学位論文（要約）

Functional analysis of a mushroom body output neuron involved in olfactory memory in *Drosophila*

（ショウジョウバエ嗅覚記憶に関わるキノコ体出力神経の機能解析）

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Table of contents

Abbreviations

MB : Mushroom body

KC : Kenyon cell

MBON : Mushroom body output neuron

DAN : Dopaminergic neuron

CS : Conditioned stimulus

US : Unconditioned stimulus

OCT : 3-octanol

MCH : 4-methylcyclohexanol

STM : short-term memory

MTM : mid-term memory

LTM : long-term memory

BGAM : blockade-of-MBON-γ1pedc-induced aversive memory

ES : electric shock

Abstract

I have analyzed the functions of the *Drosophila melanogaster* neural circuits involved in olfactory aversive memory formation, in which odor and electric shocks are associated. The structure of the *Drosophila* brain called the mushroom body (MB) is thought to be the center for olfactory memory. The MB consists of ~2,000 olfactory tertiary neurons called Kenyon cells (KCs), dopaminergic neurons (DANs), and MB output neurons (MBONs). KCs are postsynaptic to DANs and presynaptic to MBONs. These neurons interact with each other and control memory processes, but the functions and circuit mechanisms have not been fully elucidated. I found that (1) MBON-γ1pedc, an MBON postsynaptic to γ and αβ KCs, was required for both the memory acquisition and retrieval processes. (2) Suppression of the MBON-γ1pedc activity formed an aversive olfactory memory (blockade-of-MBON-γ1pedc-induced aversive memory, BGAM), even without electric shocks. (3) BGAM required the activities of DANs. This indicates that the MBONs, which were thought to be downstream of DANs, can modify the effects of DANs. Taken together, KCs, DANs, and MBONs consist of multi-layered circuits and control each other's effects, thereby regulating the memory formation process.

1. INTRODUCTION

Animals survive and prosper by altering their behaviors according to various external environments. This experience-dependent behavioral plasticity is called memory, and is observed in a wide range of vertebrate and invertebrate species. Memory involves several processes, including acquisition, consolidation and retrieval, and the functions of the neurons and circuits involved in each process have been examined in various species. However, it is still unclear where and how the information is processed in neural circuits to regulate the memory formation, consolidation and retrieval.

A simple experimental animal, the fruit fly *Drosophila melanogaster*, is ideal to study the details of neuronal functions in the memory processes, for the following reasons.

1) Various memory experiments, including classical conditioning, are available (Quinn et al., 1974).

2) The number of neurons is as small as on the order of 100,000 in the whole brain, and thus it is easy to analyze the neuronal functions at a cellular level, as compared to vertebrates.

3) Artificial manipulation of neurons at the single cell level is possible (Venken et al., 2011).

In particular, the neurons pre- and postsynaptic to the mushroom body (MB), the Kenyon cells (KCs), have been extensively mapped anatomically. Their functions in olfactory memory have been studied, since KCs are thought to encode odor information and function as a coincidence detector when olfactory memory is formed (Heisenberg et al., 1985; Heisenberg, 2003). Olfactory memory has long been studied in *Drosophila*, and is the first experimental system in which the memory formation process has been genetically dissected (Davis, 2005). In this study, I aimed to clarify the function of a type of neuron postsynaptic to KCs in olfactory aversive memory.

1-1. Classical olfactory conditioning

In this study, Pavlovian classical conditioning was used, since it has been extensively studied in the field of *Drosophila* memory. In classical conditioning, a conditioned stimulus (CS) and an unconditioned stimulus (US) are associated when presented simultaneously. The US has biologically important value for survival or prosperity, and the CS has the ability to trigger a new response that is observed after the association with the US, but not before the association. The olfactory or visual stimuli are used as the CS and several distinct stimuli including electric shocks, sucrose ingestion and bitter taste can be used experimentally as the US in typical classical conditioning in *Drosophila* (Quinn et al., 1974; Siegel and Hall, 1979; Tempel et al., 1984; Schnaitmann et al., 2010; Kaun et al., 2011; Lin et al., 2014b). However, the mechanisms by which flies select the US or tune the threshold for accepting a stimulus as the US remain largely unknown. In this study, the functions of neural circuits involved in the olfactory aversive memory in *Drosophila* were examined, by using odors as the CS and electric shocks (ESs) as the US. Based on the results, I have proposed a mechanism for the tuning of the threshold for the US.

