Technau 1984; Balling, Technau et al. 1987). 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 1975 labeled Kenyon cells on average, but not significantly higher than OK107 or 238y alone ($P > 0.05$; $n \geq 7$; Fig. 8B).

Likewise, the numbers of GAL4-expressing Kenyon cells were counted in MB-GAL4s with expression in specific subdivisions of the lobes (Fig. 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 (Fig. 1, 2E and 2F), whereas the number of GAL4-expressing Kenyon cells of NP3061 was significantly less than that of c739 ($P <0.001$; $n \geq 5$; Fig. 7B). Similarly, combination of MB247/NP6649 only slightly increased the number of labeled cells compared to MB247 alone, indicating that weaker expression in the α/βc 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/βp (Tanaka, Tanimoto et al. 2008) (Fig. 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 1975. Since we counted, on average, 1002, 370 and 671 cells for c739 (α/β), c305a (α'/β'), and H24 (γ), respectively, the sum of these lines was 2043 cells, which matches the number in OK107/238y with ~3 % of a mean difference (Fig. 7). The sum of MB247/NP6649 (α/β and γ; 1630 cells) and c305a (α'/β'; 370 cells) also matched the number

in OK107/238y (2000 vs. 1975 cells). Taken together, we conclude the average total number of Kenyon cells of the mushroom body that can be genetically labeled is 2000.

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 2000 Kenyon cells per hemisphere (Fig. 8B). This is roughly on average 500 cells fewer than previous reports, where the numbers of cell bodies (2380-2640) and axon fibers (2140-2990) were estimated and counted, respectively (Hinke 1961; Technau and Heisenberg 1982; Technau 1984; Balling, Technau et al. 1987).

We demonstrated that any combinations of MB-GAL4s with complementary or overlapping patterns covering the entire lobe systems significantly failed to exceed 2000 (Fig. 8B). This is consistent with the number of cell bodies in one MB neuroblast clone using elav-GAL4 or OK107 (i.e. ~500 cells; (Lee, 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 (ABF and YA, unpublished observation). In any event, this study suggests that approximately 2000 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 GABAergic neuron(s) have thin parallel fibers projecting through the pedunculus as Kenyon cells do (Yasuyama, Meinertzhagen et al. 2002; Tanaka, Tanimoto et al. 2008). In our confocal micrographs, we counted at least 45 fibers at the caudal pedunculus derived from a single MB-APL (KG and YA, 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 and 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; Fig. 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 800-1200 till the adult (Ito and Hotta 1992). Although we have no clear explanation, the deviation might again be technical, such as inefficient BrdU incorporation.

Considering the production of 2000 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 (2000 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 mushroom body undergo significant changes with many developmental and environmental factors, such as different wild-type strains (Technau 1984; Balling, Technau et al. 1987; Heisenberg, Heusipp et al. 1995). 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 2000 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 ~1000 presynaptic boutons, which comprises the cores of microglomeruli (Stocker, Lienhard et al. 1990; Jefferis, Potter et al. 2007; Turner, Bazhenov et al. 2008). Based on the electron microscopic data (Yasuyama, Meinertzhagen et al. 2002), Turner et al. estimated 30 active zones on average at a single bouton (Turner, Bazhenov et al. 2008). Since each Kenyon cell has dendritic arbors on 5 microglomeruli on average (Zhu, Chiang et al. 2003), each projection neuron bouton could be presynaptic to 10 different Kenyon cell claws (= 2000 cells X 5 terminals / 1000 microglomeruli). Given that several postsynaptic sites can be assigned to a single presynaptic release site (Yasuyama, Meinertzhagen et al. 2002; Prokop and Meinertzhagen 2005), each Kenyon cell claw, on average, may have more than 6 postsynaptic sites (= multiple postsynapses [≥ 2] X 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% (1002 cells) of the total labeled Kenyon cells (Fig. 8). This composition fits well with the volumetric ratio of the cell bodies of MB neuroblast clones: 35%, 23% and 42%, respectively (Lee, 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, Cheng 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/βc and α/βp neurons in the α/β lobes have at least 203 (10%; NP7175) and 76 (4%; NP3208) cells, respectively. Given that the α/βp neurons exceptionally arborize in the accessory calyx, the α/βc lobes might contain the least Kenyon cells that project to the main calyx. For the γd 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 γd and α'/β'a neurons to be approximately 55 and 98, respectively (H24 – 1471 and NP1131 – H24 for the γd 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 1000 presynaptic boutons with 5 dendritic terminals of each Kenyon cell, at least 200 Kenyon cells are theoretically required (= 1000 microglomeruli / 5 terminals). Therefore, 203 of the α/βc 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 X 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 (Tanaka, Awasaki et al. 2004; Jefferis, Potter et al. 2007; Lin, Lai et al. 2007). Consistently, we also found microglomeruli not innervated in MB-GAL4s that label 1000 Kenyon cells (e.g. arrows and arrowheads in the calyx of c739 in Fig. 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 (Fig. 2). However, the numbers of labeled Kenyon cells are significantly different (ca. 300 cells more in c739; Fig. 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 (~6%) represent each odor (Wang, Guo et al. 2004; Turner, Bazhenov et al. 2008), 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, Wilson et al. 2006), although both label the α/β neurons (Fig. 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, Peabody et al. 2006) or GAL80 enhancer trap system (Suster, Seugnet 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, Awasaki 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 GAL80[ts] system (Osterwalder, Yoon et al. 2001; Roman, Endo et al. 2001; McGuire, Le et al. 2003)).

Figures

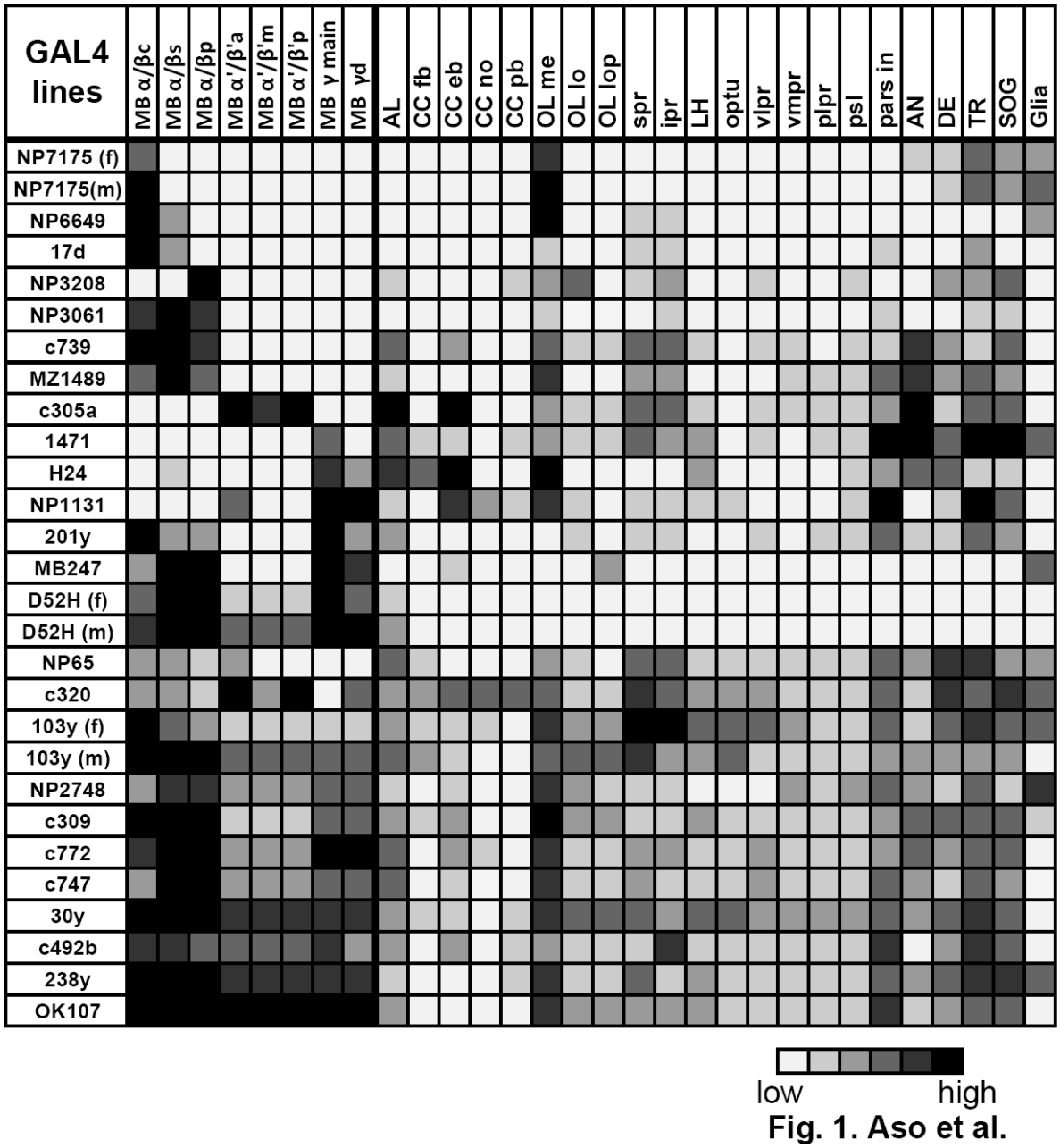


Fig. 1. Expression pattern of 25 GAL4 lines

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 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 where these GAL4 drivers have been used are listed in the supplementary Fig. 1.

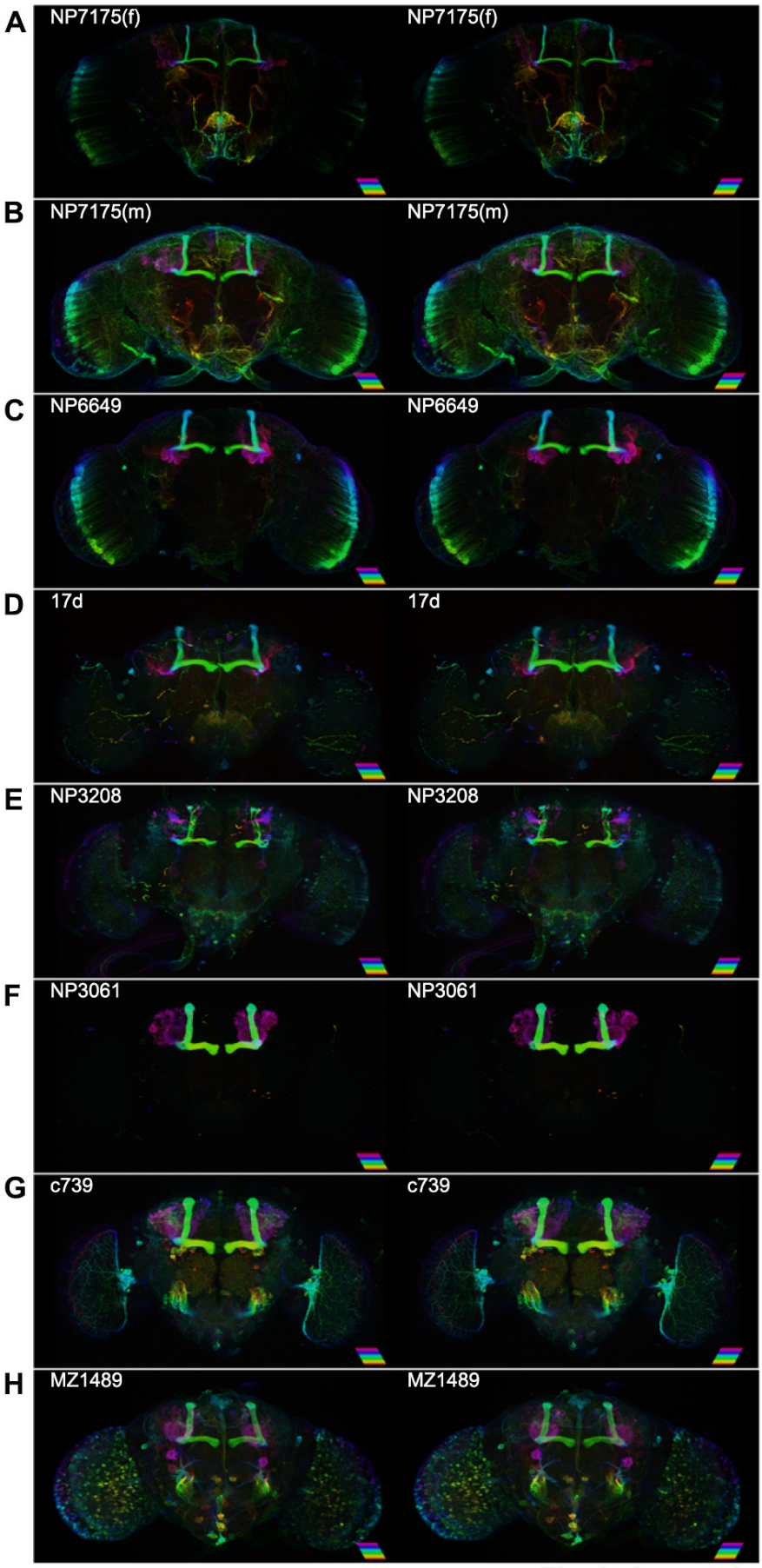


Fig. 2 Aso et al.

Fig. 2. Drivers labeling the α/β neurons

Stereopairs showing reconstructions of MB-GAL4s preferentially labeling the α/β neurons. The applied color illustrates the depth (see scale bar [25 μm] 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 male and female, unless stated.

(A) Female NP7175 exclusively innervated the α/βc lobes in the MB. It also labeled the subesophageal ganglion, the cells in the medulla, and the processes in the tritocerebrum. See Fig. 7A for the expression pattern in the male.

(B) NP6649 strongly labeled the α/βc, while the expression in the α/βs was weak. As in NP7175, the cells in the medulla were strongly labeled. It also has expression in the dorsal giant interneuron (DGI) (Ito, Sass et al. 1997).

(C) As in NP6649, the α/βc were strongly labeled in 17d, while the expression in the α/βs was weak. In addition, weak labeling was occasionally detected in the pars intercerebralis, cells located around the subesophageal ganglion, DGI, large neurons located around the calyx and protocerebral bridge. One very large neuron located ventral to the calyx project anteriorly and terminate 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/βp. 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 Fig.1).

(G) MZ1489 innervated the entire α/β lobe, though the signal in the α/βc 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 Fig. 1).

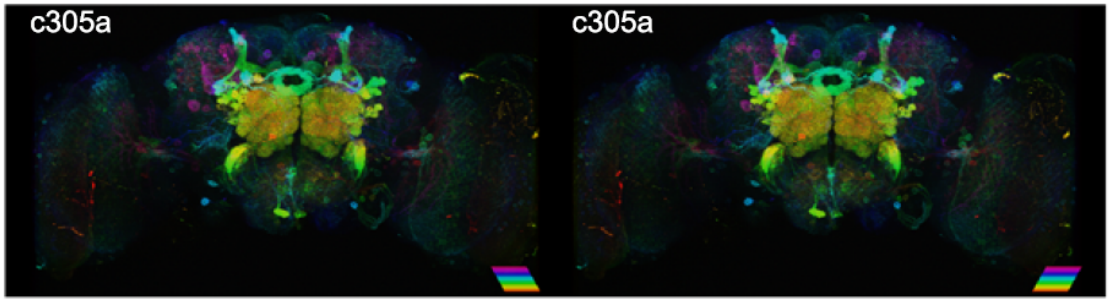


Fig. 3 Aso et al.

Fig. 3. Driver labeling the α'/β' neurons

Stereopair showing a reconstruction of c305a preferentially labeling the entire α'/β' lobes. The applied color illustrates the depth (see scale bar [25 μm] 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 Fig. 1).

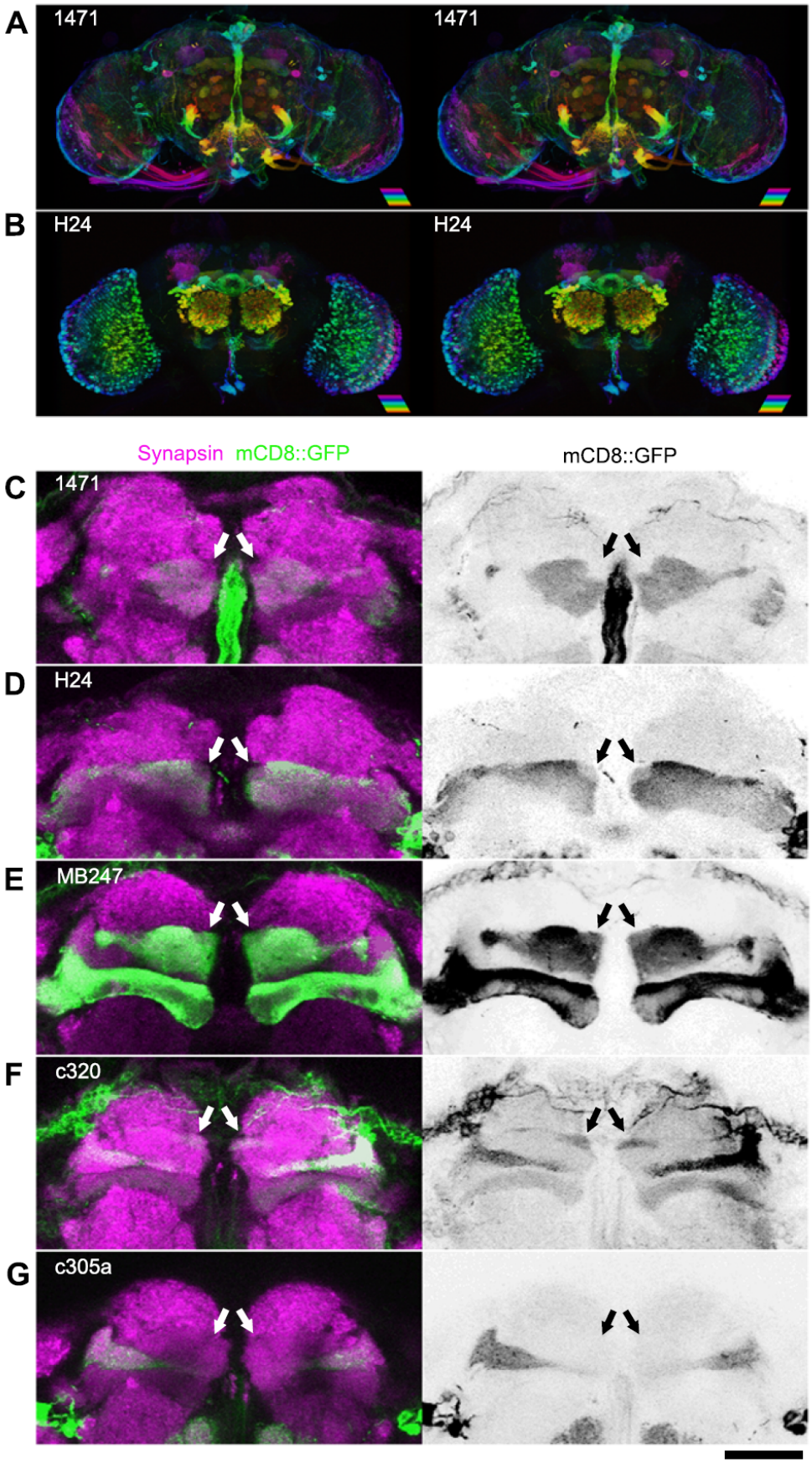


Fig. 4 Aso et al.

Fig. 4. Drivers labeling the γ neurons

(A, B) Stereopairs showing reconstructions of MB-GAL4s preferentially labeling the γ lobe. The applied color illustrates the depth (see scale bar [25 μm] 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 γd 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 ventral 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 ventral to the subesophageal ganglion showed the reporter signals.

(C-G) Frontal views of the projections of three consecutive confocal slices including the γd 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 β lobe occupies the most ventral part. The β' lobe typically lies in between them. Arrows indicate the medial end of the γd subdivision. 1471 labeled the main part of the γ lobe, but not the γd subdivision (C), whereas the expression both in the γd subdivision and the main part of the γ lobe was seen in H24 and MB247 (D and E).

(F) c320 labeled the γd subdivision without labeling the main γ lobe.

(G) c305a specifically labeled the α'/β' lobe, but not the γd subdivision.

Scale bar=25μm for (C-G).

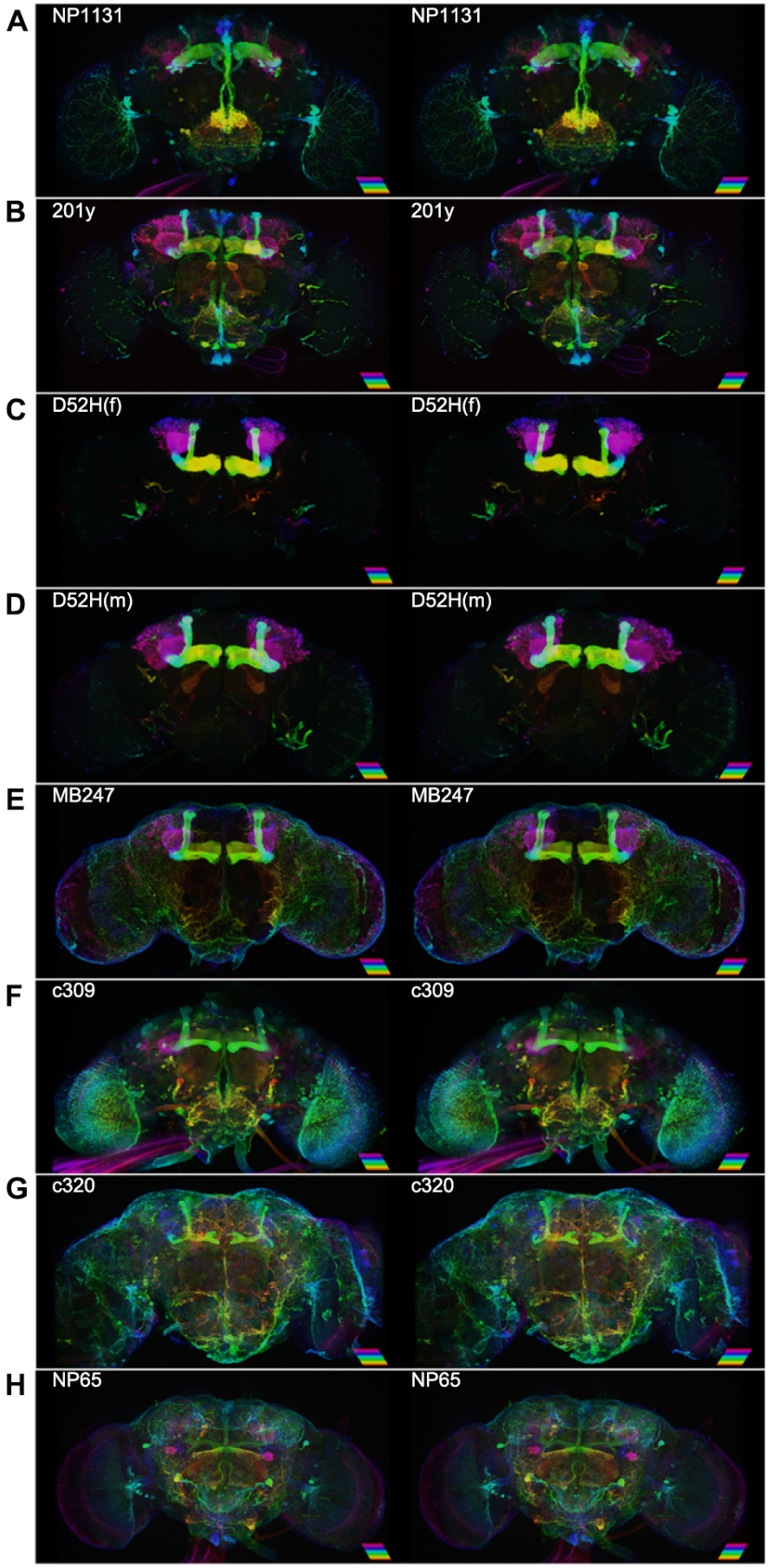


Fig. 5 Aso et al.

Fig. 5. Drivers labeling multiple types of Kenyon cells

Stereopairs showing reconstructions of MB-GAL4s preferentially labeling the multiple types of Kenyon cells. The applied color illustrates the depth (see scale bar [25 μm] 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 subdivision was remarkably weak. See also original confocal stacks for detail (http://mushroombody.net).

(A) NP1131 labeled the α'/β'a 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 Fig. 1).

(B) 201y labeled the α/βc 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 α/βc 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 α/βc was weaker than in the other α/β subdivisions. See Fig. 7B for the expression pattern in the male.

(E) NP65 labeled the α/βs, α/βc and α'/β'a 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 Fig. 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 (Fig.3F). Expression in the α'/β'm 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 Fig.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 Fig. 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.