An odor (CS) is presented to flies simultaneously with electric shocks (US), followed by the presentation of another odor (CS) without the US. In this paradigm, the CS associated with the US is called CS+ and the other CS presented alone is called CS- (Figure 1). After the conditioning, flies are allowed to choose which of the two odors to approach or avoid. The numbers of flies on each side of the test tubes are counted, and the Performance Index is calculated. The memory acquired by this protocol is classified by the retention time, as short-term memory (STM), mid-term memory (MTM) and long-term memory (LTM). In *Drosophila*, STM is usually a memory retrieved a few minutes~30 min after memory

acquisition, MTM is 2~3 hr memory and LTM is over 1 day memory. In this study, STM (~5 min memory) and MTM (~2 hr memory) were examined.

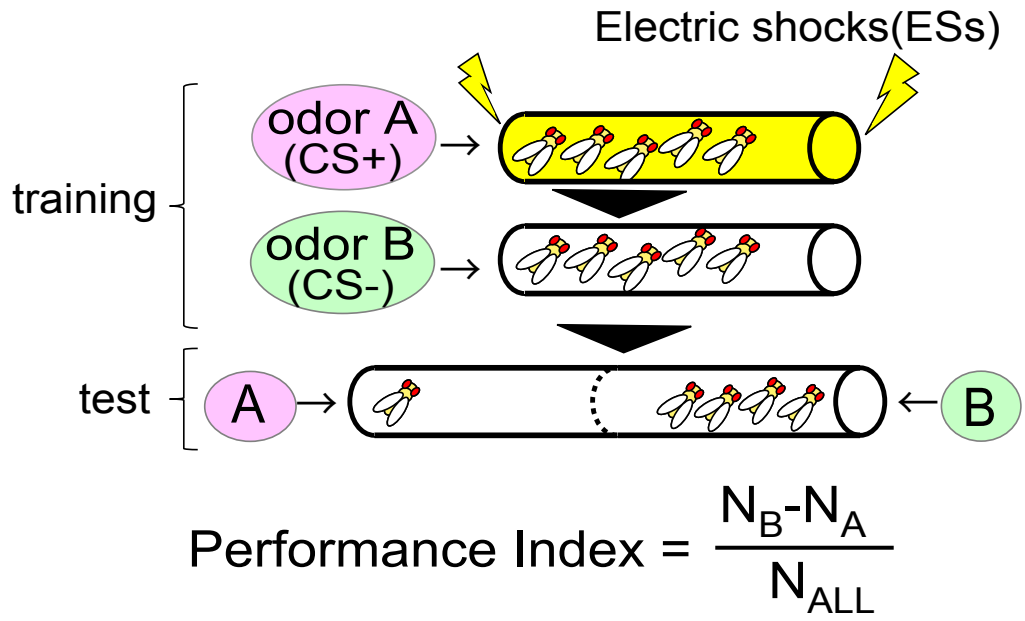


Figure 1. Olfactory aversive conditioning in *Drosophila* and evaluation

After the CS+ odor is presented with ESs and CS- without ESs, flies avoid the CS+ odor. The numbers of flies attracted to the CS+/- odors were used to calculate the Performance Index.

1-2. Mushroom body

The neuropil called the mushroom body (MB) has been extensively studied anatomically (Tanaka et al., 2008; Aso et al., 2009; 2014b) and functionally, as the center for olfactory aversive memory (Heisenberg et al., 1985; Davis, 2005; McGuire et al., 2005; Waddell, 2013). The MB consists of dopaminergic neurons (DANs), mushroom body output neurons (MBONs) and ~2,000 intrinsic neurons called Kenyon cells (KCs) in each hemisphere (Aso et al., 2009) (Figure 2B).

Odor presentation activates subsets of KCs sparsely (Turner et al., 2008; Honegger et al., 2011; Campbell et al., 2013; Lin et al., 2014a), and the pattern of activated neurons (~100) in the ~2,000 KCs represents the odor information. The odor information is modified by aversive stimuli conveyed by dopaminergic neurons (DANs) upon the classical olfactory conditioning (Davis, 2005; Claridge-Chang et al., 2009; Aso et al., 2010; Perisse et al., 2013a). The modified information then converges on the MB output neurons (MBONs) (Séjourné et al., 2011; Aso et al., 2014b), and the output from the MBONs controls the memory-based behavior (Séjourné et al., 2011; Aso et al., 2014a) (Figure 2B).