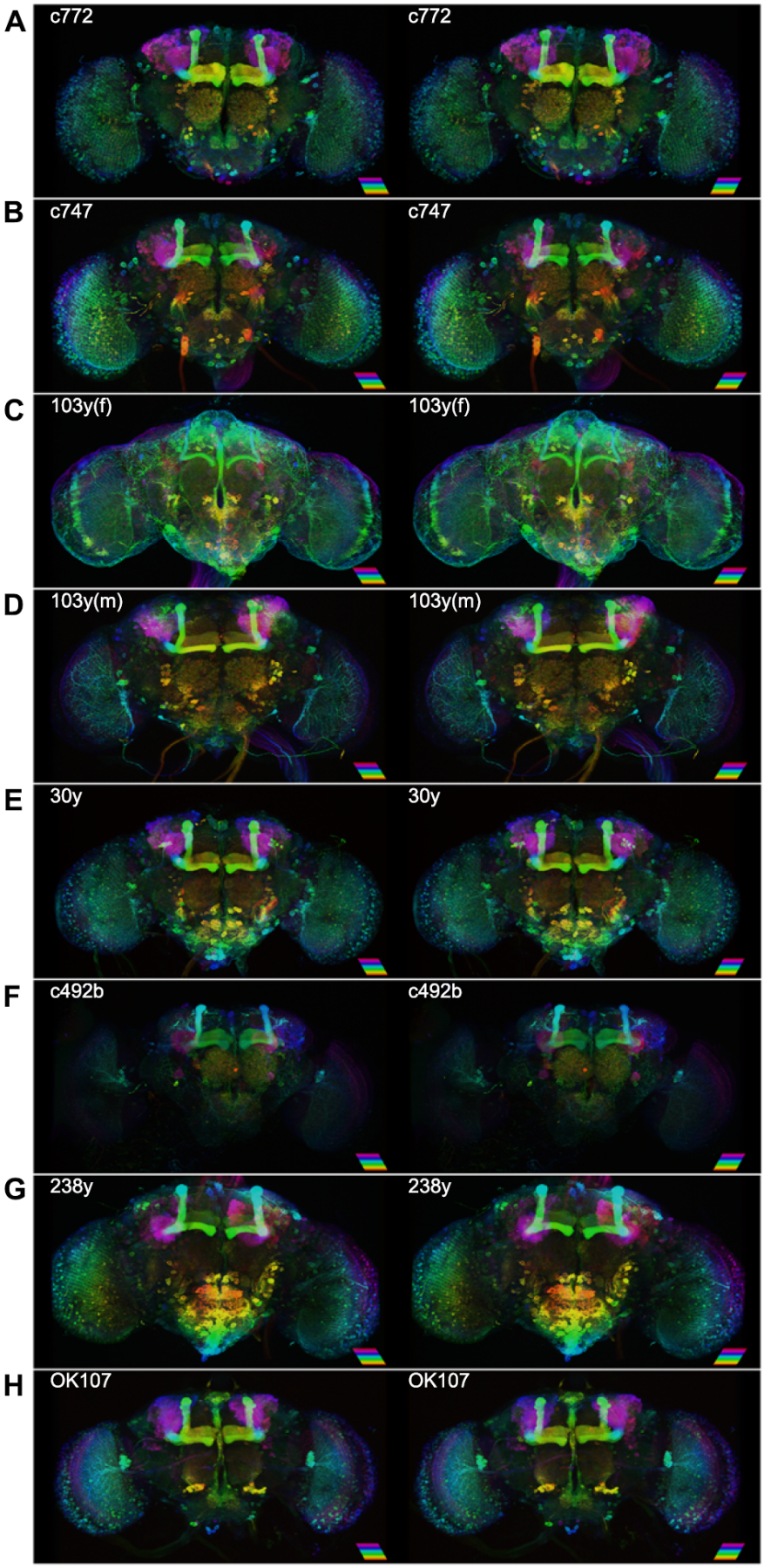


Fig. 6 Aso et al.

Fig. 6. GAL4 strains labeling all of the lobes

Stereopairs showing reconstructions of MB-GAL4s labeling all of the lobes. The applied color illustrates the depth (see scale bar [25 μm] for the color code). Since most of lines in this category exhibited expression in many neuropils, we describe only strong signals here (see also Fig.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, optic lobes (see also Fig. 1). Additionally, many different sensory nerves and the cervical connectives were stained.

(B) c772 preferentially labeled the α/βp, α/βs and γ neurons with weaker expression in the α/βc 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/βp, α/βs and γ neurons strongly and the α/βc 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 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 γd neurons was relatively weak. Compare to other lines in this category, expressions outside the MB were less pronounced. We observed reporter signals in the pars intercerebralis, local inter neurons in the antennal lobe, deutocerebrum, subesophageal ganglion, and large paired neurons located ventral to the calyx (see also the legend of Fig. 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.

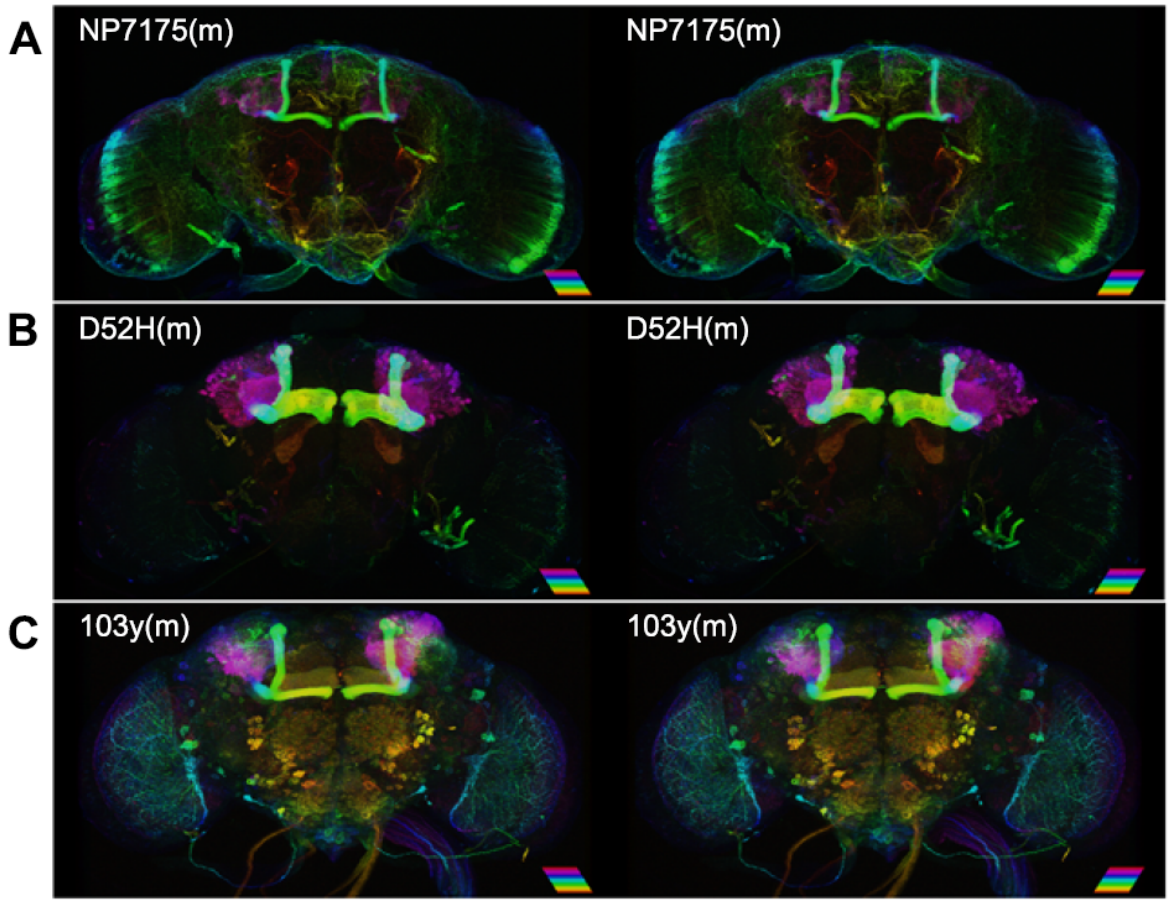


Fig. 7 Aso et al.

Fig. 7. GAL4 strains with sex-dependent difference

Stereopairs showing reconstructions of MB-GAL4s with sex-specific reporter expression. The applied color illustrates the depth (see scale bar [25 μm] 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/βc (Fig.2 A). In addition to the background expression seen in the female, surface glia was strongly labeled.

(B) In contrast to 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 female, the expression in the α'/β' and γ lobes was very faint, if any (Fig. 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.

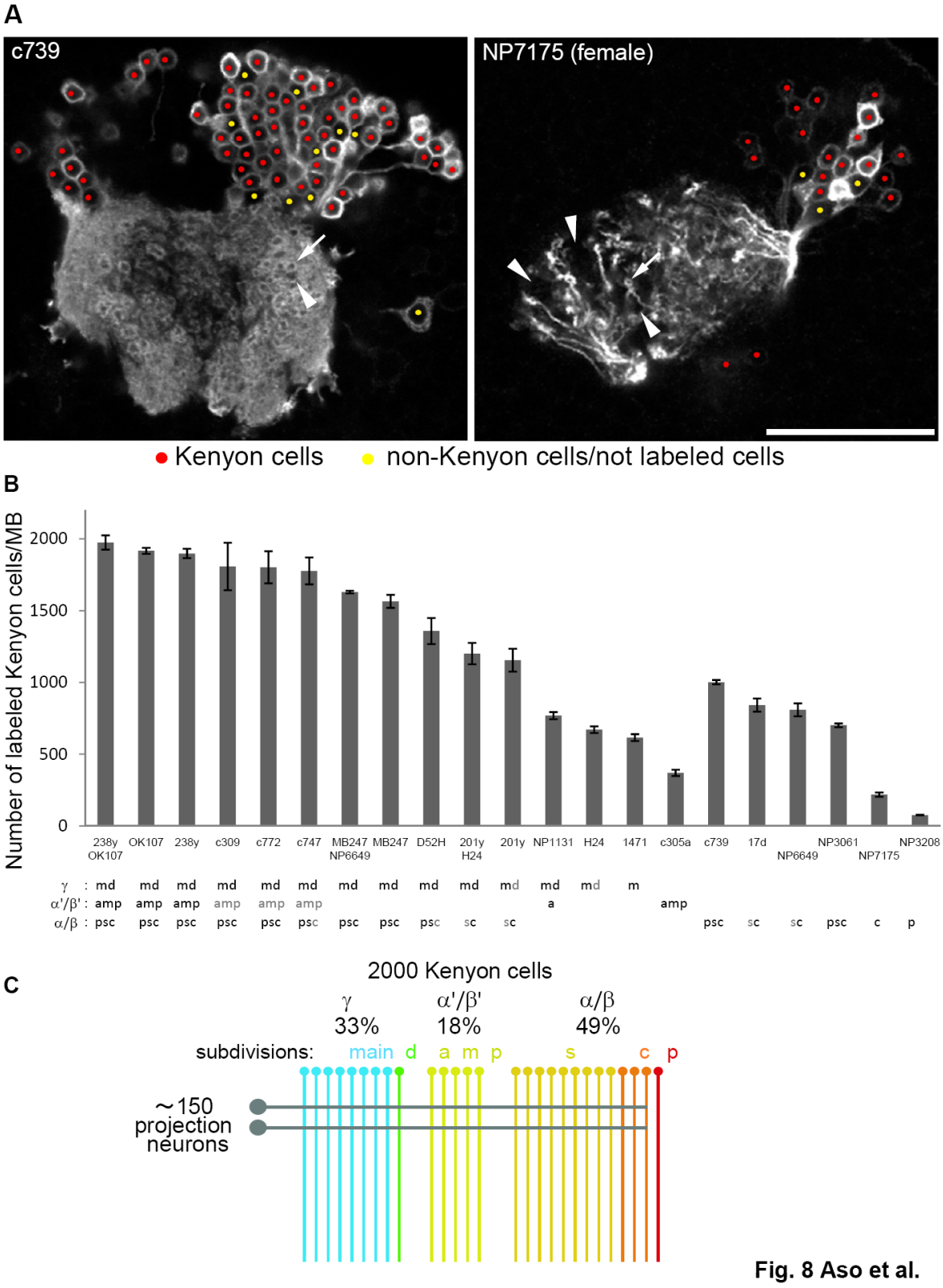


Fig. 8 Aso et al.

Fig. 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 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 μm

(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 Fig.1). The label in gray shows weak expression. See the legend of Fig.1 for abbreviations. *n*= 5-8. Error bars represent S.E.M.

(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 ~2000 Kenyon cells. One circle and line represents ~75 cells. Approximately 150 iACT projections neurons terminate on the Kenyon cells except for the α/βp neurons (right most line).

References

Akalal, D. B., Wilson, C. F., Zong, L., Tanaka, N. K., Ito, K., & Davis, R. L. (2006). Roles for *Drosophila* mushroom body neurons in olfactory learning and memory. *Learn Mem, 13*(5), 659-668.

Balling, A., Technau, G. M., & Heisenberg, M. (1987). Are the structural changes in adult *Drosophila* mushroom bodies memory traces? Studies on biochemical learning mutants. *J Neurogenet, 4*(2-3), 65-73.

Crittenden, J. R., Skoulakis, E. M., Han, K. A., Kalderon, D., & Davis, R. L. (1998). Tripartite mushroom body architecture revealed by antigenic markers. *Learn Mem, 5*(1-2), 38-51.

Fahrbach, S. E. (2006). Structure of the mushroom bodies of the insect brain. *Annu Rev Entomol, 51*, 209-232.

Heisenberg, M. (1980). Mutants of brain structure and function: what is the significance of the mushroom bodies for behavior? *Basic Life Sci, 16*, 373-390.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat Rev Neurosci, 4*(4), 266-275.

Heisenberg, M., Heusipp, M., & Wanke, C. (1995). Structural plasticity in the *Drosophila* brain. *J Neurosci, 15*(3 Pt 1), 1951-1960.

Hinke, W. (1961). Das relative postembryonale Wachstum der Hirnteile von *Culex pipiens*, *Drosophila melanogaster* und *Drosophila*-Mutanten. *Z Morph Ökol Tiere, 50*, 81-118.

Isabel, G., Pascual, A., & Preat, T. (2004). Exclusive consolidated memory phases in *Drosophila*. *Science, 304*(5673), 1024-1027.

Ito, K., & Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of *Drosophila melanogaster*. *Dev Biol, 149*(1), 134-148.

Ito, K., Okada, R., Tanaka, N. K., & Awasaki, T. (2003). Cautionary observations on preparing and interpreting brain images using molecular biology-based staining techniques. *Microsc Res Tech, 62*(2), 170-186.

Ito, K., Sass, H., Urban, J., Hofbauer, A., & Schneuwly, S. (1997). GAL4-responsive UAS-tau as a tool for studying the anatomy and development of the Drosophila central nervous system. *Cell Tissue Res, 290*(1), 1-10.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., & Strausfeld, N. J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen. *Learn Mem, 5*(1-2), 52-77.

Jefferis, G. S., Marin, E. C., Watts, R. J., & Luo, L. (2002). Development of neuronal connectivity in *Drosophila* antennal lobes and mushroom bodies. *Curr Opin Neurobiol, 12*(1), 80-86.

Jefferis, G. S., Potter, C. J., Chan, A. M., Marin, E. C., Rohlfing, T., Maurer, C. R., Jr., et al. (2007). Comprehensive maps of *Drosophila* higher olfactory centers: spatially segregated fruit and pheromone representation. *Cell, 128*(6), 1187-1203.

Keene, A. C., & Waddell, S. (2007). *Drosophila* olfactory memory: single genes to complex neural circuits. *Nat Rev Neurosci, 8*(5), 341-354.

Keleman, K., Kruttner, S., Alenius, M., & Dickson, B. J. (2007). Function of the *Drosophila* CPEB protein Orb2 in long-term courtship memory. *Nat Neurosci, 10*(12), 1587-1593.

Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., et al. (1996). Invertebrate synapsins: a single gene codes for several isoforms in *Drosophila*. *J Neurosci, 16*(10), 3154-3165.

Kurusu, M., Awasaki, T., Masuda-Nakagawa, L. M., Kawauchi, H., Ito, K., & Furukubo-Tokunaga, K. (2002). Embryonic and larval development of the *Drosophila* mushroom bodies: concentric layer subdivisions and the role of fasciclin II. *Development, 129*(2), 409-419.

Lee, T., Lee, A., & Luo, L. (1999). Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development, 126*(18), 4065-4076.

Leitch, B., & Laurent, G. (1996). GABAergic synapses in the antennal lobe and mushroom body of the locust olfactory system. *J Comp Neurol, 372*(4), 487-514.

Lin, H. H., Lai, J. S., Chin, A. L., Chen, Y. C., & Chiang, A. S. (2007). A map of olfactory representation in the *Drosophila* mushroom body. *Cell, 128*(6), 1205-1217.

Luan, H., Peabody, N. C., Vinson, C. R., & White, B. H. (2006). Refined spatial manipulation of neuronal function by combinatorial restriction of transgene expression. *Neuron, 52*(3), 425-436.

Mader, M. T. (2004). *Analyse von Expressionsmustern in den Pilzkörpern von Drosophila melanogaster (Diploma thesis).* University of Würzburg, Würzburg Germany.

Maurange, C., Cheng, L., & Gould, A. P. (2008). Temporal transcription factors and their targets schedule the end of neural proliferation in *Drosophila*. *Cell, 133*(5), 891-902.

McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K., & Davis, R. L. (2003). Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science, 302*(5651), 1765-1768.

Mobbs, P. G. (1984). Neural networks in the mushroom bodies of the honeybee. *J Insect Physiol, 30*(1), 43-58.

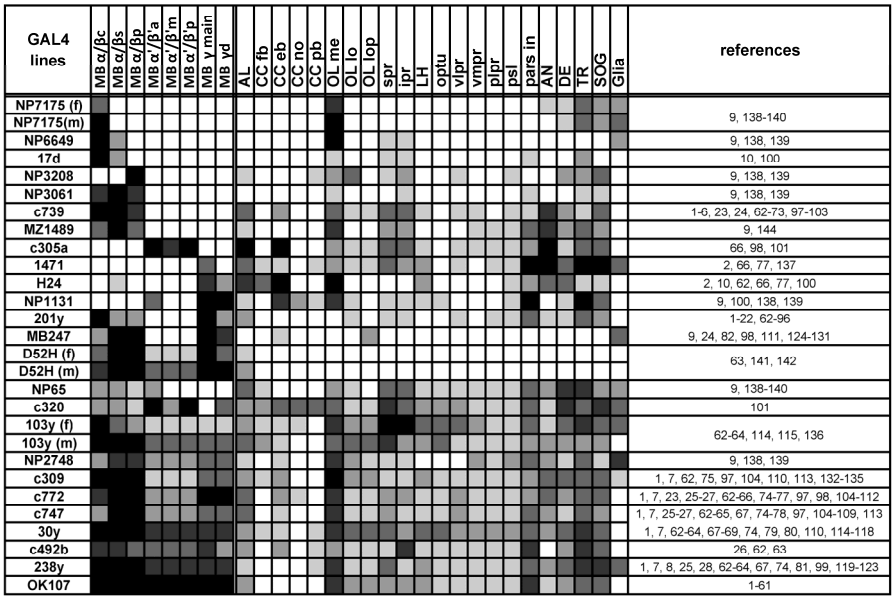
Neder, R. (1959). Allometrisches Wachstum von Hirnteilen bei drei verschieden großen Schabenarten. *Zool Jahrb Abt Anat Ontogenie Tiere, 4*, 411-464.

Osterwalder, T., Yoon, K. S., White, B. H., & Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proc Natl Acad Sci U S A, 98*(22), 12596-12601.

Prokop, A., & Meinertzhagen, I. A. (2005). Development and structure of synaptic contacts in *Drosophila*. [10.1016/j.semcdb.2005.11.010]. *Semin Cell Dev Biol*.

Roman, G., Endo, K., Zong, L., & Davis, R. L. (2001). P[Switch], a system for spatial and temporal control of gene expression in *Drosophila* melanogaster. *Proc Natl Acad Sci U S A, 98*(22), 12602-12607.

Rybak, J., & Menzel, R. (1993). Anatomy of the mushroom bodies in the honey bee brain: the neuronal connections of the alpha-lobe. *J Comp Neurol, 334*(3), 444-465.
Sjöholm, M., Sinakevitch, I., Strausfeld, N. J., Ignell, R., & Hansson, B. S. (2006). Functional division of intrinsic neurons in the mushroom bodies of male *Spodoptera littoralis* revealed by antibodies against aspartate, taurine, FMRF-amide, Mas-allatotropin and DC0. *Arthropod Struct Dev, 35*(3), 153-168.
Stocker, R. F., Lienhard, M. C., Borst, A., & Fischbach, K. F. (1990). Neuronal architecture of the antennal lobe in Drosophila melanogaster. *Cell Tissue Res, 262*(1), 9-34.
Strausfeld, N. J. (2002). Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. *J Comp Neurol, 450*(1), 4-33.
Strausfeld, N. J., Sinakevitch, I., & Vilinsky, I. (2003). The mushroom bodies of *Drosophila melanogaster*: an immunocytological and golgi study of Kenyon cell organization in the calyces and lobes. *Microsc Res Tech, 62*(2), 151-169.
Suster, M. L., Seugnet, L., Bate, M., & Sokolowski, M. B. (2004). Refining GAL4-driven transgene expression in Drosophila with a GAL80 enhancer-trap. *Genesis, 39*(4), 240-245.
Tanaka, N. K., Awasaki, T., Shimada, T., & Ito, K. (2004). Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr Biol, 14*(6), 449-457.
Tanaka, N. K., Tanimoto, H., & Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. *J Comp Neurol, 508*(5), 711-755.
Technau, G., & Heisenberg, M. (1982). Neural reorganization during metamorphosis of the corpora pedunculata in *Drosophila melanogaster*. *Nature, 295*(5848), 405-407.
Technau, G. M. (1984). Fiber number in the mushroom bodies of adult *Drosophila melanogaster* depends on age, sex and experience. *J Neurogenet, 1*(2), 113-126.
Turner, G. C., Bazhenov, M., & Laurent, G. (2008). Olfactory representations by *Drosophila* mushroom body neurons. *J Neurophysiol, 99*(2), 734-746.
Venken, K. J., & Bellen, H. J. (2005). Emerging technologies for gene manipulation in *Drosophila melanogaster*. *Nat Rev Genet, 6*(3), 167-178.
Wang, Y., Guo, H. F., Pologruto, T. A., Hannan, F., Hakker, I., Svoboda, K., et al. (2004). Stereotyped odor-evoked activity in the mushroom body of *Drosophila* revealed by green fluorescent protein-based Ca2+ imaging. *J Neurosci, 24*(29), 6507-6514.
Witthöft, W. (1967). Absolute Anzahl und Verteilung derZellen im Hirn der Honigbiene. *Zoomorphology, 61*(1), 160-184.
Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J., & Kaiser, K. (1995). Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron, 15*(1), 45-54.
Yasuyama, K., Meinertzhagen, I. A., & Schürmann, F. W. (2002). Synaptic organization of the mushroom body calyx in *Drosophila melanogaster*. *J Comp Neurol, 445*(3), 211-226.
Zhu, S., Chiang, A. S., & Lee, T. (2003). Development of the *Drosophila* mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. *Development, 130*(12), 2603-2610.



Supplemental Fig. 1. Aso et al.

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 et al. 2001); 2: (Peng, Xi et al. 2007); 3: (Ramaekers, Magnenat et al. 2005); 4: (Masuda-Nakagawa, Tanaka et al. 2005); 5: (Wu, Xia

et al. 2007); 6: (Fushima and Tsujimura 2007); 7: (Connolly, Roberts et al. 1996); 8: (Kurusu, Awasaki et al. 2002); 9: (Tanaka, Tanimoto et al. 2008); 10: (Manoli, Foss et al. 2005); 11: (Awasaki and Ito 2004); 12: (Boyle, Nighorn et al. 2006); 13: (Gu and O'Dowd 2006); 14: (Nicolai, Lasbleiz et al. 2003); 15: (Wang, Ma et al. 2004); 16: (Wang, Zugates et al. 2002); 17: (Watts, Hoopfer et al. 2003); 18: (Yang, Edenberg et al. 2005); 19: (Zheng, Wang et al. 2003); 20: (Zheng, Zugates et al. 2006); 21: (Zhu, Chiang et al. 2003); 22: (Schuldiner, Berdnik et al. 2008); 23: (Liu, Krause et al. 2007); 24: (Grillenzoni, Flandre et al. 2007); 25: (DeZazzo, Sandstrom et al. 2000); 26: (Kitamoto 2002); 27: (Suh, Wong et al. 2004); 28: (Wang, Mamiya et al. 2008); 29: (Orihara-Ono, Suzuki et al. 2005); 30: (Adachi, Hauck et al. 2003); 31: (Agrawal, Pallos et al. 2005); 32: (Billuart, Winter et al. 2001); 33: (Gu, Yang et al. 2004); 34: (Kim, Lee et al. 2003); 35: (Kobayashi, Michaut et al. 2006); 36: (Komiyama, Sweeney et al. 2007); 37: (Lee, Lee et al. 1999); 38: (Lin, Lai et al. 2007); 39: (Liu, Steward et al. 2000); 40: (Martini, Roman et al. 2000); 41: (Ng and Luo 2004); 42: (Ng, Nardine et al. 2002); 43: (Niimi, Clements et al. 2002); 44: (Pan, Zhang et al. 2004); 45: (Pascual and Preat 2001); 46: (Pascual, Huang et al. 2005); 47: (Plaza, Prince et al. 2001); 48: (Scott, Lee et al. 2001); 49: (Su and O'Dowd 2003); 50: (Wang, Guo et al. 2004); 51: (Wang, Lee et al. 2006); 52: (Whited, Robichaux et al. 2007); 53: (Wojtowicz, Flanagan et al. 2004); 54: (Zelhof and Hardy 2004); 55: (Zhu, Lin et al. 2006); 56: (Zhu, Perez et al. 2005); 57: (Nishimura, Sakoda et al. 2008); 58: (Chen, Li et al. 2008); 59: (Johard, Enell et al. 2008); 60: (Campusano, Su et al. 2007); 61: (Martin, Rogers et al. 2007); 62: (Joiner, Crocker et al. 2006); 63: (Tettamanti, Armstrong et al. 1997); 64: (Zars, Wolf et al. 2000); 65: (Kraft, Levine et al. 1998); 66: (Keleman, Kruttner et al. 2007); 67: (Yang, Armstrong et al. 1995); 68: (Mery, Belay et al. 2007); 69: (Kaun, Hendel et al. 2007); 70: (Cheng, Endo et al. 2001); 71: (Moreau-Fauvarque, Taillebourg et al. 1998); 72: (O'Dell, Armstrong et al. 1995); 73: (Kido and Ito 2002); 74: (Zars, Fischer et al. 2000); 75: (Rodan, Kiger et al. 2002); 76: (McGuire, Le et al. 2003); 77: (Acevedo, Froudarakis et al. 2007); 78: (Rodan, Kiger et al. 2002); 79: (Joiner and Griffith 2000); 80: (Mehren and Griffith 2004); 81: (Wang, Chiang et al. 2003); 82: (Shi, Lin et al. 2007); 83: (Chang, Shi et al. 2003); 84: (Diegelmann, Fiala et al. 2002); 85: (Enerly,

Larsson et al. 2003); 86: (Goldstein, Jan et al. 2005); 87: (Honjo and Furukubo-Tokunaga 2005); 88: (Koushika, Lisbin et al. 1996); 89: (Lee, Marticke et al. 2000); 90: (Luo, Lee et al. 1999); 91: (Michel, Kraft et al. 2004); 92: (Soller, Haussmann et al. 2006); 93: (Verkhusha, Otsuna et al. 2001); 94: (Walker, Tchoudakova et al. 2006); 95: (Watts, Schuldiner et al. 2004); 96: (Peng and Guo 2007); 97: (Manseau, Baradaran et al. 1997); 98: (Krashes and Waddell 2008); 99: (Yeh, Gustafson et al. 1995); 100: (Akalal, Wilson et al. 2006); 101: (Krashes, Keene et al. 2007); 102: (Casares, Calleja et al. 1996); 103: (Parker, Fessler et al. 1995); 104: (Waddell, Armstrong et al. 2000); 105: (Astle, Kozlova et al. 2003); 106: (Broughton, Kitamoto et al. 2004); 107: (Grammenoudi, Kosmidis et al. 2006); 108: (Presente, Boyles et al. 2004); 109: (Reeve, Bassetto et al. 2005); 110: (Pitman, McGill et al. 2006); 111: (Schwaerzel, Heisenberg et al. 2002); 112: (Sakai and Kitamoto 2006); 113: (Dubnau, Grady et al. 2001); 114: (Gatti, Ferveur et al. 2000); 115: (Belgacem and Martin 2002); 116: (Amrein and Axel 1997); 117: (McBride, Giuliani et al. 1999); 118: (Rosay, Armstrong et al. 2001); 119: (Comas, Petit et al. 2004); 120: (Kammermeier, Leemans et al. 2001); 121: (Noveen, Daniel et al. 2000); 122: (Schneeberger and Raabe 2003); 123: (Yu, Baird et al. 2003); 124: (Schwaerzel, Monastirioti et al. 2003); 125: (Acevedo, Froudarakis et al. 2007); 126: (Zhang, Guo et al. 2007); 127: (Schulz, Chromey et al. 1996); 128: (Xi, Peng et al. 2008); 129: (Thum, Jenett et al. 2007); 130: (Kim, Lee et al. 2007); 131: (Baker, Beckingham et al. 2007); 132: (Lee, Stultz et al. 2007); 133: (Koushika, Soller et al. 2000); 134: (Nitz, van Swinderen et al. 2002); 135: (Villella, Ferri et al. 2005); 136: (Garcia-Lopez, Monferrer et al. 2008); 137: (Isabel, Pascual et al. 2004); 138: (Yoshihara and Ito 2000); 139: (Hayashi, Ito et al. 2002); 140: (Tanaka, Awasaki et al. 2004); 141: (Qiu and Davis 1993); 142: *dunce* promoter GAL4, courtesy of R. Davis via T. Zars. See reference 63 and 141 for the dunce promoter; 143: (Wilson and Laurent 2005); 144: (Ito, Urban et al. 1995)

References

References

Acevedo, S. F., Froudarakis, E. I., Kanellopoulos, A., & Skoulakis, E. M. (2007). Protection from premature habituation requires functional mushroom bodies in *Drosophila*. *Learn Mem, 14*(5), 376-384.