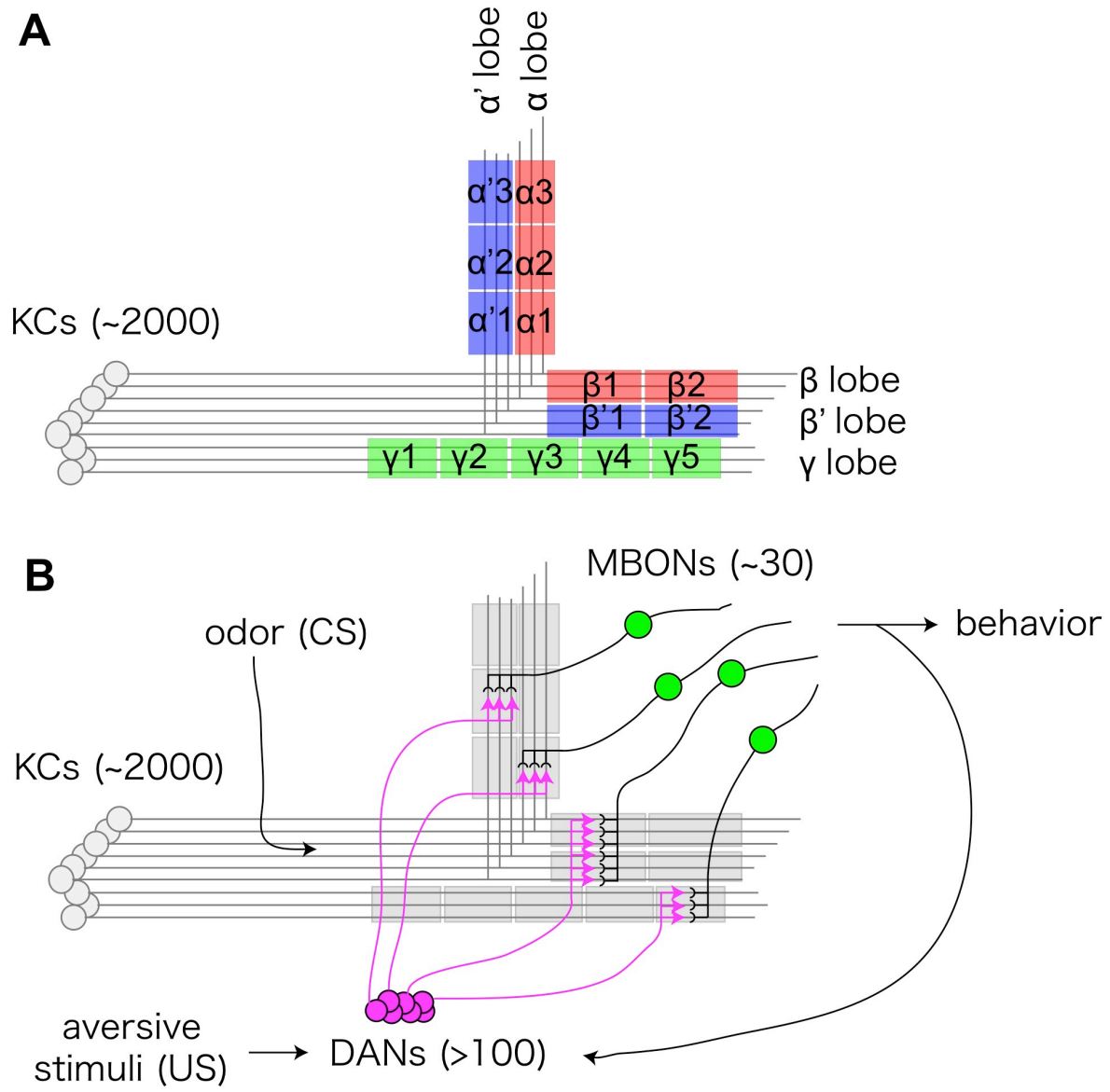


Figure 2. KCs, DANs and MBONs comprise the MB neuronal circuit

(A) Construction of Kenyon cells (KCs). αβ, α'β', and γ cells constitute the α, β, α', β' and γ lobes, and the lobes are divided into 15 compartments.