Acevedo, S. F., Froudarakis, E. I., Tsiorva, A. A., & Skoulakis, E. M. (2007). Distinct neuronal circuits mediate experience-dependent, non-associative osmotactic responses in *Drosophila*. *Mol Cell Neurosci, 34*(3), 378-389.
Adachi, Y., Hauck, B., Clements, J., Kawauchi, H., Kurusu, M., Totani, Y., et al. (2003). Conserved cis-regulatory modules mediate complex neural expression patterns of the *eyeless* gene in the *Drosophila* brain. *Mech Dev, 120*(10), 1113-1126.
Agrawal, N., Pallos, J., Slepko, N., Apostol, B. L., Bodai, L., Chang, L. W., et al. (2005). Identification of combinatorial drug regimens for treatment of Huntington's disease using *Drosophila*. *Proc Natl Acad Sci U S A, 102*(10), 3777-3781.
Akalal, D. B., Wilson, C. F., Zong, L., Tanaka, N. K., Ito, K., & Davis, R. L. (2006). Roles for *Drosophila* mushroom body neurons in olfactory learning and memory. *Learn Mem, 13*(5), 659-668.
Amrein, H., & Axel, R. (1997). Genes expressed in neurons of adult male *Drosophila*. *Cell, 88*(4), 459-469.
Astle, J., Kozlova, T., & Thummel, C. S. (2003). Essential roles for the Dhr78 orphan nuclear receptor during molting of the *Drosophila* tracheal system. *Insect Biochem Mol Biol, 33*(12), 1201-1209.
Awasaki, T., & Ito, K. (2004). Engulfing action of glial cells is required for programmed axon pruning during *Drosophila* metamorphosis. *Curr Biol, 14*(8), 668-677.
Baker, D. A., Beckingham, K. M., & Armstrong, J. D. (2007). Functional dissection of the neural substrates for gravitaxic maze behavior in *Drosophila melanogaster*. *J Comp Neurol, 501*(5), 756-764.
Belgacem, Y. H., & Martin, J. R. (2002). Neuroendocrine control of a sexually dimorphic behavior by a few neurons of the pars intercerebralis in *Drosophila*. *Proc Natl Acad Sci U S A, 99*(23), 15154-15158.
Billuart, P., Winter, C. G., Maresh, A., Zhao, X., & Luo, L. (2001). Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. *Cell, 107*(2), 195-207.
Boyle, M., Nighorn, A., & Thomas, J. B. (2006). *Drosophila* Eph receptor guides specific axon branches of mushroom body neurons. *Development, 133*(9), 1845-1854.
Broughton, S. J., Kitamoto, T., & Greenspan, R. J. (2004). Excitatory and inhibitory switches for courtship in the brain of *Drosophila melanogaster*. *Curr Biol, 14*(7), 538-547.
Campusano, J. M., Su, H., Jiang, S. A., Sicaeros, B., & O'Dowd, D. K. (2007). nAChR-mediated calcium responses and plasticity in *Drosophila* Kenyon cells. *Dev Neurobiol, 67*(11), 1520-1532.
Casares, F., Calleja, M., & Sanchez-Herrero, E. (1996). Functional similarity in appendage specification by the *Ultrabithorax* and *abdominal-A Drosophila* HOX genes. *EMBO J, 15*(15), 3934-3942.
Chang, K. T., Shi, Y. J., & Min, K. T. (2003). The *Drosophila* homolog of Down's syndrome critical region 1 gene regulates learning: implications for mental retardation. *Proc Natl Acad Sci U S A, 100*(26), 15794-15799.
Chen, G., Li, W., Zhang, Q. S., Regulski, M., Sinha, N., Barditch, J., et al. (2008). Identification of synaptic targets of *Drosophila* pumilio. *PLoS Comput Biol, 4*(2), e1000026.
Cheng, Y., Endo, K., Wu, K., Rodan, A. R., Heberlein, U., & Davis, R. L. (2001). *Drosophila* fasciclinII is required for the formation of odor memories and for normal sensitivity to alcohol. *Cell, 105*(6), 757-768.
Comas, D., Petit, F., & Preat, T. (2004). *Drosophila* long-term memory formation involves regulation of cathepsin activity. *Nature, 430*(6998), 460-463.
Connolly, J. B., Roberts, I. J., Armstrong, J. D., Kaiser, K., Forte, M., Tully, T., et al. (1996). Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. *Science, 274*(5295), 2104-2107.
DeZazzo, J., Sandstrom, D., de Belle, S., Velinzon, K., Smith, P., Grady, L., et al. (2000). nalyot, a mutation of the *Drosophila* myb-related Adf1 transcription factor, disrupts synapse formation and olfactory memory. *Neuron, 27*(1), 145-158.
Diegelmann, S., Fiala, A., Leibold, C., Spall, T., & Buchner, E. (2002). Transgenic flies expressing the fluorescence calcium sensor Cameleon 2.1 under UAS control. *Genesis, 34*(1-2), 95-98.
Dubnau, J., Grady, L., Kitamoto, T., & Tully, T. (2001). Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature, 411*(6836), 476-480.
Enerly, E., Larsson, J., & Lambertsson, A. (2003). Silencing the *Drosophila* ribosomal protein L14 gene using targeted RNA interference causes distinct somatic anomalies. *Gene, 320*, 41-48.
Fushima, K., & Tsujimura, H. (2007). Precise control of fasciclin II expression is required for adult mushroom body development in *Drosophila*. *Dev Growth Differ, 49*(3), 215-227.
Garcia-Lopez, A., Monferrer, L., Garcia-Alcover, I., Vicente-Crespo, M., Alvarez-Abril, M. C., & Artero, R. D. (2008). Genetic and chemical modifiers of a CUG toxicity model in *Drosophila*. *PLoS ONE, 3*(2), e1595.
Gatti, S., Ferveur, J. F., & Martin, J. R. (2000). Genetic identification of neurons controlling a sexually dimorphic behaviour. *Curr Biol, 10*(11), 667-670.
Goldstein, A. Y., Jan, Y. N., & Luo, L. (2005). Function and regulation of Tumbleweed (RacGAP50C) in neuroblast proliferation and neuronal morphogenesis. *Proc Natl Acad Sci U S A, 102*(10), 3834-3839.
Grammenoudi, S., Kosmidis, S., & Skoulakis, E. M. (2006). Cell type-specific processing of human Tau proteins in *Drosophila*. *FEBS Lett, 580*(19), 4602-4606.
Grillenzoni, N., Flandre, A., Lasbleiz, C., & Dura, J. M. (2007). Respective roles of the DRL receptor and its ligand WNT5 in *Drosophila* mushroom body development. *Development, 134*(17), 3089-3097.
Gu, G., Yang, J., Mitchell, K. A., & O'Tousa, J. E. (2004). *Drosophila* ninaB and ninaD act outside of retina to produce rhodopsin chromophore. *J Biol Chem, 279*(18), 18608-18613.
Gu, H., & O'Dowd, D. K. (2006). Cholinergic synaptic transmission in adult *Drosophila* Kenyon cells in situ. *J Neurosci, 26*(1), 265-272.

Hayashi, S., Ito, K., Sado, Y., Taniguchi, M., Akimoto, A., Takeuchi, H., et al. (2002). GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis, 34*(1-2), 58-61.
Honjo, K., & Furukubo-Tokunaga, K. (2005). Induction of cAMP response element-binding protein-dependent medium-term memory by appetitive gustatory reinforcement in *Drosophila* larvae. *J Neurosci, 25*(35), 7905-7913.
Isabel, G., Pascual, A., & Preat, T. (2004). Exclusive consolidated memory phases in *Drosophila*. *Science, 304*(5673), 1024-1027.
Ito, K., Urban, J., & Technau, G. (1995). Distribution, classification, and development of *Drosophila* glial cells in the late embryonic and early larval ventral nerve cord. *Dev Genes Evol, 204*(5), 284-307.
Johard, H. A., Enell, L. E., Gustafsson, E., Trifilieff, P., Veenstra, J. A., & Nässel, D. R. (2008). Intrinsic neurons of *Drosophila* mushroom bodies express short neuropeptide F: relations to extrinsic neurons expressing different neurotransmitters. *J Comp Neurol, 507*(4), 1479-1496.
Joiner, M. A., & Griffith, L. C. (2000). Visual input regulates circuit configuration in courtship conditioning of *Drosophila melanogaster*. *Learn Mem, 7*(1), 32-42.
Joiner, W. J., Crocker, A., White, B. H., & Sehgal, A. (2006). Sleep in *Drosophila* is regulated by adult mushroom bodies. *Nature, 441*(7094), 757-760.
Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., et al. (2001). Differential expression and function of the *Drosophila Pax6* genes *eyeless* and *twin of eyeless* in embryonic central nervous system development. *Mech Dev, 103*(1-2), 71-78.
Kaun, K. R., Hendel, T., Gerber, B., & Sokolowski, M. B. (2007). Natural variation in *Drosophila* larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn Mem, 14*(5), 342-349.
Keleman, K., Kruttner, S., Alenius, M., & Dickson, B. J. (2007). Function of the *Drosophila* CPEB protein Orb2 in long-term courtship memory. *Nat Neurosci, 10*(12), 1587-1593.
Kido, A., & Ito, K. (2002). Mushroom bodies are not required for courtship behavior by normal and sexually mosaic *Drosophila*. *J Neurobiol, 52*(4), 302-311.
Kim, Y. C., Lee, H. G., & Han, K. A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. *J Neurosci, 27*(29), 7640-7647.
Kim, Y. C., Lee, H. G., Seong, C. S., & Han, K. A. (2003). Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of *Drosophila melanogaster*. *Gene Expr Patterns, 3*(2), 237-245.
Kitamoto, T. (2002). Conditional disruption of synaptic transmission induces male-male courtship behavior in Drosophila. *Proc Natl Acad Sci U S A, 99*(20), 13232-13237.
Kobayashi, M., Michaut, L., Ino, A., Honjo, K., Nakajima, T., Maruyama, Y., et al. (2006). Differential microarray analysis of *Drosophila* mushroom body transcripts using chemical ablation. *Proc Natl Acad Sci U S A, 103*(39), 14417-14422.
Komiyama, T., Sweeney, L. B., Schuldiner, O., Garcia, K. C., & Luo, L. (2007). Graded expression of semaphorin-1a cell-autonomously directs dendritic targeting of olfactory projection neurons. *Cell, 128*(2), 399-410.
Koushika, S. P., Lisbin, M. J., & White, K. (1996). ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. *Curr Biol, 6*(12), 1634-1641.
Koushika, S. P., Soller, M., & White, K. (2000). The neuron-enriched splicing pattern of *Drosophila erect wing* is dependent on the presence of ELAV protein. *Mol Cell Biol, 20*(5), 1836-1845.
Kraft, R., Levine, R. B., & Restifo, L. L. (1998). The steroid hormone 20-hydroxyecdysone enhances neurite growth of *Drosophila* mushroom body neurons isolated during metamorphosis. *J Neurosci, 18*(21), 8886-8899.
Krashes, M. J., Keene, A. C., Leung, B., Armstrong, J. D., & Waddell, S. (2007). Sequential use of mushroom body neuron subsets during *Drosophila* odor memory processing. *Neuron, 53*(1), 103-115.
Krashes, M. J., & Waddell, S. (2008). Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in *Drosophila*. *J Neurosci, 28*(12), 3103-3113.
Kurusu, M., Awasaki, T., Masuda-Nakagawa, L. M., Kawauchi, H., Ito, K., & Furukubo-Tokunaga, K. (2002). Embryonic and larval development of the *Drosophila* mushroom bodies: concentric layer subdivisions and the role of fasciclin II. *Development, 129*(2), 409-419.
Lee, H., Stultz, B. G., & Hursh, D. A. (2007). The Zic family member, *odd-paired*, regulates the *Drosophila* BMP, decapentaplegic, during adult head development. *Development, 134*(7), 1301-1310.
Lee, T., Lee, A., & Luo, L. (1999). Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development, 126*(18), 4065-4076.
Lee, T., Marticke, S., Sung, C., Robinow, S., & Luo, L. (2000). Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron, 28*(3), 807-818.
Lin, H. H., Lai, J. S., Chin, A. L., Chen, Y. C., & Chiang, A. S. (2007). A map of olfactory representation in the *Drosophila* mushroom body. *Cell, 128*(6), 1205-1217.
Liu, X., Krause, W. C., & Davis, R. L. (2007). GABAA receptor RDL inhibits *Drosophila* olfactory associative learning. *Neuron, 56*(6), 1090-1102.
Liu, Z., Steward, R., & Luo, L. (2000). Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration and axonal transport. *Nat Cell Biol, 2*(11), 776-783.
Luo, L., Lee, T., Nardine, T., Null, B., & Reuter, J. (1999). Using the MARCM system to positively mark mosaic clones in *Drosophila*. *Dros Inf Serv, 82*, 102-105.

Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C., & Baker, B. S. (2005). Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour. *Nature, 436*(7049), 395-400.
Manseau, L., Baradaran, A., Brower, D., Budhu, A., Elefant, F., Phan, H., et al. (1997). GAL4 enhancer traps expressed in the embryo, larval brain, imaginal discs, and ovary of *Drosophila*. *Dev Dyn, 209*(3), 310-322.
Martin, J. R., Rogers, K. L., Chagneau, C., & Brulet, P. (2007). In vivo bioluminescence imaging of Ca signalling in the brain of *Drosophila*. *PLoS ONE, 2*(3), e275.
Martini, S. R., Roman, G., Meuser, S., Mardon, G., & Davis, R. L. (2000). The retinal determination gene, *dachshund*, is required for mushroom body cell differentiation. *Development, 127*(12), 2663-2672.
Masuda-Nakagawa, L. M., Tanaka, N. K., & O'Kane, C. J. (2005). Stereotypic and random patterns of connectivity in the larval mushroom body calyx of Drosophila. *Proc Natl Acad Sci U S A, 102*(52), 19027-19032.
McBride, S. M., Giuliani, G., Choi, C., Krause, P., Correale, D., Watson, K., et al. (1999). Mushroom body ablation impairs short-term memory and long-term memory of courtship conditioning in *Drosophila melanogaster*. *Neuron, 24*(4), 967-977.
McGuire, S. E., Le, P. T., & Davis, R. L. (2001). The role of *Drosophila* mushroom body signaling in olfactory memory. *Science, 293*(5533), 1330-1333.
McGuire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K., & Davis, R. L. (2003). Spatiotemporal rescue of memory dysfunction in *Drosophila*. *Science, 302*(5651), 1765-1768.
Mehren, J. E., & Griffith, L. C. (2004). Calcium-independent calcium/calmodulin-dependent protein kinase II in the adult *Drosophila* CNS enhances the training of pheromonal cues. *J Neurosci, 24*(47), 10584-10593.
Mery, F., Belay, A. T., So, A. K., Sokolowski, M. B., & Kawecki, T. J. (2007). Natural polymorphism affecting learning and memory in *Drosophila*. *Proc Natl Acad Sci U S A, 104*(32), 13051-13055.
Michel, C. I., Kraft, R., & Restifo, L. L. (2004). Defective neuronal development in the mushroom bodies of *Drosophila fragile X mental retardation 1* mutants. *J Neurosci, 24*(25), 5798-5809.
Moreau-Fauvarque, C., Taillebourg, E., Boissoneau, E., Mesnard, J., & Dura, J. M. (1998). The receptor tyrosine kinase gene *linotte* is required for neuronal pathway selection in the *Drosophila* mushroom bodies. *Mech Dev, 78*(1-2), 47-61.
Ng, J., & Luo, L. (2004). Rho GTPases regulate axon growth through convergent and divergent signaling pathways. *Neuron, 44*(5), 779-793.
Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., et al. (2002). Rac GTPases control axon growth, guidance and branching. *Nature, 416*(6879), 442-447.
Nicolai, M., Lasbleiz, C., & Dura, J. M. (2003). Gain-of-function screen identifies a role of the Src64 oncogene in *Drosophila* mushroom body development. *J Neurobiol, 57*(3), 291-302.
Niimi, T., Clements, J., Gehring, W. J., & Callaerts, P. (2002). Dominant-negative form of the *Pax6* homolog *eyeless* for tissue-specific loss-of-function studies in the developing eye and brain in *Drosophila*. *Genesis, 34*(1-2), 74-75.
Nishimura, I., Sakoda, J. Y., & Yoshikawa, K. (2008). *Drosophila* MAGE controls neural precursor proliferation in postembryonic neurogenesis. *Neuroscience, 154*(2), 572-581.
Nitz, D. A., van Swinderen, B., Tononi, G., & Greenspan, R. J. (2002). Electrophysiological correlates of rest and activity in *Drosophila melanogaster*. *Curr Biol, 12*(22), 1934-1940.
Noveen, A., Daniel, A., & Hartenstein, V. (2000). Early development of the *Drosophila* mushroom body: the roles of eyeless and dachshund. *Development, 127*(16), 3475-3488.
O'Dell, K. M., Armstrong, J. D., Yang, M. Y., & Kaiser, K. (1995). Functional dissection of the *Drosophila* mushroom bodies by selective feminization of genetically defined subcompartments. *Neuron, 15*(1), 55-61.
Orihara-Ono, M., Suzuki, E., Saito, M., Yoda, Y., Aigaki, T., & Hama, C. (2005). The *slender lobes* gene, identified by retarded mushroom body development, is required for proper nucleolar organization in *Drosophila*. *Dev Biol, 281*(1), 121-133.
Pan, L., Zhang, Y. Q., Woodruff, E., & Broadie, K. (2004). The *Drosophila fragile X* gene negatively regulates neuronal elaboration and synaptic differentiation. *Curr Biol, 14*(20), 1863-1870.
Parker, C. G., Fessler, L. I., Nelson, R. E., & Fessler, J. H. (1995). *Drosophila* UDP-glucose:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denatured and native proteins. *EMBO J, 14*(7), 1294-1303.
Pascual, A., Huang, K. L., & Preat, T. (2005). Conditional UAS-targeted repression in *Drosophila*. *Nucleic Acids Res, 33*(1), e7.
Pascual, A., & Preat, T. (2001). Localization of long-term memory within the *Drosophila* mushroom body. *Science, 294*(5544), 1115-1117.
Peng, Y., & Guo, A. (2007). Novel stimulus-induced calcium efflux in *Drosophila* mushroom bodies. *Eur J Neurosci, 25*(7), 2034-2044.
Peng, Y., Xi, W., Zhang, W., Zhang, K., & Guo, A. (2007). Experience improves feature extraction in *Drosophila*. *J Neurosci, 27*(19), 5139-5145.
Pitman, J. L., McGill, J. J., Keegan, K. P., & Allada, R. (2006). A dynamic role for the mushroom bodies in promoting sleep in *Drosophila*. *Nature, 441*(7094), 753-756.
Plaza, S., Prince, F., Jaeger, J., Kloter, U., Flister, S., Benassayag, C., et al. (2001). Molecular basis for the inhibition of *Drosophila* eye development by Antennapedia. *EMBO J, 20*(4), 802-811.
Presente, A., Boyles, R. S., Serway, C. N., de Belle, J. S., & Andres, A. J. (2004). Notch is required for long-term memory in *Drosophila*. *Proc Natl Acad Sci U S A, 101*(6), 1764-1768.

Qiu, Y., & Davis, R. L. (1993). Genetic dissection of the learning/memory gene *dunce* of *Drosophila melanogaster. Genes Dev, 7*(7B), 1447-1458.
Ramaekers, A., Magnenat, E., Marin, E. C., Gendre, N., Jefferis, G. S., Luo, L., et al. (2005). Glomerular Maps without Cellular Redundancy at Successive Levels of the *Drosophila* Larval Olfactory Circuit. *Curr Biol, 15*(11), 982-992.
Reeve, S. P., Bassetto, L., Genova, G. K., Kleyner, Y., Leyssen, M., Jackson, F. R., et al. (2005). The Drosophila fragile X mental retardation protein controls actin dynamics by directly regulating profilin in the brain. *Curr Biol, 15*(12), 1156-1163.
Rodan, A. R., Kiger, J. A., Jr., & Heberlein, U. (2002). Functional dissection of neuroanatomical loci regulating ethanol sensitivity in *Drosophila*. *J Neurosci, 22*(21), 9490-9501.
Rosay, P., Armstrong, J. D., Wang, Z., & Kaiser, K. (2001). Synchronized neural activity in the *Drosophila* memory centers and its modulation by amnesiac. *Neuron, 30*(3), 759-770.
Sakai, T., & Kitamoto, T. (2006). Differential roles of two major brain structures, mushroom bodies and central complex, for *Drosophila* male courtship behavior. *J Neurobiol, 66*(8), 821-834.
Schneeberger, D., & Raabe, T. (2003). Mbt, a *Drosophila* PAK protein, combines with Cdc42 to regulate photoreceptor cell morphogenesis. *Development, 130*(3), 427-437.
Schuldiner, O., Berdnik, D., Levy, J. M., Wu, J. S., Luginbuhl, D., Gontang, A. C., et al. (2008). piggyBac-based mosaic screen identifies a postmitotic function for cohesin in regulating developmental axon pruning. *Dev Cell, 14*(2), 227-238.
Schulz, R. A., Chromey, C., Lu, M. F., Zhao, B., & Olson, E. N. (1996). Expression of the D-MEF2 transcription in the *Drosophila* brain suggests a role in neuronal cell differentiation. *Oncogene, 12*(8), 1827-1831.
Schwaerzel, M., Heisenberg, M., & Zars, T. (2002). Extinction antagonizes olfactory memory at the subcellular level. *Neuron, 35*(5), 951-960.
Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., & Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J Neurosci, 23*(33), 10495-10502.
Scott, E. K., Lee, T., & Luo, L. (2001). enok encodes a *Drosophila* putative histone acetyltransferase required for mushroom body neuroblast proliferation. *Curr Biol, 11*(2), 99-104.
Shi, L., Lin, S., Grinberg, Y., Beck, Y., Grozinger, C. M., Robinson, G. E., et al. (2007). Roles of *Drosophila* Krüppel-homolog 1 in neuronal morphogenesis. *Dev Neurobiol, 67*(12), 1614-1626.
Soller, M., Haussmann, I. U., Hollmann, M., Choffat, Y., White, K., Kubli, E., et al. (2006). Sex-peptide-regulated female sexual behavior requires a subset of ascending ventral nerve cord neurons. *Curr Biol, 16*(18), 1771-1782.
Su, H., & O'Dowd, D. K. (2003). Fast synaptic currents in *Drosophila* mushroom body Kenyon cells are mediated by alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors and picrotoxin-sensitive GABA receptors. *J Neurosci, 23*(27), 9246-9253.
Suh, G. S., Wong, A. M., Hergarden, A. C., Wang, J. W., Simon, A. F., Benzer, S., et al. (2004). A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature, 431*(7010), 854-859.
Tanaka, N. K., Awasaki, T., Shimada, T., & Ito, K. (2004). Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr Biol, 14*(6), 449-457.
Tanaka, N. K., Tanimoto, H., & Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. *J Comp Neurol, 508*(5), 711-755.
Tettamanti, M., Armstrong, J. D., Endo, K., Yang, M. Y., Furukubo-Tokunaga, K., Kaiser, K., et al. (1997). Early development of the *Drosophila* mushroom bodies, brain centres for associative learning and memory. *Development Genes and Evolution, 207*(4), 242-252.
Thum, A. S., Jenett, A., Ito, K., Heisenberg, M., & Tanimoto, H. (2007). Multiple memory traces for olfactory reward learning in *Drosophila*. *J Neurosci, 27*(41), 11132-11138.
Verkhusha, V. V., Otsuna, H., Awasaki, T., Oda, H., Tsukita, S., & Ito, K. (2001). An enhanced mutant of red fluorescent protein DsRed for double labeling and developmental timer of neural fiber bundle formation. *J Biol Chem, 276*(32), 29621-29624.
Villella, A., Ferri, S. L., Krystal, J. D., & Hall, J. C. (2005). Functional analysis of *fruitless* gene expression by transgenic manipulations of *Drosophila* courtship. *Proc Natl Acad Sci U S A, 102*(46), 16550-16557.
Waddell, S., Armstrong, J. D., Kitamoto, T., Kaiser, K., & Quinn, W. G. (2000). The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell, 103*(5), 805-813.
Walker, J. A., Tchoudakova, A. V., McKenney, P. T., Brill, S., Wu, D., Cowley, G. S., et al. (2006). Reduced growth of *Drosophila neurofibromatosis 1* mutants reflects a non-cell-autonomous requirement for GTPase-Activating Protein activity in larval neurons. *Genes Dev, 20*(23), 3311-3323.
Wang, J., Lee, C. H., Lin, S., & Lee, T. (2006). Steroid hormone-dependent transformation of polyhomeotic mutant neurons in the *Drosophila* brain. *Development, 133*(7), 1231-1240.
Wang, J., Ma, X., Yang, J. S., Zheng, X., Zugates, C. T., Lee, C. H., et al. (2004). Transmembrane/juxtamembrane domain-dependent Dscam distribution and function during mushroom body neuronal morphogenesis. *Neuron, 43*(5), 663-672.
Wang, J., Zugates, C. T., Liang, I. H., Lee, C. H., & Lee, T. (2002). *Drosophila* Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron, 33*(4), 559-571.
Wang, Y., Chiang, A. S., Xia, S., Kitamoto, T., Tully, T., & Zhong, Y. (2003). Blockade of neurotransmission in *Drosophila* mushroom bodies impairs odor attraction, but not repulsion. *Curr Biol, 13*(21), 1900-1904.