(B) Circuits around the KCs. The compartments are projected by specific dopaminergic neurons (DANs) and specific mushroom body output neurons (MBONs).

1-3. Kenyon cells

Kenyon cells (KCs) are intrinsic neurons of the MB. Odorant molecules bind to odorant receptors expressed in the primary olfactory neurons (Goldman et al., 2005). The odor information is conveyed randomly to KCs via the secondary olfactory neurons, projection neurons (Caron et al., 2013), and the information is sparsely represented in KCs (Turner et al., 2008; Honegger et al., 2011; Campbell et al., 2013; Lin et al., 2014a). KCs have cell bodies on the posterior side and project their axons towards the anterior side. The KCs are classified into α/β, α'/β', and γ cells anatomically and developmentally. After branching at the heel, α/β cells construct the α lobe at the vertical (dorsal) part and the β lobe at the horizontal (medial) part, while α'/β' cells similarly form the α' lobe at the vertical part and the β' lobe at the horizontal part. The γ cells do not branch, and are projected only in the horizontal direction. These lobes are variously involved in the formation of different memories (Yu et al., 2006; Krashes et al., 2007; Trannoy et al., 2011; Qin et al., 2012; Perisse et al., 2013b) (Figure 2A).

Each lobe is further subdivided along the longitudinal axis into compartments (Figure 2A, B), into which a specific set of DANs and MBONs project and form a microcircuit (Mao and Davis, 2009; Aso et al., 2014b). Altogether, 15 compartments tile the entire MB lobes, and each compartment is proposed to perform a distinct function in various processes of memory formation (Perisse et al., 2013a; Aso et al., 2014a; 2014b) .

1-4. Dopaminergic neurons

There are 20 types of DANs projecting their axons into each compartment of the lobe, and they consist of 3 clusters (PAM, PPL1, and PPL2ab) (Mao and Davis, 2009; Aso et al., 2014b) . In particular, PAM is necessary for reward learning using sucrose as the US (Liu et al., 2012; Aso et al., 2014a) , and PPL1 is necessary for aversive learning using ESs as the US (Aso et al., 2010; 2012; 2014a). These DANs project to specific compartments and modify the input from KCs to MBONs in each compartment, and this synaptic plasticity results in behavioral plasticity (Tomchik and Davis, 2009; Gervasi et al., 2010; Boto et al., 2014). Since the simultaneous presentation of an odor conveyed from KCs and the US conveyed from DANs changes the response to the odor co-presented with the US, the simultaneous activation of KCs and DANs changes the synaptic output from KCs.

In addition, the DAN activity is dynamically changed by external stimuli or internal physiological states (Plaçais et al., 2012; Berry et al., 2015; Cohn et al., 2015), and the output from MBONs is also known to affect the DAN activity (Cohn et al., 2015), suggesting that the circuits consisting of KCs, DANs and MBONs form dynamic neuronal networks including multiple layers of feedforward and feedback regulation.

1-5. Mushroom body output neurons

Recent anatomical studies on the cellular identification of MBONs have provided intriguing information. They revealed that the odor information represented by ~2,000 KCs converges on only 34 MBONs, composed of 21 anatomically distinct cell types (Aso et al., 2014b). This result permits the study of the neuronal mechanisms underlying odor coding and olfactory memory formation in the reduced dimension, at the level of fourth-order olfactory neurons. Specifically, split-Gal4 drivers identifying each MBON at a cellular resolution enable the manipulation of each MBON (Aso et al., 2014a; 2014b). Thus I have started to witness the progress in understanding the roles of MBONs in the memory (Séjourné et al., 2011; Aso et al., 2014b; Bouzaiane et al., 2015; Owald et al., 2015; Hige et al., 2015b).

I focused on the role of the MBON called MBON-γ1pedc, because it reportedly plays a pivotal role in aversive memory (Aso et al., 2014a; Perisse et al., 2016), and memory traces were also observed in MBON-γ1pedc after the association of odors with electric shocks or the activation of DANs (Hige et al., 2015a; Perisse et al., 2016). In addition, MBON-γ1pedc reflects the internal and physiological states of flies, and inhibits the activities of other MBONs (Perisse et al., 2016). These previous reports suggested the possibility that MBON-γ1pedc plays multiple roles in memory formation, and I found that MBON-γ1pedc is required for the acquisition of memory. Furthermore, during memory formation, MBON-γ1pedc suppresses the acquisition of aversive memory for CS-, but not for CS+.