Wang, Y., Guo, H. F., Pologruto, T. A., Hannan, F., Hakker, I., Svoboda, K., et al. (2004). Stereotyped odor-evoked activity in the mushroom body of *Drosophila* revealed by green fluorescent protein-based Ca2+ imaging. *J Neurosci, 24*(29), 6507-6514.
Wang, Y., Mamiya, A., Chiang, A. S., & Zhong, Y. (2008). Imaging of an early memory trace in the *Drosophila* mushroom body. *J Neurosci, 28*(17), 4368-4376.
Watts, R. J., Hoopfer, E. D., & Luo, L. (2003). Axon pruning during *Drosophila* metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron, 38*(6), 871-885.
Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C., & Luo, L. (2004). Glia engulf degenerating axons during developmental axon pruning. *Curr Biol, 14*(8), 678-684.
Whited, J. L., Robichaux, M. B., Yang, J. C., & Garrity, P. A. (2007). Ptpmeg is required for the proper establishment and maintenance of axon projections in the central brain of *Drosophila*. *Development, 134*(1), 43-53.
Wilson, R. I., & Laurent, G. (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *J Neurosci, 25*(40), 9069-9079.
Wojtowicz, W. M., Flanagan, J. J., Millard, S. S., Zipursky, S. L., & Clemens, J. C. (2004). Alternative splicing of *Drosophila Dscam* generates axon guidance receptors that exhibit isoform-specific homophilic binding. *Cell, 118*(5), 619-633.
Wu, C. L., Xia, S., Fu, T. F., Wang, H., Chen, Y. H., Leong, D., et al. (2007). Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila* ellipsoid body. *Nat Neurosci, 10*(12), 1578-1586.
Xi, W., Peng, Y., Guo, J., Ye, Y., Zhang, K., Yu, F., et al. (2008). Mushroom bodies modulate salience-based selective fixation behavior in *Drosophila*. *Eur J Neurosci, 27*(6), 1441-1451.
Yang, M. Y., Armstrong, J. D., Vilinsky, I., Strausfeld, N. J., & Kaiser, K. (1995). Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. *Neuron, 15*(1), 45-54.
Yang, Z., Edenberg, H. J., & Davis, R. L. (2005). Isolation of mRNA from specific tissues of *Drosophila* by mRNA tagging. *Nucleic Acids Res, 33*(17), e148.
Yeh, E., Gustafson, K., & Boulianne, G. L. (1995). Green fluorescent protein as a vital marker and reporter of gene expression in *Drosophila*. *Proc Natl Acad Sci U S A, 92*(15), 7036-7040.
Yoshihara, M., & Ito, K. (2000). Improved Gal4 screening kit for large-scale generation of enhancer-trap strains. *Dros Inf Serv, 83*, 199-202.
Yu, D., Baird, G. S., Tsien, R. Y., & Davis, R. L. (2003). Detection of calcium transients in *Drosophila* mushroom body neurons with camgaroo reporters. *J Neurosci, 23*(1), 64-72.
Zars, T., Fischer, M., Schulz, R., & Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. *Science, 288*(5466), 672-675.
Zars, T., Wolf, R., Davis, R., & Heisenberg, M. (2000). Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. *Learn Mem, 7*(1), 18-31.
Zelhof, A. C., & Hardy, R. W. (2004). WASp is required for the correct temporal morphogenesis of rhabdomere microvilli. *J Cell Biol, 164*(3), 417-426.
Zhang, K., Guo, J. Z., Peng, Y., Xi, W., & Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. *Science, 316*(5833), 1901-1904.
Zheng, X., Wang, J., Haerry, T. E., Wu, A. Y., Martin, J., O'Connor, M. B., et al. (2003). TGF-beta signaling activates steroid hormone receptor expression during neuronal remodeling in the *Drosophila* brain. *Cell, 112*(3), 303-315.
Zheng, X., Zugates, C. T., Lu, Z., Shi, L., Bai, J. M., & Lee, T. (2006). Baboon/dSmad2 TGF-beta signaling is required during late larval stage for development of adult-specific neurons. *EMBO J, 25*(3), 615-627.
Zhu, S., Chiang, A. S., & Lee, T. (2003). Development of the *Drosophila* mushroom bodies: elaboration, remodeling and spatial organization of dendrites in the calyx. *Development, 130*(12), 2603-2610.
Zhu, S., Lin, S., Kao, C. F., Awasaki, T., Chiang, A. S., & Lee, T. (2006). Gradients of the *Drosophila* Chinmo BTB-zinc finger protein govern neuronal temporal identity. *Cell, 127*(2), 409-422.
Zhu, S., Perez, R., Pan, M., & Lee, T. (2005). Requirement of Cul3 for axonal arborization and dendritic elaboration in *Drosophila* mushroom body neurons. *J Neurosci, 25*(16), 4189-4197.

4. **Chapter II**

Specific dopaminergic neurons for the formation of labile aversive memory

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Running title: Dopamine neurons for aversive reinforcement

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Research highlights:

- Thermo-genetic stimulation/suppression of specific sets of dopaminergic neurons
- MB-M3 is preferentially required for labile olfactory memory
- Local stimulation in the mushroom body is sufficient for aversive memory formation
- Multiple types of dopaminergic neurons mediate aversive reinforcement

Summary

A paired presentation of an odor and electric shock induces aversive odor memory in *Drosophila melanogaster* (Tully and Quinn 1985; McGuire, Deshazer et al. 2005). Electric shock reinforcement is mediated by dopaminergic neurons (Schwaerzel, Monastirioti et al. 2003; Schroll, Riemensperger et al. 2006; Vergoz, Roussel et al. 2007), and it converges with the odor signal in the mushroom body (MB) (Heisenberg 2003; Gerber, Tanimoto et al. 2004; McGuire, Deshazer et al. 2005; Keene and Waddell 2007). Dopamine is synthesized in ~280 neurons that form distinct cell clusters (Nässel and Elekes 1992; Friggi-Grelin, Coulom et al. 2003; Mao and Davis 2009) and is involved in a variety of brain functions (Friggi-Grelin, Coulom et al. 2003; Riemensperger, Voller et al. 2005; Zhang, Guo et al. 2007; Andretic, Kim et al. 2008; Seugnet, Suzuki et al. 2008; Zhang, Yin et al. 2008; Krashes, DasGupta et al. 2009; Lebestky, Chang et al. 2009; Liu, Dartevelle et al. 2009; Selcho, Pauls et al. 2009). Recently, one of the dopaminergic clusters (PPL1) that includes MB-projecting neurons was shown to signal reinforcement for aversive odor memory (Claridge-Chang, Roorda et al. 2009). As each dopaminergic cluster contains multiple types of neurons with different projections and physiological characteristics (Krashes, DasGupta et al. 2009; Mao and Davis 2009), functional understanding of the circuit for aversive memory requires cellular identification. We here show that MB-M3, a specific type of dopaminergic neurons in cluster PAM, is preferentially required for the formation of labile memory. Strikingly, flies formed significant aversive odor memory without electric shock, when MB-M3 was selectively stimulated together with odor presentation. We additionally identified another type of dopaminergic neurons in the PPL1 cluster, MB-MP1, which can induce aversive odor memory. As MB-M3 and MB-MP1 target the distinct subdomains of the MB, these reinforcement circuits might

induce different forms of aversive memory in spatially segregated synapses in the MB.

Results and Discussion

Dopaminergic neurons that project to the mushroom body

To functionally manipulate restricted neurons for the induction of aversive odor memory, we searched for GAL4 expression drivers that label specific subsets of the MB-projecting dopaminergic neurons. A recent anatomical study using GAL4 drivers systematically described the neurons connecting the MB and other brain regions (Tanaka, Tanimoto et al. 2008). By immunolabeling of tyrosine hydroxylase (TH) (Neckameyer, Woodrome et al. 2000), an enzyme required for dopamine biosynthesis, we found at least five different types of MB-projecting dopaminergic neurons distributed to two clusters: MB-M3 and MB-MVP1 in the PAM cluster, and MB-V1, MB-MV1 and MB-MP1 in the PPL1 cluster (see Table S2 for the summary). Their terminals in the mushroom body (MB) are restricted in distinct subdomains. As different types of the major MB intrinsic neurons Kenyon cells have their own roles in dynamics of short- (Zars 2000; Isabel, Pascual et al. 2004; Akalal, Wilson et al. 2006) and long-lasting (Isabel, Pascual et al. 2004; Blum, Li et al. 2009) odor memories, these dopaminergic neurons may signal different forms of reinforcement. In contrast, MB-M4, MB-V2, MB-V3, MB-V4, MB-CP1, MB-MV2, and MB-MVP2 were not labeled by the anti-TH antibody (data not shown). Based on the specificity of GAL4 drivers, we started our behavioral analysis with a specific type of dopaminergic neurons: MB-M3 (Figure 1A).

Two independent drivers, *NP1528* and *NP5272*, selectively label three dopaminergic MB-M3 neurons per brain hemisphere on average (Figure 1A-D). They

are labeled also in the *TH-GAL4* driver (Figure 1C)(Tanaka, Tanimoto et al. 2008), which covers many more dopaminergic neurons (total ca. 130 cells in the brain) including at least six and two types of neurons projecting to the lobes and calyx of the MB, respectively (Figure 1D) (Friggi-Grelin, Coulom et al. 2003; Mao and Davis 2009). Presynaptic sites of MB-M3 are preferentially localized in the distal tip of the β lobe (β_{s2}) and sparsely in the limited region of the distal β' lobe (Figure 1B; see (Tanaka, Tanimoto et al. 2008) for nomenclature), suggesting that they receive input from the anterior and middle inferior medial protocerebrum and give output in these subdomains of the MB lobes.

Requirement of MB-M3 output for shock-induced memory

To address the role of the three MB-M3 neurons in aversive reinforcement of electric shock, we blocked output of these neurons by expressing Shi^{ts1}, a dominant negative temperature sensitive variant of Dynamin that blocks synaptic vesicle endocytosis at high temperature (Kitamoto 2001). Blocking not only many types of dopaminergic neurons but also MB-M3 neurons alone impaired aversive memory tested at 30 min after conditioning (Figure 1E). Notably, the effect of blocking MB-M3 on aversive odor memory was significant, but less pronounced than that with *TH-GAL4*. Consistent with the previous report (Schwaerzel, Monastirioti et al. 2003), blocking MB-M3 and other dopaminergic neurons did not significantly affect reflexive avoidance of the electric shock (Table S1). We then contrasted aversive and appetitive memory with the same odorants, because the requirement of the reinforcement circuit should be selective. As expected, appetitive odor memory induced by sugar was not disturbed (Figure 1F), suggesting that odor discrimination

and locomotion of these flies that are required for the task should not be affected significantly under the blockade of GAL4-expressing cells.

We next asked whether the output of the MB-M3 neurons is required specifically during memory formation by blocking them only transiently during training. We found significant impairment of 30 min-memory by the transient block of MB-M3 (Figure 1G). By contrast, the block after the training period (i.e. during the retention interval and the test period) and the experiment at continuous permissive temperature did not significantly impair odor memory (Figure S1). These results together indicate that the phenotype is attributed to the impairment of memory formation rather than the memory retention, retrieval, or the effect of the genetic background.

Aversive reinforcement for a distinct memory component

Given the partial requirement of MB-M3 for 30 min memory (Figure 1E and 1G), we hypothesized that the output of MB-M3 may be responsible for a specific memory component. We blocked MB-M3 or neurons labeled in *TH-GAL4* during training and examined memory retention up to 9 hours. Blocking with *TH-GAL4* significantly impaired aversive odor memory at all time points (Figure 2A). Intriguingly, the effect of the MB-M3 block was most pronounced in the middle-term memory (2 h after training); memory tested immediately and 9 h after training was only slightly impaired, if any. The dynamics of memory decay was different from the block with *TH-GAL4* and control groups (Figure 2A). This result is consistent with the previous report showing that immediate memory is not affected by blocking many of PAM cluster neurons (Claridge-Chang, Roorda et al. 2009).

What type of memory is impaired by blocking the MB-M3? Initially labile odor memory in *Drosophila* is consolidated gradually and becomes resistant to retrograde amnesia (Quinn and Dudai 1976; Folkers, Drain et al. 1993). At two hours after training, labile anesthesia-sensitive memory (ASM) and consolidated anesthesia-resistant memory (ARM) coexist (Quinn and Dudai 1976; Folkers, Drain et al. 1993). ARM can be measured by erasing ASM with short cold anesthesia of flies. Manipulation of various signaling molecules, such as Amnesiac, AKAP, DC0, Rac or NMDAR (Tully and Quinn 1985; Li, Tully et al. 1996; Dubnau and Tully 1998; Schwaerzel, Jaeckel et al. 2007; Wu, Xia et al. 2007; Shuai, Lu et al. 2010) (but see (Horiuchi, Yamazaki et al. 2008) for the role of DC0 for ARM), affects ASM and causes memory dynamics similar to that caused by the MB-M3 block (Figure 2A).

To address whether the MB-M3 neurons are required selectively for ASM, we trained flies with their MB-M3 neurons blocked and, two hours later, measured their total memory and ARM. The output of MB-M3 during training was required for the total 2 h memory, whereas ARM was not significantly affected (Figure 2B). This suggests that the MB-M3 neurons preferentially contribute to the formation of ASM. In contrast, the block with *TH-GAL4* significantly impaired both total memory and ARM (Figure 2B). Although the scores of ARM were small, subtle differences in ARM were detectable, because unpaired conditioning resulted in the significantly lower memory in every genotype (Figure 2B). Taken together, these results imply that multiple types of reinforcement neurons are recruited for the formation of different forms of memory.

Aversive odor memory formed by the activation of MB-M3

We next examined whether selective stimulation of the MB-M3 neurons can induce aversive odor memory without electric shock. *Drosophila* heat-activated cation channel dTRPA1 (also known as dANKTM1), allows transient depolarization of targeted neurons by raising temperature (Viswanath, Story et al. 2003; Hamada, Rosenzweig et al. 2008). To selectively activate the corresponding dopaminergic neurons, flies that express *dTrpA1* by the above-described GAL4 drivers were trained with odor presentation and a concomitant temperature shift instead of electric shock (Figure 3A). To minimize the noxious effect of heat itself, we used moderate temperature (30°C) for activation. When examined immediately (approximately 2 min) after training, robust aversive memory was formed by the activation with *TH-GAL4* (Figure 3B). Strikingly, selective activation of MB-M3 also caused significant aversive odor memory (Figure 3B). Unpaired presentation of an odor and dTRPA1-dependent activation did not cause significant associative memory (Figure 3C), indicating the importance of stimulus contingency.

Although the drivers for MB-M3 have a selective expression pattern, *NP5272* and *NP1528* have additional faint labeling of nerves in the abdominal ganglion and, only occasionally, other neurons in the brain (Figures 3F and S2A). To confirm that the activation of MB-M3 neurons was the cause of dTRPA1-induced odor memory, we expressed a GAL4 inhibitor, GAL80, in dopaminergic neurons using *TH-GAL80* (Sitaraman, Zars et al. 2008). Indeed, *TH-GAL80* suppressed reporter expression in dopaminergic neurons in *NP5272* and *TH-GAL4* (Figure 3D-G) and dTRPA1-induced odor memory to the control level (Figure 3H). These data suggest that selective activation of MB-M3 can induce immediate aversive memory, while blocking MB-M3 had a limited effect on immediate shock-induced memory (Figure 2A). This may suggest that the contribution of MB-M3 is redundant with other dopaminergic neurons

in shock-induced immediate memory. Alternatively, the activation of MB-M3 by dTrpA1 might not fully recapitulate that in electric shock conditioning in terms of a temporal pattern and intensity.

We also measured dTRPA1-induced memory at 2 hours after training. With *TH-GAL4* aversive memory was still significant, whereas the memory induced with MB-M3 activation diminished by 2 hours, indicating the labile nature of the memory (Figure 3H). Given the selective requirement of MB-M3 (Figure 2A), the contribution of MB-M3 to 2-hour memory might be interdependent with other dopaminerigic neurons. A similar interaction has been shown at the level of different subsets of Kenyon cells (Zars, Fischer et al. 2000; Akalal, Wilson et al. 2006; Krashes, Keene et al. 2007; Blum, Li et al. 2009; Shuai, Lu et al. 2010) and might thus be a potential consequence of the synergistic action of dopaminergic neurons.

Other individual dopaminergic neurons for aversive reinforcement

To explore the function of other types of MB-projecting dopaminergic neurons for aversive memory formation, we individually stimulated 4 different cell types (MB-MP1, MB-V1, MB-MVP1 and unnamed type that project to the β' lobe) using selective GAL4 driver lines (*NP2758*, *c061*; *MB-GAL80*, *MZ840* and *NP6510*; Figures 4 and S2; see Table S2 for the summary of labeled neurons).

c061; MB-GAL80 labels three dopaminergic neurons in the PPL1 cluster, including one MB-MP1 neuron that is labeled also in *NP2758* (Figures 4A, 4C, S2B, S2E and Table S2) (Krashes, DasGupta et al. 2009). Activation with *c061; MB-GAL80* induced robust immediate memory (Figures 4B). Furthermore, we found that $Cha^{3.3kb}$*-GAL80* strongly silenced reporter expression in two of three PPL1 neurons in *c061; MB-GAL80* and the effect on aversive memory to the control level (Figure S2B-

C, 4B) (Kitamoto 2002). As one remaining dopaminergic neuron projects to the anterior inferior medial protocerebrum, but not to the MB (Figure S2C)(Mao and Davis 2009), MB-MP1 is more likely to be responsible for the formation of aversive memory. This suppression of transgene expression in dopaminergic neurons might be due to the incomplete recapitulation of $Cha^{3.3kb}$ enhancer (Figure S2B-C).The addition of *TH-GAL80* also suppressed the effect of *c061; MB-GAL80* to the control level (Figure 4B). Consistently, significant memory was induced with *NP2758* (Figure 4C-D), although the suppression by *TH-GAL80* was partial (Figure 4D), presumably through expression in non-dopaminergic neurons in *NP2758* or incomplete suppression of dTRPA1 expression (Figure S2E-F). The formed memory with *c061; MB-GAL80* decayed significantly but lasted for 2 hours (Figure 4B). Taken together, these results revealed that the specific cell type within the PPL1 cluster, MB-MP1, can mediate aversive reinforcement. Intriguingly, the recent work using the same driver reported that MB-MP1 has another important role for suppressing the retrieval of appetitive memory depending on the feeding states (Krashes, DasGupta et al. 2009). As the output of MB-MP1 is dispensable for 3-hour memory induced by electric shock (Krashes, DasGupta et al. 2009), MB-MP1 might mainly induce short-lasting odor memory. Alternatively, MB-MP1 neurons might be recruited to mediate aversive reinforcement other than electric shock.

MZ840 and *NP6510* that label the single PPL1 neuron (MB-V1) and 15 PAM neurons (MB-MVP1 and unnamed cell type), respectively (Fig. 4E, G, S2G-J). Thermo-activation with these drivers did not cause significant memory (Figures 4E-H and S2G-J). This may indicate PAM and PPL1 cluster contain functionally heterogeneous population in terms of aversive reinforcement signals. Consistently, each type of PPL1 neurons differentially responds to odors and electric shock (Mao

and Davis 2009). It is noteworthy that MB-MVP1 synapse onto the restricted subdomains adjacent to the terminals of MB-M3. Thus, the activation of specific sets of dopaminergic neurons rather than the total amount of dopamine input in the MB may be critical for memory formation. Despite the particular importance of the dopamine signal in the vertical lobes of the MB (Mao and Davis 2009; Tomchik and Davis 2009; Gervasi, Tchenio et al. 2010), we could not examine them, except for MB-V1, due to the lack of reasonably specific GAL4 drivers.

Parallel reinforcement input to the mushroom body

In a current circuit model of aversive odor memory, associative plasticity is generated in the output site of the MB (i.e. the presynaptic terminals of Kenyon cells) upon internal convergence of neuronal signals of odor and electric shock (Heisenberg 2003; Gerber, Tanimoto et al. 2004; McGuire, Deshazer et al. 2005). Type I adenylyl cyclase, Rutabaga, is an underlying molecular coincidence detector to form a memory trace (Zars, Fischer et al. 2000; Gerber, Tanimoto et al. 2004; Tomchik and Davis 2009; Gervasi, Tchenio et al. 2010). Rutabaga in different types of Kenyon cells (e.g. γ and α/β neurons) together acts to form complete aversive memory (Zars, Fischer et al. 2000; Akalal, Wilson et al. 2006; Blum, Li et al. 2009). Thus, local, but spatially segregated, Rutabaga stimulation through multiple dopaminergic pathways may induce distinct memory traces (Yu, Akalal et al. 2006; Mao and Davis 2009; Tomchik and Davis 2009; Gervasi, Tchenio et al. 2010).

We showed the selective requirement of MB-M3 for middle-term ASM, whereas blocking many more dopaminergic neurons impaired all memory phases examined in this study (Figure 2). Therefore, electric shock recruits a set of distinct dopaminergic neurons that forms a parallel reinforcement circuit in the subdomains of

the MB. Compartmentalized synaptic organization along the trajectory of Kenyon cell axons may well explain how the MB as a single brain structure can support "pleiotropic" behavioral functions (Isabel, Pascual et al. 2004; Riemensperger, Voller et al. 2005; Yu, Akalal et al. 2006).

EXPERIMENTAL PROCEDURES

Fly strains

All flies were raised on standard medium. For behavioral assay, F1 progenies of the crosses between females of *UAS-shi^{ts1} (X, III)*(Kitamoto 2001), *UAS-dTrpA1(II)* (Hamada, Rosenzweig et al. 2008), *UAS-dTrpA1 TH-GAL80 (II)* (Sitaraman, Zars et al. 2008), *UAS-dTrpA1; Cha$^{3.3kb}$-GAL80 (II, III)* (Kitamoto 2002) or *white* and males of *NP5272 (II)* (Tanaka, Tanimoto et al. 2008), *NP1528 (II) / CyO* (Tanaka, Tanimoto et al. 2008), *NP2758 (X)* (Tanaka, Tanimoto et al. 2008), *NP6510 (III)* (Tanaka, Tanimoto et al. 2008), *MZ840 (III)* or *TH-GAL4 (III)* (Friggi-Grelin, Coulom et al. 2003) were employed. For the experiments with *c061; MB-GAL80 (X and III),* female of this strain was used for crosses. After measurement, flies without a GAL4 driver (i.e. those with the balancer or male of *NP2758* crosses) were excluded from calculation. Accordingly, for experiments with *NP2758* (Figure 4D), only the learning indices of females were compared. For experiments with *UAS-shi^{ts1}* and *UAS-dTrpA1*, flies were raised at 18°C and 25°C, 60% relative humidity and used during 8-14 and 7-12 days after eclosion, respectively to allow sufficient accumulation of effecter genes without age-related memory impairment. For anatomical assay, F1 progenies of the crosses between females of *UAS-mCD8::GFP(X, II, III)* (Lee and Luo 1999)*, UAS-Syt::HA(X); UAS-mCD8:GFP(X, II)* (Robinson, Ranjan et al. 2002)*, UAS-mCD8::GFP(X); UAS-mCD8::GFP(III), UAS-mCD8::GFP(X); TH-GAL80(II); UAS-*

mCD8::GFP(III), *UAS-Cameleon2.1,* or *NP5272 UAS-Cameleon2.1 (II)* and males of *MZ604, MZ840*, *NP242 (III) NP2150(X), NP2297 (II), NP2492 (X), NP2583 (II), NP3212 (III), NP7251 (X)* (Tanaka, Tanimoto et al. 2008)*,* or lines used for behavioral assays were employed. The progeny of these crosses were all heterozygous for transgenes and homozygous for *white* in hybrid genetic backgrounds of original strains.

Behavioral assays

Standard protocol for olfactory conditioning with two odors (4-methylcyclohexanol and 3-octanol) was used. Flies were trained by receiving one odor (CS+) for 1 min with 12 pulses of electric shocks (90V DC) or, for appetitive learning, dried filter paper having absorbed with 2 M sucrose solution (Tully and Quinn 1985; Schwaerzel, Monastirioti et al. 2003; Thum, Jenett et al. 2007). Subsequently, they received another odor (CS-) for 1 min, but without electroshock or sugar. After a given retention time, the conditioned response of the trained flies was measured with a choice between CS+ and CS- for 2 min in a T-maze. No or a few percentage of flies was trapped in the middle compartment and did not choose either odor. A learning index (LI) was then calculated by taking the mean preference of two groups, which had been trained reciprocally in terms of the two odors used as CS+ and CS- (Tully and Quinn 1985). To cancel the effect of the order of reinforcement (Tully and Quinn 1985; Kim, Lee et al. 2007), the first odor was paired with reinforcement in a half of experiments and the second odor was paired with reinforcement in another half.

For rigorous comparison of aversive and appetitive memory (Figures 1E-F), flies were starved for 40-68 hours at 18°C (calibrated with 5-10% of the mortality rate) and treated equally with only a difference being the type of reinforcement (sugar

or electric shock). Unlike previous reports, flies for appetitive memory were trained only once for one minute instead of twice.

To measure ARM, trained flies were anesthetized by transferring them into pre-cooled tubes (on ice) for 60 seconds at 100 min after training (Figure 2B; Quinn and Dudai, 1976). For conditioning with thermo-activation by dTRPA1 (Figures 3B-C, 3H, 4B, 4D, 4F and 4H), we trained flies in the same way as electric shock conditioning except that flies were transferred to the pre-warmed T-maze in the climate box (30-31°C) only during the presentation of one of the two odorants (60 seconds). To minimize the noxious effect of heat itself, we used moderate temperature (30-31°C) for activation. This temperature shift by itself only occasionally induced small aversive odor memory that is significantly higher than the chance level (see the control groups in Figures 3 and 4). *MZ604/UAS-dTrpA1* was not tested for olfactory learning because of obvious motor dysfunction at high temperature.

Statistics

Statistical analyses were performed using Prism (GraphPad Software). Because all groups tested did not violate the assumption of the normal distribution and the homogeneity of variance, mean performance indices were compared with one-way ANOVA followed by planned multiple pair-wise comparisons (Bonferroni correction).

Immunohistochemistry

Female F1 progenies (5-10 days after eclosion at 25°C) from the crosses described above were examined. The brain and thoracicoabdominal ganglion were prepared for immunolabeling and analyzed as previously described (Thum, Jenett et al. 2007; Aso, Grubel et al. 2009). The brains were dissected in Ringer's solution,

fixed in phosphate-buffered saline containing 0.1% Triton X-100 (PBT) and 2% formaldehyde (Sigma) for 1 hour at room temperature, and subsequently rinsed with PBT three times (3x 10min). 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 employed primary antibodies were the rabbit polyclonal antibody to GFP (1:1000; Molecular Probes), mouse monoclonal antibody to the presynaptic protein Synapsin (1:20; 3C11) (Klagges, Heimbeck et al. 1996) or HA (1:200; 16B12; Covance; MMS-101P).The brains were washed with PBT for 20min three times and incubated with secondary antibodies in the blocking solution at 4°C overnight. The employed secondary antibodies were Alexa Fluor488-conjugated goat anti-rabbit (1:1000 or 1:500; Molecular Probes) or anti-mouse IgG (1000; Molecular Probes) and Cy3-conjugated goat anti-mouse (1:250; Jackson ImmunoResearch) or anti-rabbit IgG (1:1000; Molecular Probes). Finally, the brains were rinsed with PBT (3x 20 min + 1x 1h) and mounted in Vectashield (Vector), and frontal optical sections of whole-mount brains were taken with a confocal microscopy, Olympus FV1000, Leica SP1 or SP2. 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 (NIH).

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References

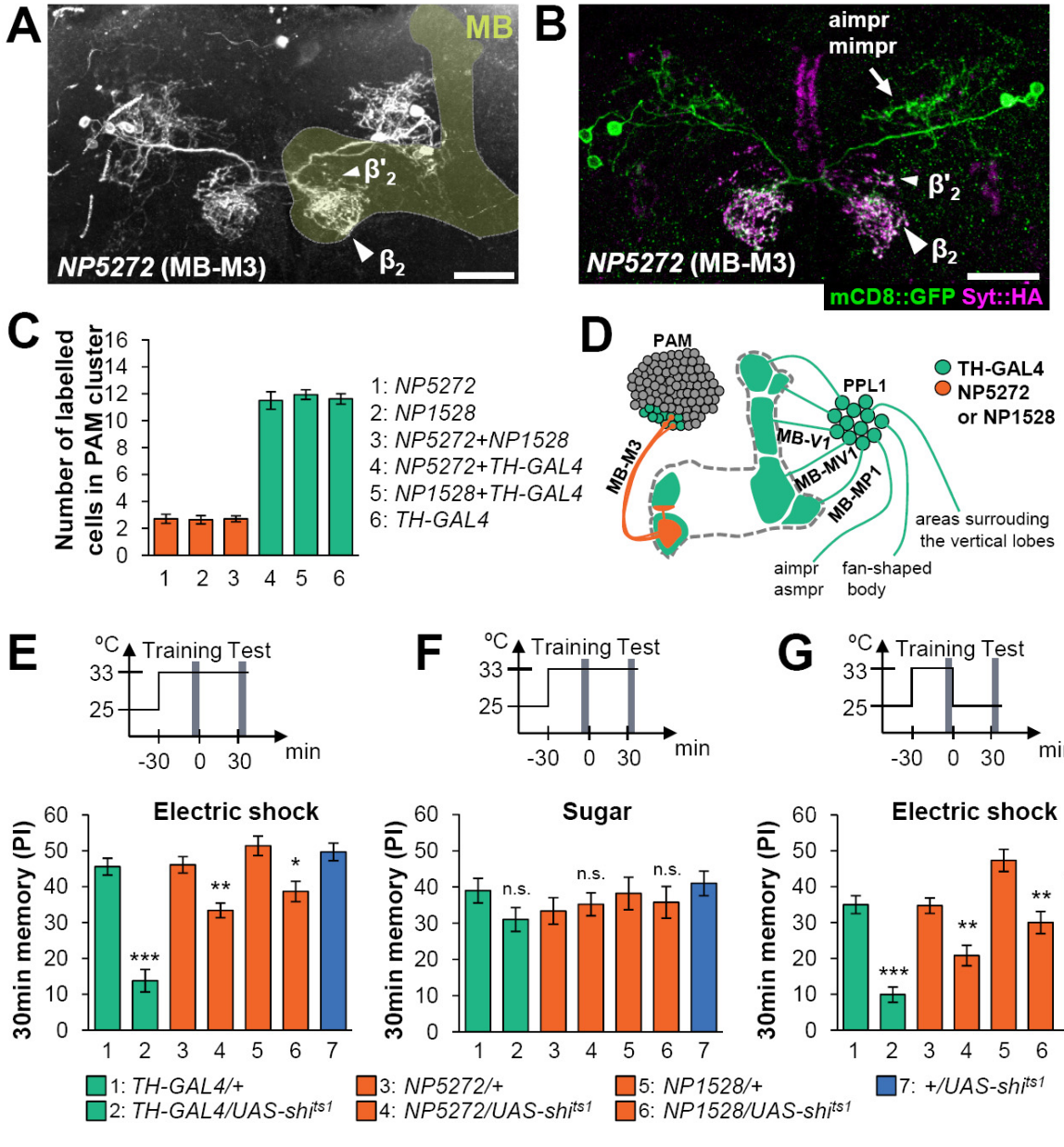
1. Tully, T., and Quinn, W.G. (1985). Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol [A] *157*, 263-277.
2. McGuire, S.E., Deshazer, M., and Davis, R.L. (2005). Thirty years of olfactory learning and memory research in *Drosophila melanogaster*. Prog Neurobiol *76*, 328-347.
3. Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci *23*, 10495-10502.
4. Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E., et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. Curr Biol *16*, 1741-1747.
5. Vergoz, V., Roussel, E., Sandoz, J.C., and Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. PLoS ONE *2*, e288.
6. Gerber, B., Tanimoto, H., and Heisenberg, M. (2004). An engram found? Evaluating the evidence from fruit flies. Curr Opin Neurobiol *14*, 737-744.
7. Heisenberg, M. (2003). Mushroom body memoir: from maps to models. Nat Rev Neurosci *4*, 266-275.
8. Keene, A.C., and Waddell, S. (2007). Drosophila olfactory memory: single genes to complex neural circuits. Nat Rev Neurosci *8*, 341-354.
9. Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J Neurobiol *54*, 618-627.
10. Nässel, D.R., and Elekes, K. (1992). Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase-immunoreactive neurons and their relationship with putative histaminergic neurons. Cell Tissue Res *267*, 147-167.
11. Mao, Z., and Davis, R.L. (2009). Eight different types of dopaminergic neurons innervate the *Drosophila* mushroom body neuropil: anatomical and physiological heterogeneity. Front Neural Circuits *3*, 5.
12. Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in Drosophila. Curr Biol *15*, 1953-1960.

13. Zhang, K., Guo, J.Z., Peng, Y., Xi, W., and Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision-making in *Drosophila*. Science *316*, 1901-1904.
14. Seugnet, L., Suzuki, Y., Vine, L., Gottschalk, L., and Shaw, P.J. (2008). D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in *Drosophila*. Curr Biol *18*, 1110-1117.
15. Zhang, S., Yin, Y., Lu, H., and Guo, A. (2008). Increased dopaminergic signaling impairs aversive olfactory memory retention in *Drosophila*. Biochem Biophys Res Commun *370*, 82-86.
16. Andretic, R., Kim, Y.C., Jones, F.S., Han, K.A., and Greenspan, R.J. (2008). *Drosophila* D1 dopamine receptor mediates caffeine-induced arousal. Proc Natl Acad Sci U S A *105*, 20392-20397.
17. Selcho, M., Pauls, D., Han, K.A., Stocker, R.F., and Thum, A.S. (2009). The role of dopamine in *Drosophila* larval classical olfactory conditioning. PLoS One *4*, e5897.
18. Lebestky, T., Chang, J.S., Dankert, H., Zelnik, L., Kim, Y.C., Han, K.A., Wolf, F.W., Perona, P., and Anderson, D.J. (2009). Two different forms of arousal in drosophila are oppositely regulated by the dopamine D1 receptor ortholog DopR via distinct neural circuits. Neuron *64*, 522-536.
19. Liu, T., Dartevelle, L., Yuan, C., Wei, H., Wang, Y., Ferveur, J.F., and Guo, A. (2009). Reduction of dopamine level enhances the attractiveness of male *Drosophila* to other males. PLoS One *4*, e4574.
20. Krashes, M.J., DasGupta, S., Vreede, A., White, B., Armstrong, J.D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in *Drosophila*. Cell *139*, 416-427.
21. Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. Cell *139*, 405-415.
22. Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. J Comp Neurol *508*, 711-755.
23. Neckameyer, W.S., Woodrome, S., Holt, B., and Mayer, A. (2000). Dopamine and senescence in Drosophila melanogaster. Neurobiol Aging *21*, 145-152.
24. Akalal, D.B., Wilson, C.F., Zong, L., Tanaka, N.K., Ito, K., and Davis, R.L. (2006). Roles for *Drosophila* mushroom body neurons in olfactory learning and memory. Learn Mem *13*, 659-668.
25. Isabel, G., Pascual, A., and Preat, T. (2004). Exclusive consolidated memory phases in Drosophila. Science *304*, 1024-1027.
26. Zars, T. (2000). Behavioral functions of the insect mushroom bodies. Curr Opin Neurobiol *10*, 790-795.
27. Blum, A.L., Li, W., Cressy, M., and Dubnau, J. (2009). Short- and long-term memory in *Drosophila* require cAMP signaling in distinct neuron types. Curr Biol *19*, 1341-1350.
28. Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol *47*, 81-92.
29. Folkers, E., Drain, P., and Quinn, W.G. (1993). Radish, a Drosophila mutant deficient in consolidated memory. Proc Natl Acad Sci U S A *90*, 8123-8127.
30. Quinn, W.G., and Dudai, Y. (1976). Memory phases in Drosophila. Nature *262*, 576-577.

31. Dubnau, J., and Tully, T. (1998). Gene discovery in Drosophila: new insights for learning and memory. Annu Rev Neurosci *21*, 407-444.
32. Schwaerzel, M., Jaeckel, A., and Mueller, U. (2007). Signaling at A-kinase anchoring proteins organizes anesthesia-sensitive memory in *Drosophila*. J Neurosci *27*, 1229-1233.
33. Wu, C.L., Xia, S., Fu, T.F., Wang, H., Chen, Y.H., Leong, D., Chiang, A.S., and Tully, T. (2007). Specific requirement of NMDA receptors for long-term memory consolidation in *Drosophila* ellipsoid body. Nat Neurosci *10*, 1578-1586.
34. Shuai, Y., Lu, B., Hu, Y., Wang, L., Sun, K., and Zhong, Y. (2010). Forgetting is regulated through Rac activity in *Drosophila*. Cell *140*, 579-589.
35. Li, W., Tully, T., and Kalderon, D. (1996). Effects of a conditional Drosophila PKA mutant on olfactory learning and memory. Learn Mem *2*, 320-333.
36. Horiuchi, J., Yamazaki, D., Naganos, S., Aigaki, T., and Saitoe, M. (2008). Protein kinase A inhibits a consolidated form of memory in *Drosophila*. Proc Natl Acad Sci U S A *105*, 20976-20981.
37. Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity, P.A. (2008). An internal thermal sensor controlling temperature preference in *Drosophila*. Nature *454*, 217-220.
38. Viswanath, V., Story, G.M., Peier, A.M., Petrus, M.J., Lee, V.M., Hwang, S.W., Patapoutian, A., and Jegla, T. (2003). Opposite thermosensor in fruitfly and mouse. Nature *423*, 822-823.
39. Sitaraman, D., Zars, M., Laferriere, H., Chen, Y.C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G.E., and Zars, T. (2008). Serotonin is necessary for place memory in *Drosophila*. Proc Natl Acad Sci U S A *105*, 5579-5584.
40. Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. Science *288*, 672-675.
41. Krashes, M.J., Keene, A.C., Leung, B., Armstrong, J.D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during drosophila odor memory processing. Neuron *53*, 103-115.
42. Kitamoto, T. (2002). Conditional disruption of synaptic transmission induces male-male courtship behavior in Drosophila. Proc Natl Acad Sci U S A *99*, 13232-13237.
43. Tomchik, S.M., and Davis, R.L. (2009). Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. *64*, 510-521.
44. Gervasi, N., Tchenio, P., and Préat, T. (2010). PKA dynamics in a *Drosophila* learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. Neuron *65*, 516-529.
45. Tomchik, S.M., and Davis, R.L. (2009). Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. Neuron *64*, 510-521.
46. Yu, D., Akalal, D.B., and Davis, R.L. (2006). *Drosophila* alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. Neuron *52*, 845-855.
47. Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451-461.
48. Robinson, I.M., Ranjan, R., and Schwarz, T.L. (2002). Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain. Nature *418*, 336-340.

49. Thum, A.S., Jenett, A., Ito, K., Heisenberg, M., and Tanimoto, H. (2007). Multiple memory traces for olfactory reward learning in *Drosophila*. J Neurosci *27*, 11132-11138.
50. Kim, Y.C., Lee, H.G., and Han, K.A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. J Neurosci *27*, 7640-7647.
51. Aso, Y., Grubel, K., Busch, S., Friedrich, A.B., Siwanowicz, I., and Tanimoto, H. (2009). The mushroom body of adult *Drosophila* characterized by GAL4 drivers. J Neurogenet *23*, 156-172.
52. Klagges, B.R., Heimbeck, G., Godenschwege, T.A., Hofbauer, A., Pflugfelder, G.O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S., and Buchner, E. (1996). Invertebrate synapsins: a single gene codes for several isoforms in Drosophila. J Neurosci *16*, 3154-3165.

Figures



Aso et al., Figure 1

Figure 1 Dopaminergic inputs into the mushroom body via MB-M3 neurons

(A) Projection of MB-M3 neurons visualized in *NP5272 UAS-mCD8::GFP.* In the MB (light green overlay based on the Synapsin counterstaining), MB-M3 neurons arbors in the medial tip of the β lobe (β_2; arrowhead). Sparse terminals are detected also in the β' lobe (β'_2; small arrowhead).

(B) In *UAS-Syt::HA; NP5272 UAS-mCD8::GFP*, the arborizations in the anterior and middle inferiormedial protocerebrum (aimpr-mimpr; arrows) are only weakly labeled by presynaptic

marker Syt::HA (magenta), if any, relative to mCD8::GFP (green), implying their dendritic nature. Scale bar 20 μm.

(C) The GAL4-expressing cells in the PAM cluster are visualized with *UAS-Cameleon2.1*. and counted. No significant difference is observed between *NP5272*, *NP1528* and the combination of them. Also, combining *NP5272* and *NP1528* does not significantly increase the number of the labeled cells as compared to *TH-GAL4* alone.

(D) The diagram illustrates the terminal areas of GAL4-expressing cells of *TH-GAL4* (green) and *NP5272/NP1528* (orange) in the MB lobes, based on cell counting and a single-cell analysis (Mao and Davis 2009). The MB lobes are shown as an outline. See also Table S2.

(E) Aversive odor memory tested at 30 min after training. The respective dopaminergic neurons are blocked with Shi^{ts1} driven by *TH-GAL4*, *NP5272* or *NP1528*. Block with these drivers significantly impairs aversive memory. *n*=17-22.

(F) Thirty-min appetitive memory of the same genotypes. Learning indices of all the experimental groups (*TH-GAL4/UAS- shi^{ts1}*, NP1528/UAS- shits1, *NP5272/UAS- shi^{ts1}*) are not significantly different from corresponding control groups (*P*>0.05; one-way ANOVA). *n*=13-16.

(G) Transient block only during aversive training. The results are essentially the same as in (E). *n*=20-22. Bars and error bars represent the mean and s.e.m., respectively. * *P*<0.05; ** *P*<0.01; *** *P*<0.001; n.s. not significant. When comparisons with multiple control groups give distinct significance levels, only the most conservative result is shown.

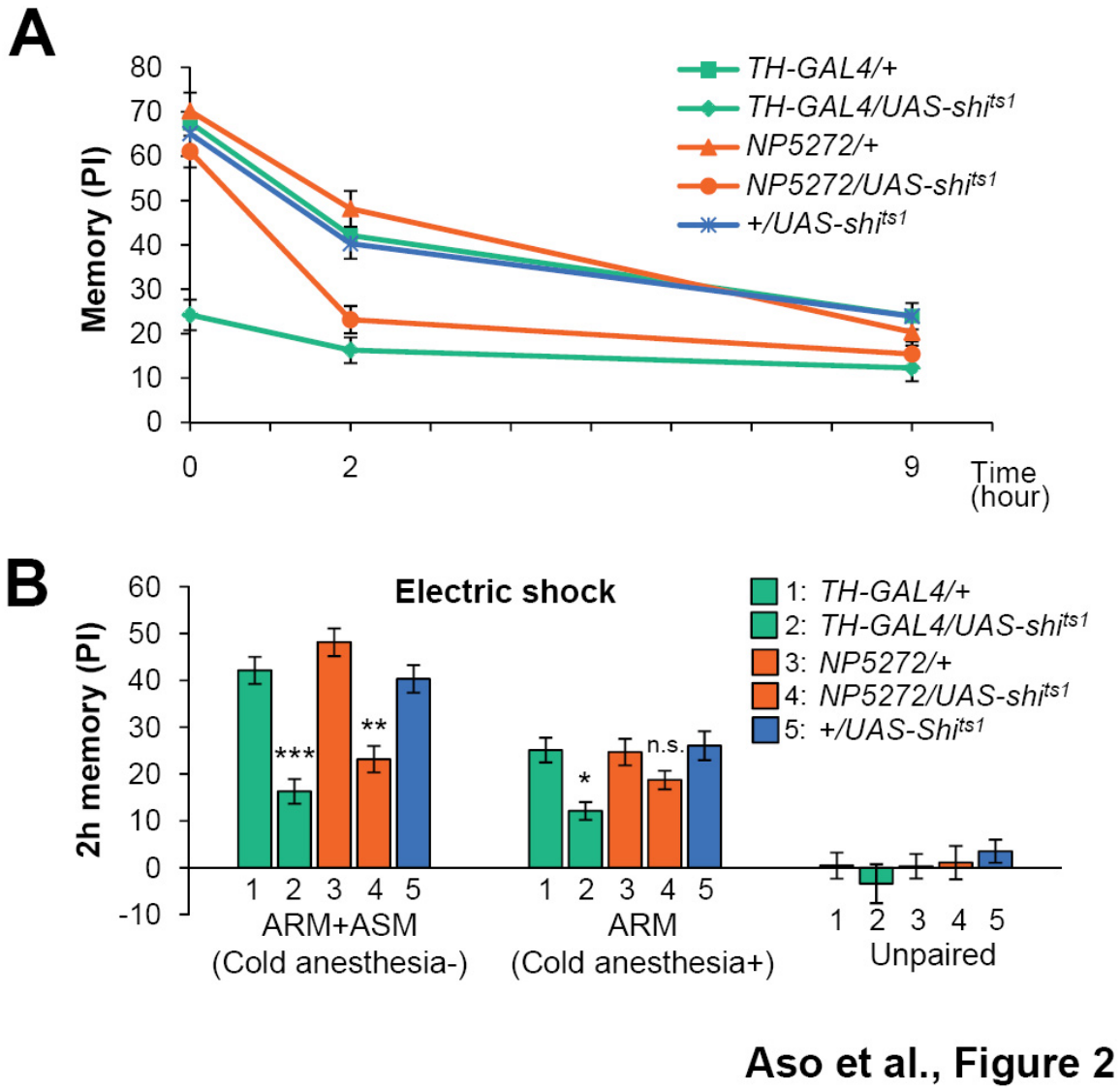
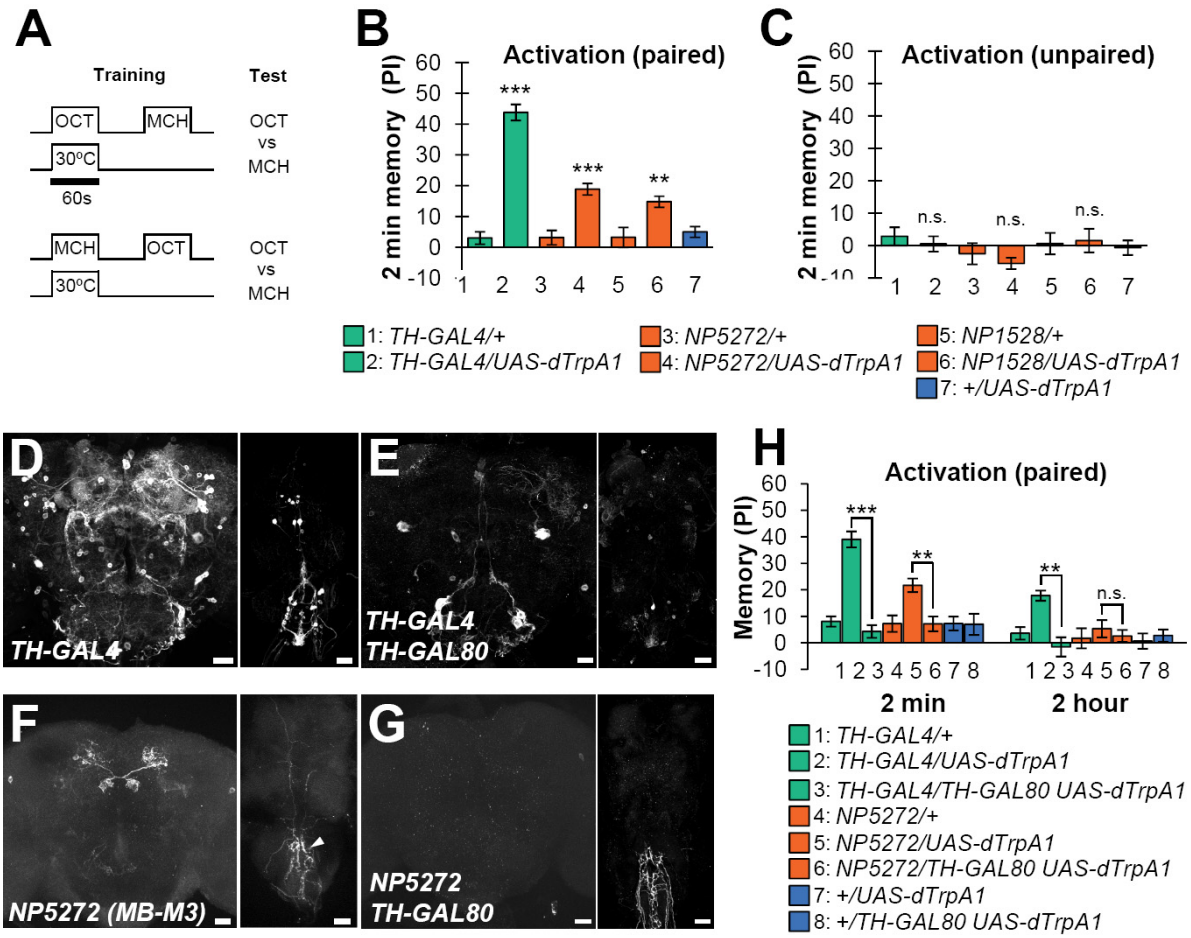


Figure 2 Preferential requirement of MB-M3 for inducing labile middle-term memory

(A) Requirement of the MB-M3 neurons for different memory phases. Flies were trained and tested immediately at restrictive temperature, or kept for two or nine hours and tested at permissive temperature. Memory is significantly impaired at all three retention intervals by *TH-GAL4*. Blocking MB-M3 slightly affects immediate and 9-hour memory, but only 2-hour memory is significantly impaired. *n*=14-20.

(B) Total memory (ASM+ARM; the same data set as in a), a consolidated memory component (ARM), and memory induced by unpaired presentation of odors and electric shock tested at 2 hours after training (*n*=12-18). Although the block with *TH-GAL4* impairs both total memory and ARM, only total memory is significantly impaired when MB-M3

neurons are blocked during training. The requirement of the MB-M3 neurons for the total memory and ARM is differential (P<0.05; significant interaction [genotype X cold shock treatment] in two-way ANOVA). Bars and error bars represent the mean and s.e.m., respectively. * P<0.05; ** P<0.01; *** P<0.001; n.s. not significant.



Aso et al., Figure 3

Figure 3 dTrpA1-dependent activation of MB-M3 can induce aversive odor memory.