2. Materials and methods

2-1. Fly strains

All flies were raised on standard cornmeal-agar food at 25ºC. In this study, the following strains were used.

CS10 (*w1118* backcrossed by *Canton-S* for 10 generations) : A control strain in this study.

MB112C : A split-Gal4 strain expressing Gal4 protein in MBON-γ1pedc. Unless otherwise indicated, this strain was used as an MBON-γ1pedc specific driver in this study.

MB060B : A split-Gal4 strain expressing Gal4 protein in PPL1-γ2α'1, α'2α2, α3, α'3.

MB504B : A split-Gal4 strain expressing Gal4 protein in PPL1-γ1pedc, γ2α'1, α'2α2, α3.

MB438B : A split-Gal4 strain expressing Gal4 protein in PPL1-γ1pedc, α'2α2, α3.

The generation and basic characterization of the split-Gal4 strains have been described (Aso et al., 2014b) and provided by the Rubin lab. Further information is available at https://www.janelia.org/split-gal4.

R83A12 : A Gal4 strain expressing Gal4 protein in MBON-γ1pedc (Perisse et al., 2016), from Bloomington (#40348).

TH-Gal4 : A Gal4 strain expressing Gal4 protein in dopaminergic neurons (Friggi-Grelin et al., 2003), provided by M. Heisenberg.

pJFRC99-20XUAS-IVS-Syn21-Shibire-ts1-p10 in VK00005 : A UAS strain expressing the temperature sensitive dominant negative form of dynamin Shi^{ts} downstream of UAS (Pfeiffer et al., 2012), from HHMI Janelia Farm Fly Facility.

UAS-dTrpA1 in attP16 : A UAS strain expressing the temperature-sensitive cation channel downstream of UAS (Hamada et al., 2008), from Bloomington (#26263).

UAS-GCaMP5 : A strain expressing the calcium indicator GCaMP5 downstream of UAS (Akerboom et al., 2012), from HHMI Janelia Farm Fly Facility (Bloomington #42037 for *UAS-GCaMP5 attP40*, #42038 for *UAS-GCaMP5 VK0005*).

UAS-myr::tdTomato in su(Hw)attP8 : A strain expressing the membrane-localized tdTomato variant of RFP downstream of UAS (http://flybase.org/reports/FBrf0212441.html), from Bloomington (#32223).

UAS-mCherry.NLS: A strain expressing the nuclear-localized mCherry variant of RFP downstream of UAS (http://flybase.org/reports/FBrf0218019.html), from Bloomington (#38424).

UAS-mCD8::GFP : A strain expressing the membrane-localized GFP (Lee and Luo, 1999), from Bloomington (#5130).

mb247dsRed : A strain expressing the RFP in the mushroom body (Riemensperger et al., 2005), provided by A. Fiala.

2-2. Gal4/UAS system

By using the Gal4/UAS system, arbitrary genes located downstream of the UAS are expressed depending on the expression of the Gal4 protein (Brand and Perrimon, 1993) (Figure 3A). Gal4 is a yeast-derived transcription factor, and the UAS (Upstream Activating Sequence) is its target sequence. In the Gal4 strain, the promoter or enhancer controls the expression of the Gal4 protein, which is expressed in the target tissue or at the specific developmental stage. The UAS strain is a strain in which the cDNA sequence of the target gene is integrated downstream of the UAS sequence. By crossing the Gal4 strain and the UAS strain, arbitrary genes are expressed dependent on the spatiotemporal expression pattern of the Gal4 protein in the next generation.

In this study, the temperature sensitive dominant negative form of dynamin, Shi^{ts} (Kitamoto, 2001; Pfeiffer et al., 2012), expressed by the Gal4/UAS system, was used to block the synaptic output from the neurons expressing Shi^{ts} during behavioral experiments. The synaptic output from neurons expressing Shi^{ts} is blocked only at the restrictive temperature (33ºC), and not at the permissive temperature (22ºC) (Figure 3B). This blockade of the synaptic output is reversible, and the blockade of the neurons is relieved when the neurons are returned to the permissive temperature.