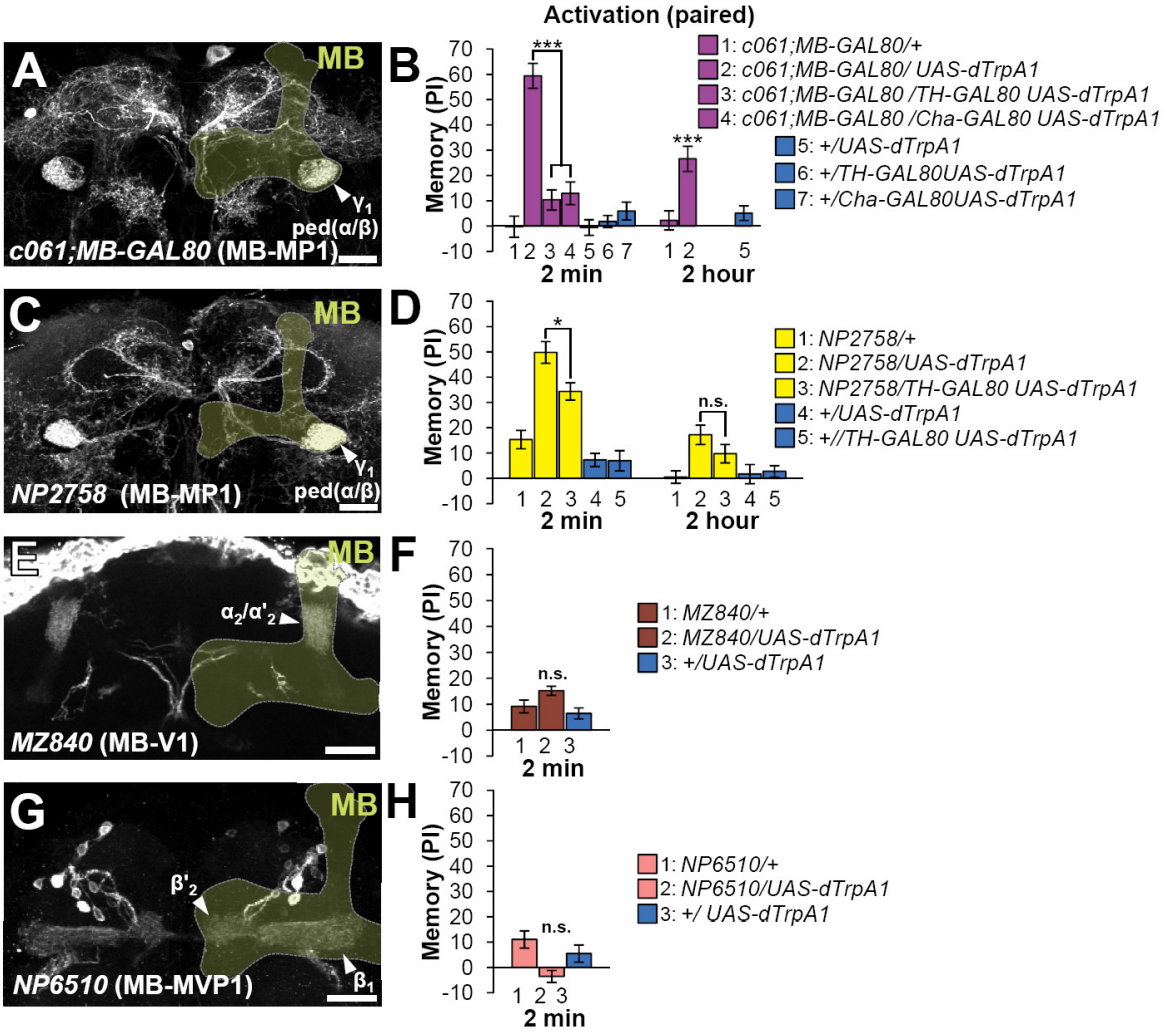
(A) The conditioning protocol for dTrpA1-induced memory (see Experimental Procedures for detail).

(B) Immediate aversive odor memory formed by odor presentation and simultaneous thermo-activation of the subsets of dopaminergic neurons expressing *dTrpA1*. *n*=18-22.

(C) Thermo-activation did not induce aversive odor memory when it is applied 60-120 seconds prior to the presentation of the odor. *n*=10-12.

(D-G) Expression pattern of *TH-GAL4* (D) and *NP5272* (F) in the brain (left panels; frontal view, dorsal up) and thoracicoabdominal ganglion (right panels; dorsal view, anterior up). *TH-GAL80* silences mCD8::GFP expression in MB-M3 (G) and the most of cells labeled by *TH-GAL4* (E). Remaining cells are presumably non-dopaminergic cells judging from their size and position (Mao and Davis 2009). Scale bars: 20 μm.

(H) Immediate aversive odor memory induced by dTrpA1-dependent activation is significantly suppressed by *TH-GAL80*, indicating that the corresponding dopaminergic neurons are responsible. *n*=15-17. Bars and error bars represent the mean and s.e.m., respectively. * *P*<0.05; ** *P*<0.01; *** *P*<0.001; n.s. not significant.



Aso et al., Figure 4

Figure 4 dTrpA1-induced memory by other dopaminergic neurons

(A, C, E, G) Projection of brain regions including the MB (light green outline; frontal view, dorsal up). Various dopaminergic neurons projecting to the MB are visualized with mCD8::GFP (arrowheads) driven by *c061; MB-GAL80* (A), *NP2758* (C), *MZ840* (E), and *NP6510* (G). Scale bar 20 μm. see also Figure S2 and Table S2.

(B, D, F, H) Memory induced by dTrpA1-dependent activation of the various types of dopaminergic neurons.

(E) With *c061;MB-GAL80*, robust immediate and 2-hour memory are formed and significantly suppressed by *TH-GAL80* and $Cha^{3.3kb}$*-GAL80*. *n*=18-22.

(F) Activation of dTrpA1-expressing cells in *NP2758* induces robust aversive odor memory, which is significantly suppressed by *TH-GAL80*. (G) Despite the tendency, no significant memory is formed with *MZ840*.

(H) With *NP6510,* the learning index of *NP6510/UAS-dTrpA1* is different from *NP6510/+* but not from *+/UAS-dTrpA1*. *n*=15-18. Bars and error bars represent the mean and s.e.m., respectively. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; n.s. not significant.

Specific dopaminergic neurons for the formation of labile aversive memory

Yoshinori Aso, Igor Siwanowicz, Lasse Bräcker, Kei Ito, Toshihiro Kitamoto, Hiromu Tanimoto*

Inventory of Supplemental Data

Figures	Supplemental data
Figure 1: Dopaminergic inputs into the mushroom body via MB-M3 neurons	**Figure S1:** Output of MB-M3 is dispensable for memory consolidation and retrieval **Table S1 :** Shock avoidance under blockade of dopaminergic neurons
Figure 2: Preferential requirement of MB-M3 for inducing labile middle-term memory.	none
Figure 3: dTrpA1-dependent activation of MB-M3 can induce aversive odor memory.	None
Figure 4: dTrpA1-induced memory by other dopaminergic neurons	**Figure S2:** Expression pattern of GAL4 drivers labeling MB-projecting dopaminergic neurons **Table S2:** GAL4 drivers for dopaminergic MB-extrinsic neurons, related to Figure 4

Supplemental Data

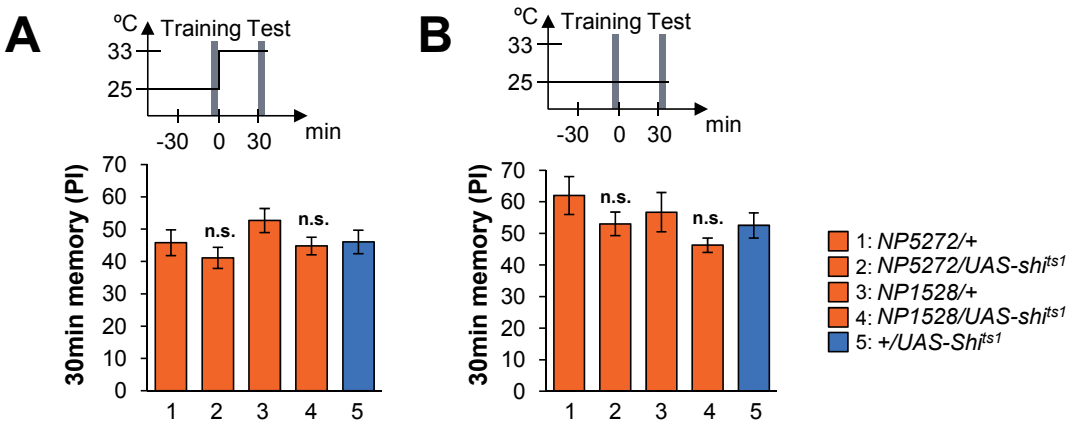


Figure S1 Output of MB-M3 is dispensable for memory consolidation and retrieval (related to Figure 1)

(A) Synaptic block of the MB-M3 neurons during the retention interval and test period does not affect shock-induced odor memory tested 30 min after training. One-way ANOVA for genotype (P>0.05). n=15-17.

(B) Similarly, at permissive temperature, there is no significant difference between experimental genotypes (GAL4/UAS-shi^{ts1}) and respective genetic controls (P>0.05). n=14

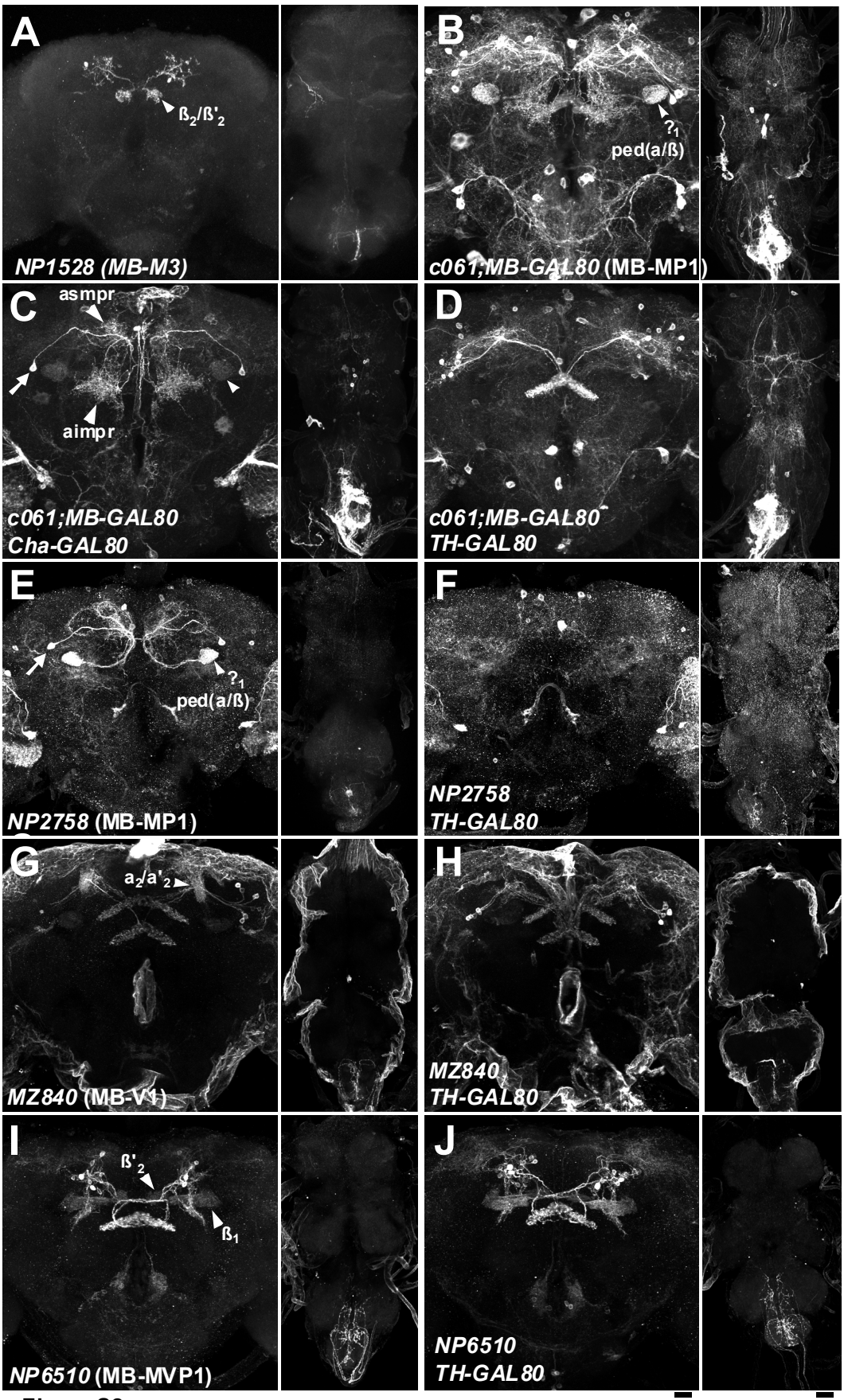

A
ß2/ß'2
NP1528 (MB-M3)
B
?1
ped(a/ß)
c061;MB-GAL80 (MB-MP1)
C
asmpr
aimpr
c061;MB-GAL80
Cha-GAL80
D
c061;MB-GAL80
TH-GAL80
E
?1
ped(a/ß)
NP2758 (MB-MP1)
F
NP2758
TH-GAL80
G
a2/a'2
MZ840 (MB-V1)
H
MZ840
TH-GAL80
I
ß'2
ß1
NP6510 (MB-MVP1)
J
NP6510
TH-GAL80

Figure S2

Figure S2 Expression pattern of GAL4 drivers labeling MB-projecting dopaminergic neurons (related to Figure 4)

Projection of confocal stacks of the brain (left panels; frontal view, dorsal up) and thoracicoabdominal ganglion (right panels; dorsal view, anterior up). The GAL4-expressing cells are visualized by *UAS-mCD8::GFP* and examined with counter-labeling with the antibody to Synapsin or Tyrosine Hydroxylase (not shown).

(A) *NP1528* has the essentially same expression pattern as NP5272 (Figure 3F-G).

(B) *c061;MB-GAL80* labels three neurons in the PPL1 cluster. In addition, it labels the neurons projecting to the fan-shaped body, the ellipsoid body, the medulla and lobula of the optic lobes, optic tubercle, inferior and superior posterior slope, scattered cells and a large neuron on the posterior cortex, faint innervations in the antennal lobe, ventral lateral protocerebrum in the brain, and scattered cells and the dense innervation to the abdominal ganglion in the thoracicoabdominal ganglion. Among them, only three PPL1 cells show obvious TH-immunoreactivity.

(C) *Cha*$^{3.3kb}$*-GAL80* significantly silences *mCD8::GFP* expression of *c061;MB-GAL80* in many neurons including two PPL1 cells and neurons projecting to the fan-shaped body. Expression in one TH-immunoreactive neuron in the PPL1 cluster (arrow) is only slightly affected, if any. The morphology of this neuron is different from MB-MP1 neuron in NP2758. It projects to the region surrounding the γ lobe of the MB (anterior inferior and superior medial protocerebrum; asmpr and aimpr; arrowheads). The faint signal in the γ1 is presumably due to the residual expression in the MB-MP1 neurons (small arrowhead).

(D) *TH-GAL80* silences *mCD8::GFP* expression of *c061;MB-GAL80* in three PPL1 cells. Expression in the other cells is largely preserved. Notably the terminals of PPL1 neurons in the aimpr and asmpr disappear (arrowheads in C).

(E) *NP2758* labels a single MB-MP1 neuron (arrow) that is one of three PPL1 cluster cells in the *c061;MB-GAL80* . It additionally labels approximately 20 other cells per brain hemisphere including a large interneuron projecting to the lobula of the optic lobes, innervations to the antennal lobe and inferior medial protocerebrum, cells located dorsal to the protocerebral bridge and scattered small cells. Importantly, only the single MB-MP1 neuron is immunoreactive to TH and project to the MB. In the thoracicoabdominal ganglion, a few innervations in the abdominal segments are detectable.

(F) *TH-GAL80* significantly reduces the reporter signal in the MB-MP1 neuron but has little effect on the other cells in *NP2758*.

(G) *MZ840* labels one PPL1 cell (MB-MV1) and neurons projecting to the fan-shaped body, medulla and lobula of the optic lobes, as well as strong signals in glial cells. Faint signals are detectable in the suboesophageal ganglion and deutocerebrum. To show the expression pattern in the brain, confocal stacks of the brain was excluded from the projection as the strong signal from glia obscures it.

(H) *TH-GAL80* significantly reduces the reporter signal in the MB-V1 neuron but has little effect on the other cells in *MZ840*.

(I) *NP6510* labels MB-MVP1 neurons and another type of MB-extrinsic neurons projecting to the tip of the β' lobe. It also labels the fan-shaped body, the pars intercerebralis, scattered small cells in the posterior cortex and a few innervations in the abdominal segments.

(J) Addition of *TH-GAL80* does not obviously change the expression pattern of *NP6510*, presumably because the utilized *TH* promoter does not have expression in the PAM cluster cells labeled in *NP6510*. Scale bars: 20 μm.

Table S1 Shock avoidance under blockade of dopaminergic neurons (related to Figure 1)

Genotype	Shock avoidance	
TH-GAL4 /+	52.3	(4.9)
UAS- shi^{ts1} /+;TH-GAL4/ UAS- shi^{ts1}	39.3	(3.2)
NP5272/+	58.5	(5.5)
UAS- shi^{ts1} /+;NP5272/+;UAS- shi^{ts1} /+	65.2	(4.5)
NP1528/+	52.7	(6.4)
UAS- shi^{ts1} /+;NP1528/+;UAS- shi^{ts1} /+	58.1	(3.6)
UAS- shi^{ts1} /+;;UAS- shi^{ts1} /+	48.3	(4.9)

Electric shock sensitivity of naïve flies is assayed by measuring the reflexive avoidance from 12 pulses of shock (1.5 sec of 90 V, DC in every 5 sec) in one minute using the same apparatus used for olfactory conditioning. Avoidance index was calculated as previously described. There is no significant difference between experimental genotypes (GAL4/shi) and respective genetic controls (P>0.05, the *post-hoc* test [Bonferroni]). Mean avoidance index is shown with s.e.m. in bracket. n=12-16

Table S2 GAL4 drivers for dopaminergic MB-extrinsic neurons (related to Figure 4)

GAL4 drivers	Labeled TH-positive neurons projecting to the MB[a]	Labeled MB-compartments[b]
TH-GAL4	12 PAM cells (MB-M3 and neurons projecting to the β' 2 and/or γ5) 12 PPL1 cells[c] (MB-V1, MB-MP1, MB-MV1, neurons projecting to the α'3 or α3) 6 PPL2ab cells (neurons projecting to the calyx)	calyx, ped, γ1, γ2, γ5, β2, β' 2, α'1, α'2, α'3, α1, α2, α3
NP5272	3 PAM cells (MB-M3) [d]	β2, β' 2
NP6510	15 PAM cells (MB-MVP1 and neurons projecting to the β' 2)	β1, β' 2, α1, ped
NP2758	1 PPL1 cell (MB-MP1[e])	γ1, ped (α/β)
c061;MB-GAL80	3 PPL1 cells [f] (MB-MP1)	γ1, ped (α/β)
MZ840	1 PPL1 cell (MB-V1)	α'2, α2

[a] Based on anti-TH immunolabeling and the data from (Mao et al. Krashes et al. Claridge-change et al.)
[b] Nomeclature of the mushroom body subdivisions and brain regions follows Tanaka et al and Otsuna et al.
Gray characters indicate weak or sparse signals of the reporter.
[c] Other PPL1 neurons project to the fan-shaped body, aimpr and asmpr, or the areas surrounding the vertical lobes (Mao et al)
[d] These MB-M3 are also labeled in TH-GAL4 (Table S1).
[e] This MB-MP1 is also labeled in c061.
[f] One of PPL1 neuron projects to the aimpr and asmpr. It is ambiguous whether there is one or two MB-MP1.

5. Chapter III

Parallel memory traces with distinct temporal dynamics constitute odor memory in Drosophila

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In *Drosophila*, dopamine mediates a reinforcing property of aversive stimuli to the mushroom body for the formation of odor memory. Many types of dopaminergic neurons with various physiological properties project to the distinct compartment of the mushroom body, but role of individual neurons in memory formation is not yet fully uncovered. By ectopic expression of thermo-sensitive channel dTRPA1, we found that activation of at least three types of dopaminergic neurons can induce aversive odor memories that differ in initial magnitude and temporal dynamics of decay. Selective blockade of each cell type partially impaired olfactory learning with electric shock. Intriguingly, memory phases impaired by blocking each cell type did not always match the memory phases that can be induced by activation of the same cell type, implying parallel memory traces constitute the memory with the functional redundancy and interaction rather than simply adding up. Indeed, their activation in the particular combinations synergistically improved memory performance for 2 hours and 9 hours retention, even though they were not additive for the immediate memory. These results cast light on how parallel memory traces in the single brain structure and their interaction constitute the distinct temporal domain of memory.

Parallel molecular and cellular pathways and distributed brain systems support encoding and storage of memory. As exemplified in classical conditioning of *Aplysia* siphon-withdrawal reflex and olfactory conditioning in *Drosophila*, such parallel processes typically underlie different temporal domains of memory (Zars, Fischer et al. 2000; Pascual and Preat 2001; Isabel, Pascual et al. 2004; Stough, Shobe et al. 2006; Blum, Li et al. 2009). In *Drosophila*, the center for olfactory learning, mushroom body (MB), comprises three types of major intrinsic neurons, Kenyon cells: the α/β, α'/β' and γ neurons named after the projection pattern of their axon bundles (Crittenden, Skoulakis et al. 1998). A series of evidences indicate that the these different population of Kenyon cells form parallel memory traces that contribute to different memory phases (Zars, Fischer et al. 2000; McGuire, Le et al. 2001;

McGuire, Le et al. 2003; Isabel, Pascual et al. 2004; Akalal, Wilson et al. 2006; Krashes, Keene et al. 2007; Blum, Li et al. 2009). Given that various dopaminergic neurons intersect distinct synaptic compartments of Kenyon cell's axon bundles, however, it is not fully understood where in the MB these memory traces are formed and how they contribute to different phase of memory.

To explore the site of memory traces in the brain, cellular identification of modulatory neurons is a powerful approach. In the *Drosophila* brain, dopaminergic neurons from the three clusters PPL1, PAM and PPL2ab project to the MB (Mao and Davis 2009). Among them, PPL1 cluster has essential role in the short-term memory (Claridge-Chang, Roorda et al. 2009), although function of individual neurons within the cluster (Krashes, DasGupta et al. 2009; Mao and Davis 2009) and contribution to different memory phases just began to be uncovered(Aso, Siwanowicz et al. 2010). To identify specific dopaminergic neurons for aversive reinforcement, we used an approach in which a thermo-sensitive cation channel dTRPA1 is expressed in a specific set of dopaminergic neurons by the GAL4/UAS system. Flies were conditioned by transient elevation of temperature (30°C) while odor was presented for 60 seconds (Fig.1A). In this way, our previous study identified that MB-M3 and MB-MP1, from PAM and PPL1 cluster respectively, can induce aversive odor memory (Aso, Siwanowicz et al. 2010). By screening a new set of GAL4 drivers for various dopaminergic neurons, we found that activation of MB-MV1+MB-V1 in the PPL1 cluster can also induce aversive odor memory (Fig. 1B, S1 and S2). The magnitude of memory depended on the activation temperature, indicating the amount of dopamine input correlates with efficacy of memory formation (Fig.1C). On the other hand, activation of many other neurons did not induce memory (Fig. S1 and S2; see Supplementary texts for the detail description of the screening) (Aso, Siwanowicz et al. 2010), highlighting the functional heterogeneity in the PAM and PPL1 cluster and importance of pattern of dopamine input to the MB. Using three drivers with which flies formed significant dTRP1-dependent memory and that is completely suppressed by selective expression of GAL4 inhibitor GAL80 in the dopaminergic neurons

(*NP5272* for MB-M3, *c061;MB-GAL80* for MB-MP1, and *5htr1b-GAL4* for MB-MV1+MB-V1; Fig. S1 and S2) (Aso, Siwanowicz et al. 2010), we investigated how memory traces induced by these dopaminergic neurons constitute the odor memory.

To deconstruct memory after electric shock conditioning with single dopamine neurons, we transiently blocked corresponding neurons by expressing Shi^{ts1}, a dominant negative temperature sensitive variant of Dynamin (Kitamoto 2001). In the previous study using *TH-GAL4*, blocking many dopaminergic neurons including MB-MP1, MB-M3, MB-MV1 and MB-V1 during training significantly impaired memory at 2 min, 2 hours and 9 hours retention, though there was small residual memory at all time points (Schwaerzel, Monastirioti et al. 2003; Aso, Siwanowicz et al. 2010) In contrast, blocking individual dopamine neuron type impaired specific temporal components of aversive memory (Fig.2). Selective blocking of MB-M3 preferentially impaired 2 hours memory, but not 2 min and 9 hours memory (Fig. 2A) (Aso, Siwanowicz et al. 2010), indicating that each dopaminergic neuron type may have a preferential role in distinct memory phases. Along with this hypothesis, we found that blocking MB-MV1+MB-V1 impaired memory only at 9 hours (Fig. 2B), whereas blocking MB-MP1 during training significantly impaired 2 min, 2 hours and 9 hours memory (Fig. 2C). The observed memory impairment should not be due to the defect in processing odor and electric shock itself (Fig. S3A). Furthermore, blocking after training or experiments at permissive temperature did not impair memory (Fig. S3B-I). Taken together, we propose that the electric shock punishment recruits multiple dopaminergic neuron types to form parallel memory traces in the mushroom body.

Given that both MB-M3 and MB-MV1+MB-V1 can induce immediate memory (Fig.1 and Fig.S1) (Aso, Siwanowicz et al. 2010) even though their requirement is pronounced in only later memory phases (Fig.2), there are likely to be functional redundancy between memory traces induced by each dopaminergic neurons. To untangle the contribution of each dopaminergic neuron type to different memory phases, we measured retention of memory formed by selective activation of MB-M3, MB-MV1+MB-V1, or MB-MP1 at 30°C. Memory

formed by the activation of individual dopaminergic pathways showed different stabilities (Fig. 2). The initially robust memory induced by MB-MP1 decayed rapidly over 9 hours. In contrast, dynamics of memory by MB-MV1+MB-V1 was unique; it started relatively low, but decayed very slowly. This differential memory decay is not due to the initial magnitude of memory, because a memory induced by MB-M3 or mild activation of MB-MP1 started at the same level but decayed completely to the chance level in 3 hours. Also, the genetic background or the effect of dTRPA1 ectopic expression at permissive temperature cannot be the cause of differential memory dynamics, because decay of electric-shock induced memory was indistinguishable in these flies (Fig. S4). Thus, these results indicate that each dopaminergic neuron type induces memory traces that decay with different temporal dynamics and thereby relative contribution to memory can be weighted depending on the retention time.

How parallel memory traces formed by distinct dopaminergic neurons constitute the shock-induced odor memory? The temporal domains of memory affected by blocking selective dopaminergic neuron type and that induced by activation do not always match (Fig.2 and Fig.3). For instance, output of MB-M3 is necessary for 2 hour memory by electric shock even though memory induced by selective activation of MB-M3 does not last 2 hours. Although such discrepancy may simply reflect the degree of activation by the electric shock and dTrpA1, it may also imply the functional redundancy and interaction between memory traces. To address how memory traces induced by MB-M3, MB-MP1 and MB-MV1+MB-V1 constitute memory when they are formed in parallel, we activated corresponding dopamine neurons in various combinations and measured memory retention after conditioning at 30°C (Fig. 4A-C). In all combinations, flies expressing dTrpA1 with single and double drivers showed indistinguishable two hour memory when they are trained with electric shock (Fig. S4A), indicating that these flies have comparable ability to form and retain memory. In comparison to activation of MB-MV1+MB-V1 alone, additional activation of MB-M3 did not change the performance for 2 min memory. For 2 hours retention, however, combinatorial activation significantly improved performance, but it had no effect on the 9 hours retention

(Fig. 4A). MB-MV1 is likely to be the interaction partner of MB-M3, because we observed similar effect by additional activation of MB-M3 together with MB-MP1+MB-MV1+MB-V1 whereas combinatorial activation of MB-M3 and MB-V1 did not induce significant 2 hour memory (Fig. 4D and 4E). Combination of (MB-M3 and MB-MP1) and (MB-MP1 and MB-MV1+MB-V1) had no significant improvement on memory performance at all retention time compared to activation of MB-MP1 alone (Fig. 4B and 4C). Although this result may imply that memory by MB-M3 or MB-MV1+MB-V1 is redundant with that by MB-MP1 in some conditions, MB-MP1 and MB-MV1+MB-V1 are not redundant for electric shock induced memory at 9 hours retention (Fig.2B and 2C). This might result from different activation level of MB-MP1 by dTrpA1 and electric shock, because requirement of MB-MP1 for immediate shock-induced memory is relatively small compared to the robust memory by dTRPA1-depedent activation of MB-MP1 at 30°C (Fig.2C and Fig. 1). By lowering activation temperature, we can calibrate magnitude of immediate memory. For MB-M3, flies formed significant memory only at 30°C, but flies formed significant memory even at 28°C for MB-MP1 and MB-MV1+MB-V1 (Fig. 1). After conditioning at 28°C, compared to activation of MB-MP1 or MB-MV1+MB-V1 alone, combinatorial activation significantly slowed down the memory decay for 9 hours memory, whereas it only tended to add up at 2 min and 2 hours retention time.

These results demonstrate that parallel memory traces have largely redundant contribution to the early memory phases, but they synergistically contribute to the maintenance of memory. Such an interaction may occur at the molecular level in the same Kenyon cell as in the sensory neurons of Aplysia in which contingent application of serotonin to the cell bodies and presynaptic terminals synergistically induce intermediate and long-term synaptic facilitation (Sherff and Carew 1999). Alternatively, it may occur at the circuit level via neurons such as DPM neurons that have an essential role in memory consolidation (Keene and Waddell 2007).

We showed that at least three dopaminergic pathways can contribute to induction of parallel memory traces in the MB. Given that temporal dynamics of memory decay is largely dependent on the type of dopaminergic neurons that induced memory (Fig. 3), different aversive stimuli can potentially induce memory with different stability by recruiting dopaminergic neurons with certain intensity and combination. Indeed, dopaminergic neurons examined in this study have dendrites in partly overlapping region in the protocerebrum near the medial and vertical lobes of the MB (aimpr) but also in non overlapping regions of the brain (Tanaka, Tanimoto et al. 2008; Mao and Davis 2009). Characterization of neurons innervating these regions will help understanding upstream circuits of these dopaminergic neurons. Studies using calcium reporters have revealed that MB-projecting dopaminergic neurons respond distinctly to odors and electric shock (Riemensperger, Voller et al. 2005; Mao and Davis 2009). In the future, it will be of great interest to examine how memory dynamics and recruited dopaminergic neurons differ when distinct aversive stimuli are used in conditioning.

References

Akalal, D. B., C. F. Wilson, et al. (2006). "Roles for Drosophila mushroom body neurons in olfactory learning and memory." Learn Mem **13**(5): 659-668.

Aso, Y., I. Siwanowicz, et al. (2010). "Specific dopaminergic neurons for the formation of labile aversive memory." Curr Biol **20**(16): 1445-1451.

Blum, A. L., W. Li, et al. (2009). "Short- and long-term memory in Drosophila require cAMP signaling in distinct neuron types." Curr Biol **19**(16): 1341-1350.

Claridge-Chang, A., R. D. Roorda, et al. (2009). "Writing memories with light-addressable reinforcement circuitry." Cell **139**(2): 405-415.

Crittenden, J. R., E. M. Skoulakis, et al. (1998). "Tripartite mushroom body architecture revealed by antigenic markers." Learn Mem **5**(1-2): 38-51.

Isabel, G., A. Pascual, et al. (2004). "Exclusive consolidated memory phases in Drosophila." Science **304**(5673): 1024-1027.

Keene, A. C. and S. Waddell (2007). "Drosophila olfactory memory: single genes to complex neural circuits." Nat Rev Neurosci **8**(5): 341-354.

Kitamoto, T. (2001). "Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons." J Neurobiol **47**(2): 81-92.

Krashes, M. J., S. DasGupta, et al. (2009). "A neural circuit mechanism integrating motivational state with memory expression in Drosophila." Cell **139**(2): 416-427.

Krashes, M. J., A. C. Keene, et al. (2007). "Sequential use of mushroom body neuron subsets during drosophila odor memory processing." Neuron **53**(1): 103-115.

Mao, Z. and R. L. Davis (2009). "Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity." Front Neural Circuits **3**: 5.

McGuire, S. E., P. T. Le, et al. (2001). "The role of Drosophila mushroom body signaling in olfactory memory." Science **293**(5533): 1330-1333.

McGuire, S. E., P. T. Le, et al. (2003). "Spatiotemporal rescue of memory dysfunction in Drosophila." Science **302**(5651): 1765-1768.

Pascual, A. and T. Preat (2001). "Localization of long-term memory within the Drosophila mushroom body." Science **294**(5544): 1115-1117.

Riemensperger, T., T. Voller, et al. (2005). "Punishment prediction by dopaminergic neurons in Drosophila." Curr Biol **15**(21): 1953-1960.

Schwaerzel, M., M. Monastirioti, et al. (2003). "Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila." J Neurosci **23**(33): 10495-10502.

Sherff, C. M. and T. J. Carew (1999). "Coincident induction of long-term facilitation in Aplysia: cooperativity between cell bodies and remote synapses." Science **285**(5435): 1911-1914.

Stough, S., J. L. Shobe, et al. (2006). "Intermediate-term processes in memory formation." Curr Opin Neurobiol **16**(6): 672-678.

Tanaka, N. K., H. Tanimoto, et al. (2008). "Neuronal assemblies of the Drosophila mushroom body." J Comp Neurol **508**(5): 711-755.

Zars, T., M. Fischer, et al. (2000). "Localization of a short-term memory in Drosophila." Science **288**(5466): 672-675.

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Figures legends

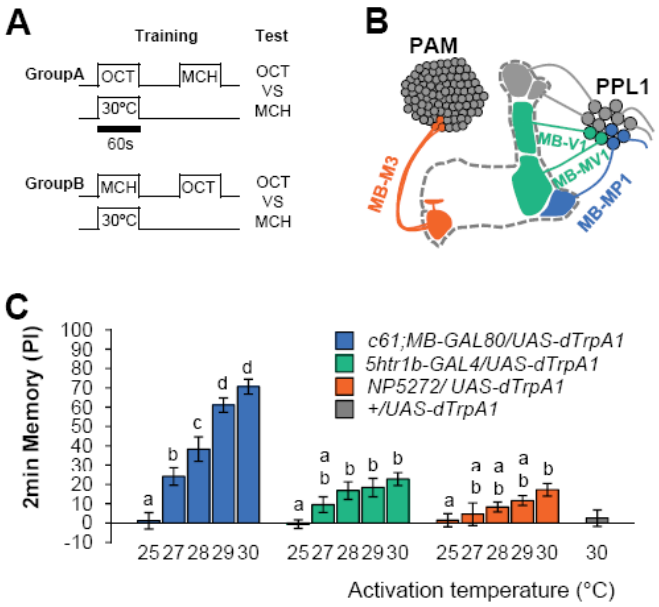

Fig. 1. Three types of dopaminergic neurons for aversive reinforcement.

(A) The conditioning protocol for dTrpA1-induced memory (see Material and Methods for detail).

(B) The diagram illustrates the terminal areas of MB-M3, MB-MP1 and MB-MV1+MB-V1 in the lobe regions of the mushroom body.

(C) Flies are trained by transient 60 seconds temperature shift from 25°C to various temperatures between 25°C and 30°C with simultaneous odor presentation, and tested immediately. *c061;MB-GAL80/UAS-dTrpA1* flies formed a significant memory at 27°C compared to flies trained at 25°C. Memory performance proportionally increased with elevation of activation temperature. With *5htr1b-GAL4 and NP5272*, the minimum activation temperature for inducing memory was 28°C and 30°C respectively. Bars and error bars represent the mean and s.e.m., respectively. Different alphabet denotes statistically distinguishable groups ($p<0.05$) by Newman-Keuls test for all pairs following one-way ANOVA. n=12-16.

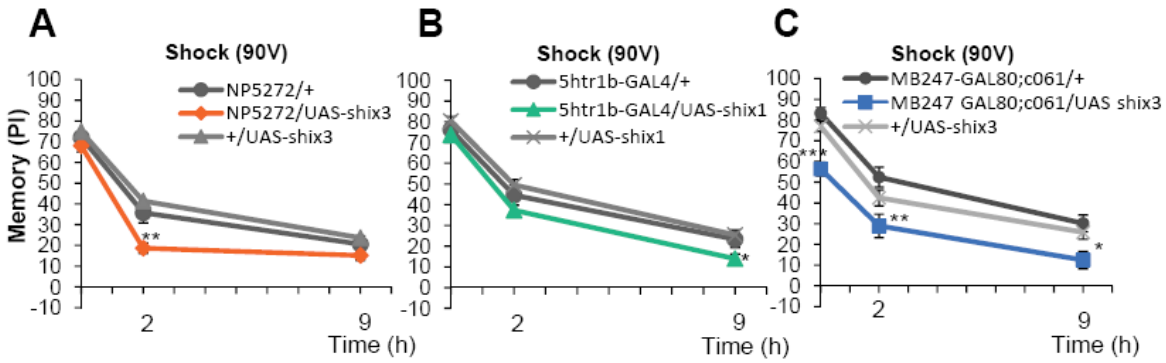

Fig. 2. Requirement of dopaminergic neurons for the shock induced memory.

Flies were trained and tested immediately at restrictive temperature, or kept for two or nine hours and tested at permissive temperature.

(A) Blocking MB-M3 significantly 2-hour memory, but effect on immediate and 9-hour memory was marginal. n=12-16.

(B) Blocking MB-MV1+MB-V1 preferentially impaired 9 hour memory, whereas 2 min and 2 hour memory was not significantly affected. Because of memory impairment at permissive temperature with three copies of *UAS-shi^{ts1}* with *5htr1b-GAL4*, one copy of *UAS-shi^{ts1}* was used. n=16-28

(C) Memory at all retention time was significantly impaired by blocking of MB-MP1. n= 16-24

Bars and error bars represent the mean and s.e.m., respectively. * P<0.05; ** P<0.01; n.s. not significant.

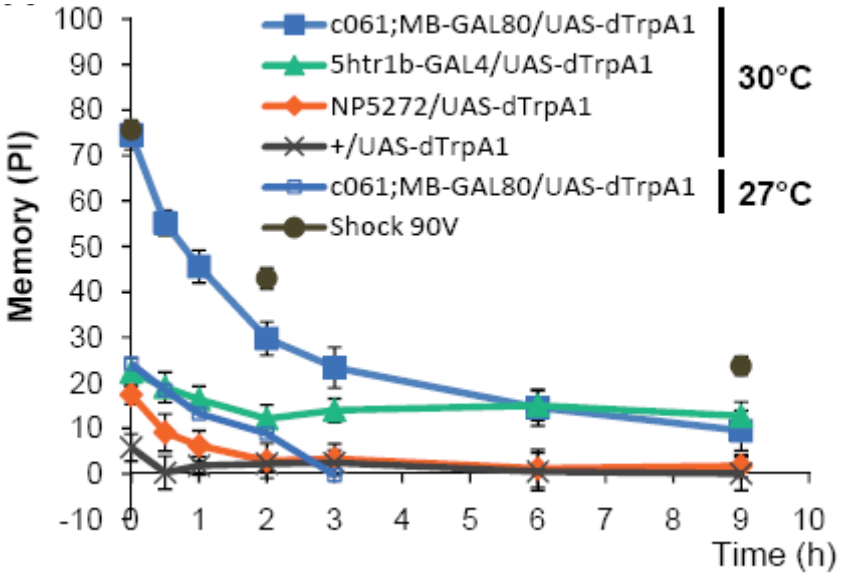

Fig. 3. Dynamics of memories induced by individual dopaminergic neurons

Flies expressing dTRPA1 in different dopaminergic neurons are trained at 30°C and tested at various retention times. *c061;MB-GAL80/UAS-dTrpA1* flies were also trained at 27°C. n=16-26. For comparison, averaged performance of these flies after electric shock conditioning is also plotted (see Fig. S4A for memory dynamics of individual genotype). Marker and error bars represent the mean and s.e.m., respectively. Memory induced by different dopaminergic neurons decayed with significantly different temporal dynamics (P<0.05; significant interaction [genotype X retention time] in two-way ANOVA).

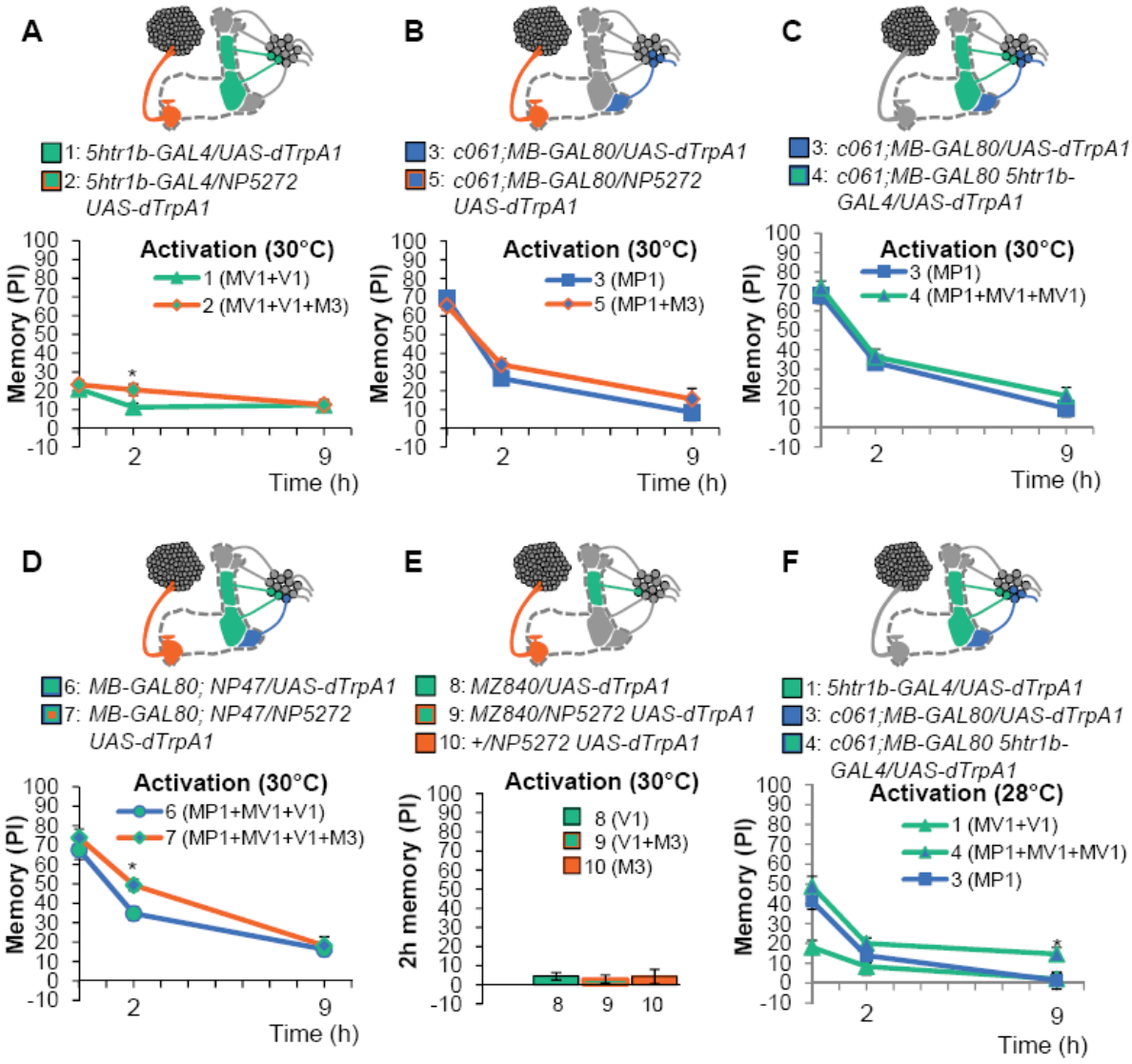

Fig. 4. Dynamics of odor memory induced by combinatorial activation of dopaminergic neurons.

dTRPA1 is targeted different combination of dopaminergic neurons. Flies are trained at 30°C (A-E) or 28°C (F) and tested at 2 min, 2 hours, and 9 hours after training. Diagram above each graph depict dopaminergic neurons targeted by the driver combination.

(A) 5htr1b-GAL4*/UAS-dTrpA1* and *5htr1b-GAL4 NP5272/UAS-dTrpA1* flies showed indistinguishable 2min and 9h memory, but 2h memory was significantly higher in 5htr1b-GAL4 NP5272/UAS-dTrpA1.

(B) Memory of *c061;MB-GAL80 NP5272/UAS-dTrpA1*was not significantly different from that of *c061;MB-GAL80/UAS-dTrpA1* at all retention time*,* although it tended to be higher at longer retention time.

(C) *c061;MB-GAL80/UAS-dTrpA1* and *c061;MB-GAL80 5htr1b-GAL4/UAS-dTrpA1* showed indistinguishable level of memory at all retention time (but see F).

(D) 2h Memory of *MB-GAL80 NP5272 ;NP47/UAS-dTrpA1*flies was significantly higher than that of *MB-GAL80 ;NP47/UAS-dTrpA1*flies, but they were not significantly different at 2min and 9h.

(E) Flies did not form significant 2h memory with *MZ840*, *NP5272* and their combination.

(F) When flies were trained at 28°C instead of 30°C, 9h memory of *c061;MB-GAL80 5htr1b-GAL4/ UAS-dTrpA1* flies was significantly higher than that of single drivers. Although it was not significant, memory at 2min and 2h tended to be higher in driver combination.

Bars and error bars represent the mean and s.e.m., respectively. * P<0.05; ** P<0.01; n.s. not significant.

Supporting Material

Materials and Methods

Supplementary Text

Fig. S1 to Fig. S4

References and Notes

Materials and Methods

Fly strains

All flies were raised on standard medium. We generated flies for behavioral and anatomical studies by crossing *MB-GAL80;NP47* (II, III), *NP7187* (X), *NP7323* (II), *MZ19;Cha$^{3.3kb}$-GAL80* (II, III), *NP5272* (II), *MZ840* (III) (Tanaka, Tanimoto et al. 2008), *c061;MB-GAL80* (X,II) (Krashes, DasGupta et al. 2009), *DDC-GAL4* (III) (Li, Chaney et al. 2000), *HL9-GAL4* (III) (Claridge-Chang, Roorda et al. 2009), *MB-GAL80;c259* (II,III) (http://www.fly-trap.org/), *5htr1b-GAL4* (II) (Ritze. 2007), *UAS-dTrpA1(II)* (Hamada, Rosenzweig et al. 2008), *UAS-dTrpA1 TH-GAL80 (II)* (Sitaraman, Zars et al. 2008), *UAS-dTrpA1; Cha$^{3.3kb}$-GAL80 (II, III)* (Kitamoto 2002), *c061; MB-GAL80 UAS-dTrpA1* (X,II), *NP5272 UAS-dTrpA1(II), UAS-shi^{ts1};UAS-shi^{ts1}* (X, III), *UAS-shi^{ts1}* (III), *UAS-mCD8::GFP(II), UAS-mCD8::GFP(X,III), UAS-mCD8::GFP(X, II, III)* (Lee and Luo 1999)*, UAS-mCD8::GFP(X); TH-GAL80(II); UAS-mCD8::GFP(III)*, *UAS-mCD8::GFP; Cha$^{3.3kb}$-GAL80 (II,III).* Because all these transgenic lines have *white* mutation*,* the control groups (only drivers or effectors) were generated by crossing drivers or effecter strains to *white.* After measurement, flies without a GAL4 driver (e.g. those with the balancer or male of *NP7187* crosses) were excluded from calculation. For experiments with *UAS-shi^{ts1}* and *UAS-dTrpA1*, flies were raised at 18°C and 25°C, 60% relative humidity and used during 8-14 and 7-12 days after eclosion, respectively, to allow sufficient accumulation of effecter genes without age-related memory impairment. For anatomical assay, females of 5-10 days after eclosion at 25°C were analyzed.

Behavioral assays

For olfactory conditioning, we used 4-methylcyclohexanol and 3-octanol diluted in the paraffin oil (1:10). One odor was presented for 1 min at high temperature or with 12 pulses of electric shocks (90V). Subsequent to 1 min pause, another odor was presented for 1 min. The reciprocal group of flies was trained by the protocol in which first and second odor were altered. After a given retention time, the conditioned odor response was measured in a T-maze for two minutes. Then, a performance index (PI) was then calculated by taking the

mean preference of two reciprocal groups (Tully and Quinn 1985). The first odor was paired with reinforcement in a half of experiments and the second odor was paired with reinforcement in another half so that the effect of the order of reinforcement is canceled (Tully and Quinn 1985). The protocol for the conditioning with thermo-activation by dTRPA1 was essentially same as standard protocol of olfactory conditioning using electric shock (Tully and Quinn 1985; Schwaerzel, Monastirioti et al. 2003; Thum, Jenett et al. 2007), except that flies were transferred to the pre-warmed T-maze in the climate box only during the presentation of one of the two odorants (60 seconds).

Statistics

Statistical analyses were performed using Prism (GraphPad Software). For the data that did not violate the assumption of the normal distribution and the homogeneity of variance, mean performance indices were compared with t-test, or Newman-Keuls test for all pairs or Bonferroni-post test for selected pairs following one-way or two-way ANOVA. Otherwise, Mann Whitney test (Fig.S4E) or Dunn's multiple comparison test following Kurkal-Wallis-test was applied (Fig. S3I).

Immunohistochemistry

The brain and thoracicoabdominal ganglion were prepared for immunolabeling and analyzed as previously described (Thum, Jenett et al. 2007; Aso, Grubel et al. 2009; Aso, Siwanowicz et al. 2010). Frontal optical sections of whole-mount brains were taken with a confocal microscopy, Olympus FV1000, Leica SP2. For the evaluating the effect of GAL80, brains for comparison were scanned with identical setting. Images of the confocal stacks were analyzed with the open-source software Image-J (NIH).

Supplementary Text

Screening dopaminergic neurons for aversive reinforcement

To indentify novel type of neurons for aversive reinforcement, we screened eight GAL4 driver lines that label distinct set of dopaminergic neurons (Fig.S1). We trained flies by simultaneous odor presentation and transient elevation of temperature to 30°C for 60 seconds, and tested immediately (2 min). The utilized drivers have GAL4 expression not only in the target dopaminergic neurons but also in other neurons. Therefore, when flies formed significant memory, we confirm that observed memory is because of activation of target neurons by co-expressing an inhibitor of GAL4, GAL80, preferentially in the dopaminergic neurons by TH-GAL80 (Sitaraman, Zars et al. 2010).

With two driver that label MB-MP1 (i.e. *MB-GAL80;NP47* and *MB-GAL80;c259*), flies formed very robust aversive memory (Fig.S1A), which is fully consistent with the previous result using other drivers *c061;MB-GAL80* and *NP2758*. *TH-GAL80* silenced GAL4 activity selectively in the dopaminergic neurons in these drivers (Fig. S2A-D), and significantly suppressed dTRPA1-dependent memory. With *5htr1b-GAL4* that labels one MB-V1 and one MB-MV1 in the PPL1 cluster, flies formed a slight but significant aversive memory and it was significantly suppressed by *TH-GAL80* (Fig.S1A). However, it was not possible to conclude which neurons are responsible for the induced memory, because TH-GAL80 silenced virtually all the cells in the brain in this driver (Fig.S2E-F)Therefore, we also tested *Cha*$^{3.3kb}$*-GAL80* that preferentially silences MB-MV1 but largely unchanged expression in MB-V1 and other GAL4-positive cells (Fig.S1D, J and S2G). In combination with *Cha*$^{3.3kb}$*-GAL80, 5-HT1BGAL4/UAS-dTrpA1* flies did not form memory (Fig.S1K). Also with *NP7187* and *MZ840* that label one MB-V1, flies did not form significant memory (Fig.S1A) (Aso, Siwanowicz et al. 2010). Thus, memory induced with *5htr1b-GAL4* is likely to be an effect of MB-MV1 activation. In accordance with this interpretation, calcium imaging revealed that MB-MV1 robustly responds to the electric shock, whereas MB-V1 preferentially responds to odors(Mao and Davis 2009). Though, considering the importance of the vertical lobes in

olfactory learning (Pascual and Preat 2001; Yu, Keene et al. 2005; Yu, Akalal et al. 2006; Gervasi, Tchenio et al. 2010), we preserve the possibility that MB-MV1 may act together with MB-V1 for the formation of odor memory.

With *DDC-GAL4* that labels majority of PAM cluster cells in addition to a few PPL2ab cells and one or two PPL1 cells (Fig.S1F and S2I), flies formed a small appetitive memory (Fig.S1). With *HL9-GAL4* that labels the majority of PAM cluster cells and a few PPL2ab cells (Fig.S1G and S2J), no memory was induced. This is consistent with previous report in which neurons were activated in light-dependent manner (Claridge-Chang, Roorda et al. 2009). With *NP7323* for MB-M2 in the PAM cluster (Fig.S1H and S2K), flies formed a slight but significant aversive memory (Fig.S1A). With *MZ19;Cha3.3kb-GAL80* that label approximately ten PAM cluster cells (Fig.S1I and S2L), activation did not have significant effect (Fig.S1A). These results imply functional heterogeneity of PAM cluster neurons. However, the induced memory can also be attributed to non-dopaminergic GAL4-positive neurons. Because *TH-GAL80* does not express in the majority of PAM cluster cells, we could not distinguish these possibilities. We therefore focused our in depth analysis on three drivers with which flies formed significant memory and that is completely suppressed with TH-GAL80: NP5272 for MB-M3, c061;MB-GAL80 for MB-MP1 and 5htr1b-GAL4 for MB-MV1+MB-V1.

Fig. S1 to Fig. S4

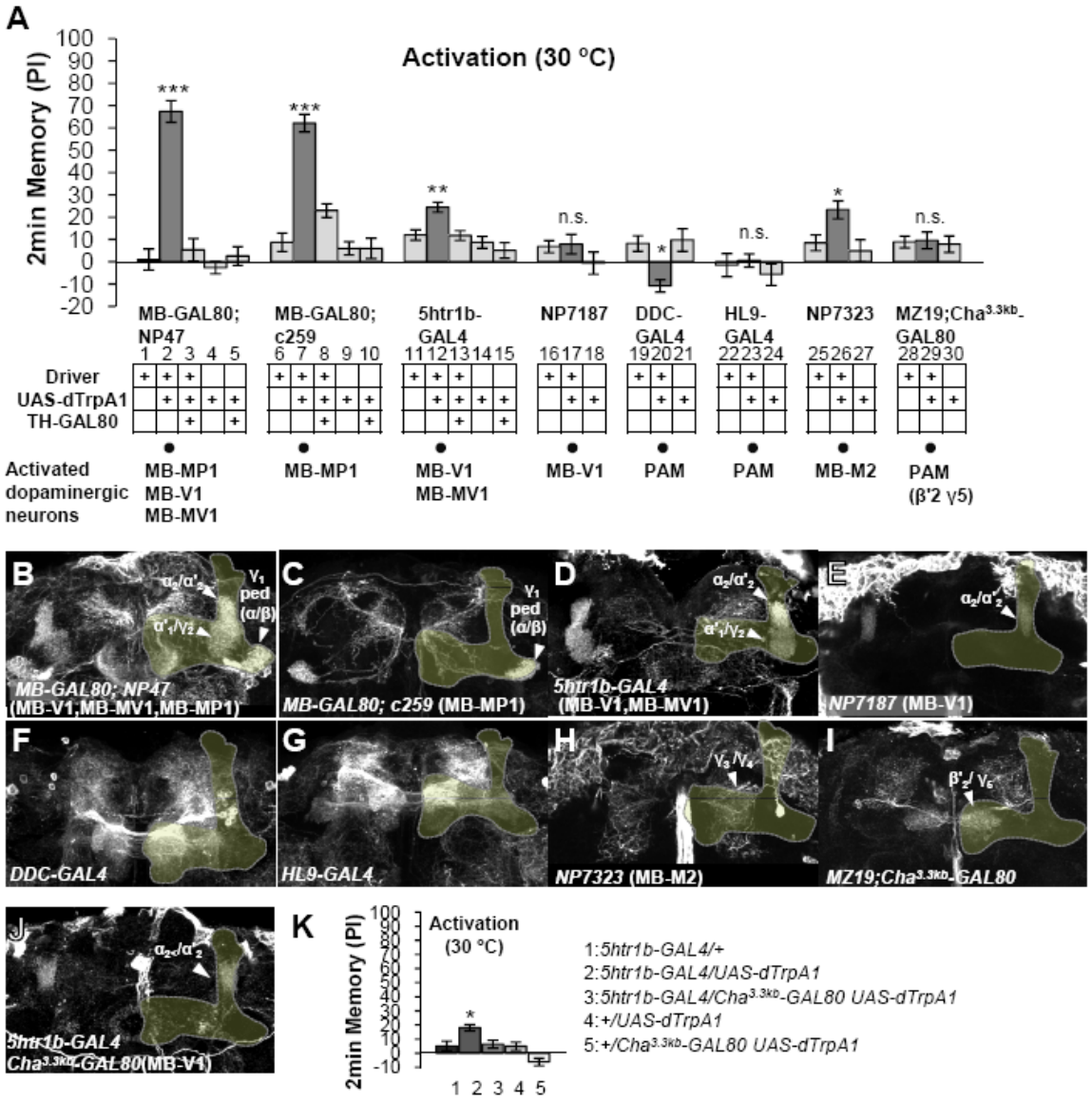

Fig. S1 Screening of driver lines for identifying the dopaminergic neurons for aversive reinforcement.

(A) Flies were conditioned by simultaneous odor presentation and transient temperature shift from 25°C to 30°C for 60 seconds. 2min memory of experimental group was compared with that of control groups (i.e. GAL4/+ , +/UAS-dTrpA1 and GAL4/TH-GAL80 UAS-dTrpA1). The name of targeted dopaminergic neurons are described at the bottom of graph. n=9-20.

(B-I) Projection of targeted dopaminergic neurons visualized with *UAS-mCD8::GFP* and drivers specified in each panel. The light green overlay of the mushroom body is

based on the Synapsin counterstaining. Arrowheads show terminals in the mushroom body. Expression patterns in other regions of brain are show in Fig. S2).

(J) *Cha*$^{3.3kb}$*-GAL80* significantly silenced reporter expression in MB-MV1, but not MB-V1.

(K) *Cha*$^{3.3kb}$*-GAL80* significantly suppressed dTRPA1 dependent memory by 5htr1b-GAL4. n= 16. Bars and error bars represent the mean and s.e.m., respectively. * P<0.05; ** P<0.01; *** P<0.001; n.s. not significant.

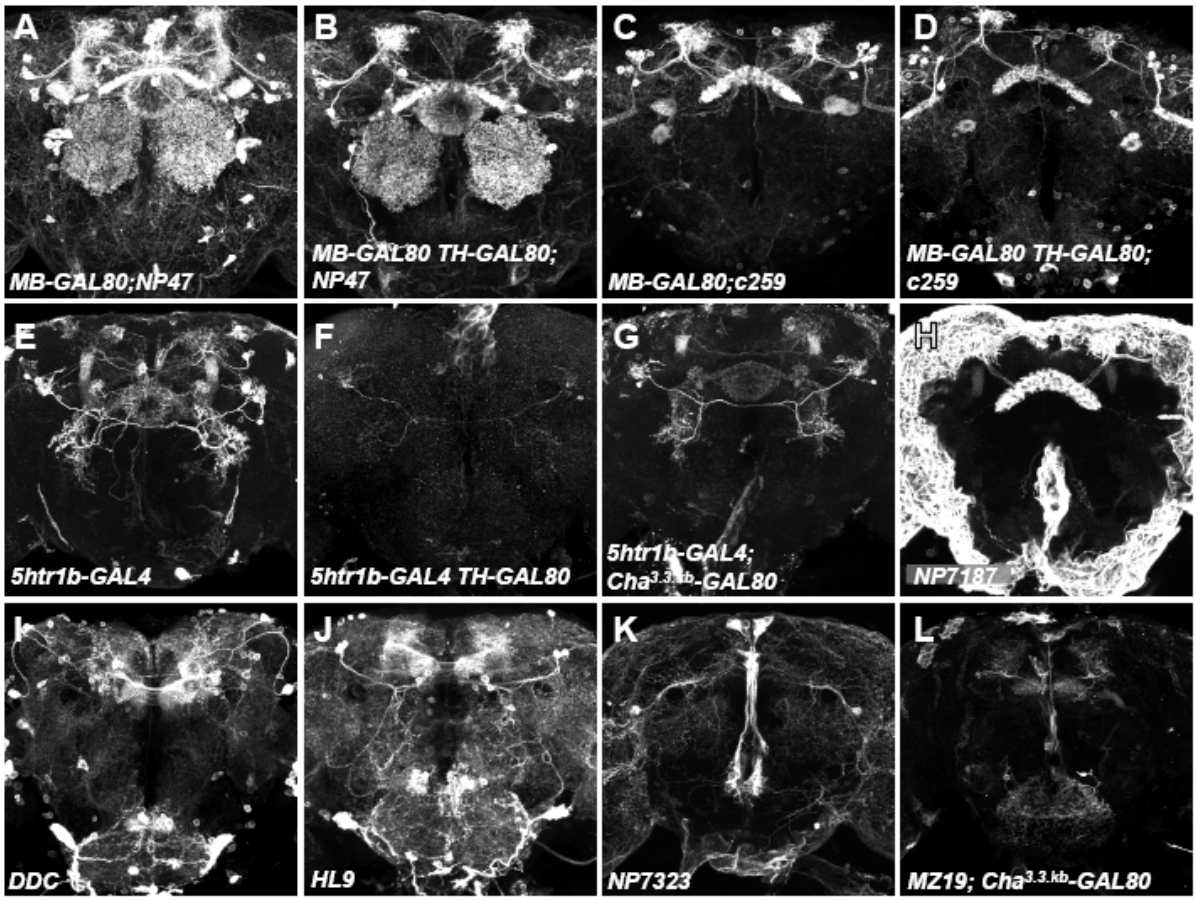

Fig. S2 Expression pattern of GAL4 drivers.

Projection of confocal slices show expression pattern in the brain visualized by UAS-mCD8::GFP and drivers specified in each panel (frontal view, dorsal up). For the drivers with strong expression in the glia, confocal slices of the brain surfaces were not included in the projection for the better presentation of the signal inside the

brain. See the Fig.S1 for the magnified view of the mushroom body and terminals of the target dopaminergic neurons in each driver.

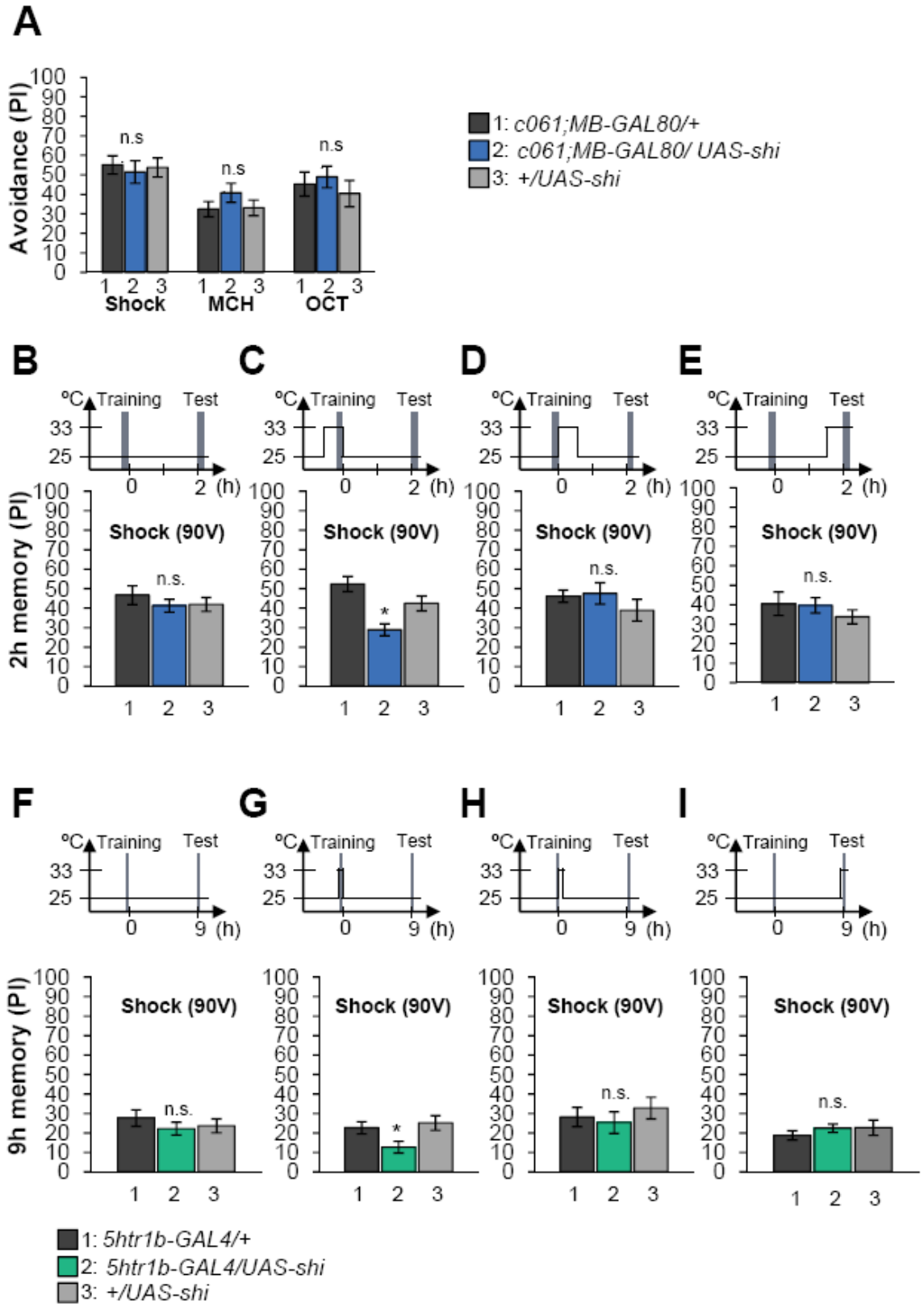

Fig. S3 Requirement of dopaminergic neurons

(A) Blocking MB-MP1 neurons with c061;MB-GAL80 did not impair avoidance of electric shock, MCH or OCT in naïve flies. n=12.

(B-E) c061;MB-GAL80/UAS-shi flies showed impaired 2h memory, only when they were trained at restrictive temperature (C). Experiments at permissive temperature (B), 30 min temperature shift after training (D) or during test (E) did not significantly affect the memory performance. n=12-20

(F-I) At permissive temperature, 5htr1b-GAL4/UAS-shi flies showed indistinguishable memory performance compared to the control genotypes (F). 30 min transient block of neurons in 5htr1b-GAL4/UAS-shi flies during training resulted in a slight but significant impairment of 9 hour memory (G), though the same 30 min blockade immediately after training (H) or during test (I) did not. n= 12-28. Bars and error bars represent the mean and s.e.m., respectively. * $P<0.05$; n.s. not significant.

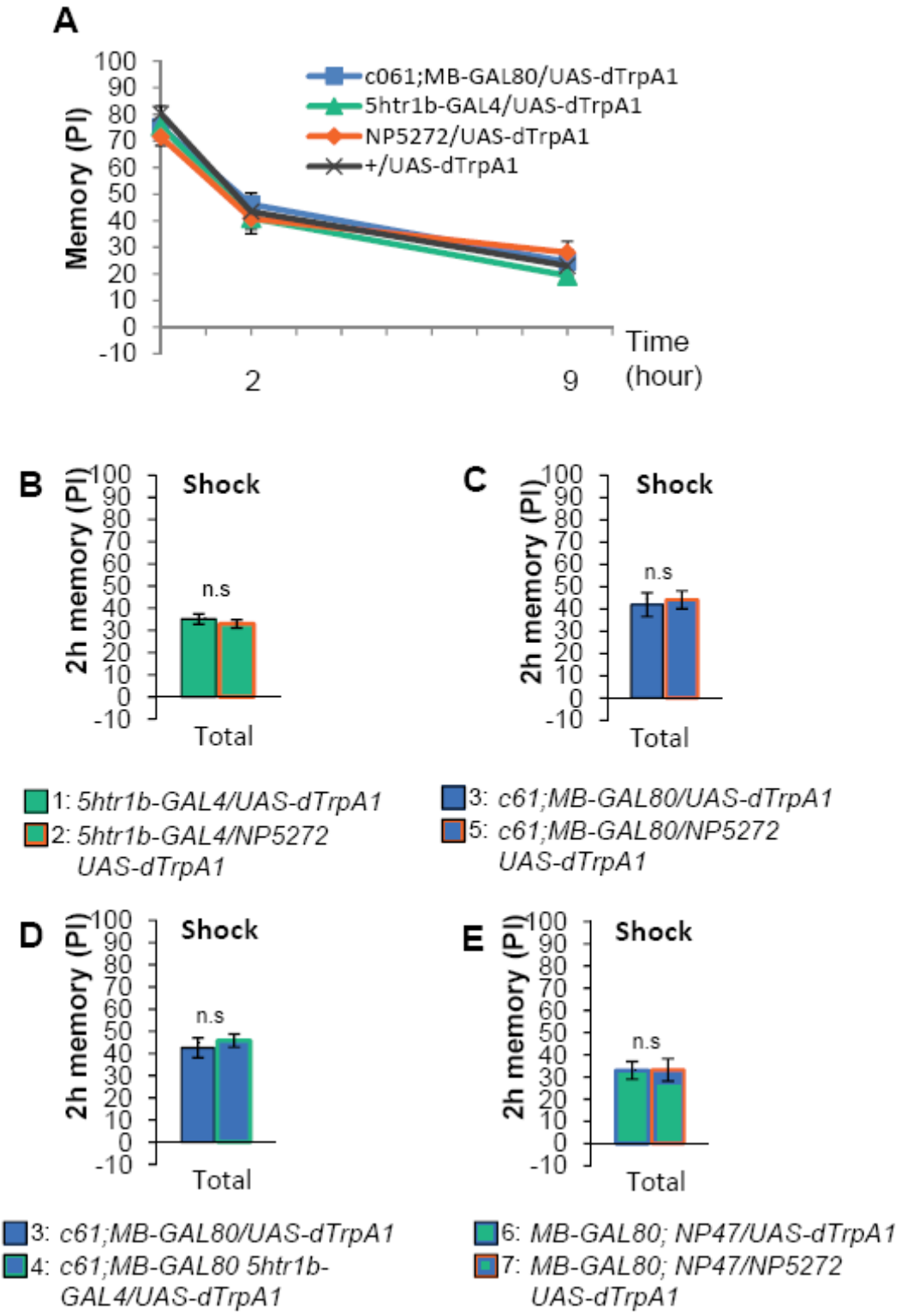

Fig. S4 Effect of dTrpA1 expression at permissive temperature

(A) Flies expressing dTrpA1 with c061;MB-GAL80, 5htr1b-GAL4, NP5272 or no driver showed indistinguishable level of memory at 2min, 2h and 9h, when they were trained with electric shock at permissive temperature (25°C). n=10-14

(B-E) At permissive temperature (25°C), 2h memory induced by electric shock was not significantly different in the flies expressing dTrpA1 with single and combination of drivers. Bars and error bars represent the mean and s.e.m., respectively. n=12-18. Bars and error bars represent the mean and s.e.m., respectively. n.s. not significant.

References and Notes

Aso, Y., K. Grubel, et al. (2009). "The mushroom body of adult Drosophila characterized by GAL4 drivers." J Neurogenet **23**(1-2): 156-172.

Aso, Y., I. Siwanowicz, et al. (2010). "Specific dopaminergic neurons for the formation of labile aversive memory." Curr Biol **20**(16): 1445-1451.

Claridge-Chang, A., R. D. Roorda, et al. (2009). "Writing memories with light-addressable reinforcement circuitry." Cell **139**(2): 405-415.

Gervasi, N., P. Tchenio, et al. (2010). "PKA dynamics in a Drosophila learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase." Neuron **65**(4): 516-529.

Hamada, F. N., M. Rosenzweig, et al. (2008). "An internal thermal sensor controlling temperature preference in Drosophila." Nature **454**(7201): 217-220.

Kitamoto, T. (2002). "Conditional disruption of synaptic transmission induces male-male courtship behavior in Drosophila." Proc Natl Acad Sci U S A **99**(20): 13232-13237.

Krashes, M. J., S. DasGupta, et al. (2009). "A neural circuit mechanism integrating motivational state with memory expression in Drosophila." Cell **139**(2): 416-427.

Lee, T. and L. Luo (1999). "Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis." Neuron **22**(3): 451-461.

Li, H., S. Chaney, et al. (2000). "Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster." Curr Biol **10**(4): 211-214.

Mao, Z. and R. L. Davis (2009). "Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity." Front Neural Circuits **3**: 5.

Pascual, A. and T. Preat (2001). "Localization of long-term memory within the Drosophila mushroom body." Science **294**(5544): 1115-1117.

Schwaerzel, M., M. Monastirioti, et al. (2003). "Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila." J Neurosci **23**(33): 10495-10502.

Sitaraman, D., M. Zars, et al. (2008). "Serotonin is necessary for place memory in *Drosophila*." Proc Natl Acad Sci U S A **105**(14): 5579-5584.

Sitaraman, D., M. Zars, et al. (2010). "Place memory formation in Drosophila is independent of proper octopamine signaling." J Comp Physiol A Neuroethol Sens Neural Behav Physiol **196**(4): 299-305.

Tanaka, N. K., H. Tanimoto, et al. (2008). "Neuronal assemblies of the Drosophila mushroom body." J Comp Neurol **508**(5): 711-755.

Thum, A. S., A. Jenett, et al. (2007). "Multiple memory traces for olfactory reward learning in Drosophila." J Neurosci **27**(41): 11132-11138.

Tully, T. and W. G. Quinn (1985). "Classical conditioning and retention in normal and mutant Drosophila melanogaster." J Comp Physiol [A] **157**(2): 263-277.

Yu, D., D. B. Akalal, et al. (2006). "Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning." Neuron **52**(5): 845-855.

Yu, D., A. C. Keene, et al. (2005). "Drosophila DPM neurons form a delayed and branch-specific memory trace after olfactory classical conditioning." Cell **123**(5): 945-957.

Ritze, Y. 2007. Die Rolle des Neurotransmitters Serotonin bei der Entwicklung von Ethanolsensitivität und Toleranz in Drosophila melanogaster. Dissertation. Universität Würzburg.

6. Curriculum Vitae

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Educational Background

Graduate studies (PhD):

University: Max Planck Institute of Neurobiology
Subjects: Behavioral Genetics
From: To: 01/04/2008-30/09/2010

University: University of Würzburg
Subjects: Behavioral Genetics
From: 01/04/2007 To: 31/03/2008

Graduate studies (Master):

University: University of Heidelberg

Subjects: Molecular and Cellular Biology. Neuroscience
From: 01/10/2005 To: 31/03/2007

University: University of Tokyo
Subjects: Neuroanatomy.
From: 01/04/2005 To: 31/09/2005

Undergraduate studies:
University: Tokyo Kogyo Daigaku (Tokyo Institute of Technology)
Subjects: Molecular and Cellular Biology. Neuroscience
From: 01/04/2001 To: 31/03/2005

Elementary Studies:
High school: Saitama Kenritsu Kasukabe Koto Gakko
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From: 01/04/1997 To: 31/03/2000

Junior high school: Misato-shiritsu Waseda Chugakko
Misato, Saitama Pref. Japan
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Primary School: Misato-shiritsu Zenma Shogakko
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From: 01/04/1988 To: 31/03/1994

Bachelar THESIS
Title: "Conditional destruction of tyrosine hydroxylase gene in mice"

Key words: Tyrosine Hydroxylase, Cre reconmbinase, Dopaminergic neurons
University: Tokyo Kogyo Daigaku (Tokyo Institute of Technology)
Department: Graduate School for Life Science
Town: Tokyo, Japan
Supervisor: Prof Dr. Hiroshi Ichinose
From: 01/04/2004 To: 31/3/2005

MASTER THESIS
Project title: "Activity dependent induction of Gadd45 family genes and promotion of survival in hippocampal neurons"
Key words: gene delivery with rAAV, neuronal cell death, nuclear calcium, CREB
University: University of Heidelberg
Department: Interdisciplinary Centre for Neurosciences
Town: 69120 Heidelberg, Germany

Supervisor: Prof. Dr. Hilmar Bading
From: 10/2006 To: 03/2007

Doctor THESIS

Project title: "Neuronal circuit underlying olfactory learning in Drosophila"
Key words: mushroom body, appetitive and aversive memory, dopamine
University: University of Würzburg/ Max Planck Institute of Neurobiology
Department: Behavioral Genetics
Town: Würzburg / Heidelberg, Germany
Supervisor: Prof. Martin Heisenberg/ Dr. Hiromu Tanimoto
From: 04/2007 To: 09/2010

PRACTICAL TRAINING

University: University of Heidelberg
Department: Interdisciplinary Center for Neurosciences
Supervisor: Prof. Dr. Hilmar Bading
Title of course: "Activity dependent neuroprotection" (4 months)

University: University of Heidelberg
Department: Interdisciplinary Centre for Neurosciences
Supervisor: Dr. Francesca Ciccolini
Title of course: "Calcium imaging of neuronal precursors" (5 weeks)

University: University of Heidelberg
Department: Interdisciplinary Center for Neurosciences
Supervisor: Prof. Dr. Christoph M. Shuster
Title of course: "Establishment of methods to record NO production at Drosophila neuromuscular junctions" (3 months)

University: University of Würzburg
Department: Department of Genetics and Neurobiology
Supervisor: Dr. Hiromu Tanimoto
Title of course: "Relation between taste preference and reward quality" (4 weeks)

University: University of Tokyo
Department: Institute for Molecular and Cellular Biosciences
Supervisor: Prof. Kei Ito
Title of course: "Identification of clonal units forming the Drosophila larval brain" (6months)

Presentation

Poster presentation

- Neurofly (06-11/09/2008; Würzburg, Germany)"
- Learning and memory (09-11/11/2008; Janelia Farm, USA)"

Oral presentation

- Heidelberg-Göttingen meeting (12/06/2006; Heidelberg)
- Drosophila Regional Meeting (09-10/10/2008; Martinsried, Germany)
- Insect Learning and Memory Workshop (17-20/06/2009;Roscoff, France)
- Japan Drosophila Research Conference (06-08/07/2009; Kakegawa, Japan)
- Behaviour and Physiology of Memory in Insects(11-13/12/2009; Martinsried, Germany)

Academic Honors

Title:	Scholarship for short-term scientific activities on abroad from Tokyo Institute of Technology
Date received:	January 2005

Title:	Scholarship for PhD study from DAAD.
Date received	April 2007-September 2010

7. List of Publications

Aso Y, Grubel K, Busch S, Friedrich AB, Siwanowicz I, Tanimoto H. The mushroom body of adult Drosophila characterized by GAL4 drivers. J Neurogenet 2009;23:156-72.

Zhang SJ, Zou M, Lu L, Lau D, Ditzel DA, Delucinge-Vivier C, Aso Y, Descombes P, Bading H. Nuclear calcium signaling controls expression of a large gene pool: identification of a gene program for acquired neuroprotection induced by synaptic activity. PLoS Genet. 2009 Aug;5(8):e1000604. Epub 2009 Aug 14.

Aso Y, Siwanowicz I, Bräcker L, Ito K, Kitamoto T, Tanimoto H. Specific Dopaminergic Neurons for the Formation of Labile Aversive Memory. Current Biology (2010), 20, 1-7, August 24

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I deeply appreciate advices from the thesis committee members: Prof. Martin Heisenberg, Prof. Mark Hübener, Dr. Ilona Kadow.

Many thanks to the co-authors of the publications and the manuscripts contained in this thesis. Especially, Prof. Kei Ito introduced me an exciting circuit level research using *Drosophila* as a former mentor during my master study. Also, without his suggestion to visit Dr. Tanimoto and Prof. Heisenberg, I did not initiate the present work.

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