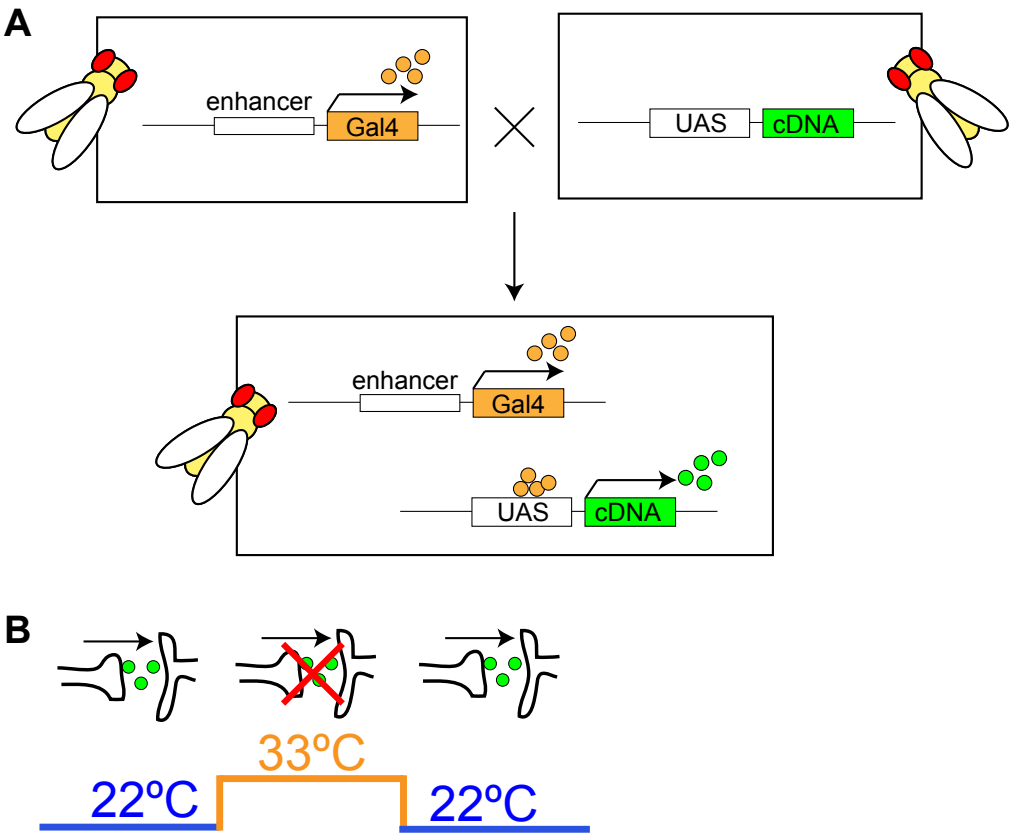


Figure 3. Neuronal manipulation using Gal4/UAS system

(A) Specific promoters or enhancers express Gal4 protein in specific neurons. The proteins encoded by the genes under the control of the UAS sequence are expressed by Gal4 protein binding to the UAS.

(B) Synaptic output from neurons expressing Shi^{ts} is suppressed at the restrictive temperature (33ºC) and restored at the permissive temperature (22ºC).

2-3. Behavioral experiments

Groups of ~50 flies (2~5 days old, both male and female) raised under a 12 hr:12 hr light-dark cycle were used for one trial in behavioral experiments. Before the behavior experiments, flies were kept in vials with Kimwipes soaked with sucrose solution. The training and test apparatus were the same as those described previously (Tully and Quinn, 1985), and the protocols were slightly modified. Flies were exposed to 60 s CS+ odor (MCH or OCT) with 12 90 V electric shocks at a 5 s interstimulus interval, 30 s clean air, followed by CS- odor (OCT or MCH) without electric shocks. After the training stage, flies were allowed to select either the CS+ or CS- odor in a T-maze at the test stage. The odors were placed in a glass 'odor cup' (8 mm diameter for OCT and 10 mm for MCH) sitting in the middle of an odor stream. The flow velocities of air and odors were 0.75 L/min in each stage.

Temperature shift

To shift the temperature between the permissive temperature (22ºC) and the restrictive temperature (33ºC), I used two climate boxes set to 22ºC or 33ºC, and all of the training tubes and T-mazes were pre-heated and set at the temperatures. The temperature shift was performed immediately. After the transfer, the flies were left in a tube with airflow at the desired temperature.

Blockade-of-MBON-γ1pedc-induced aversive memory (BGAM) training and test

Flies were exposed to 60 s odor1 at 22ºC, instantly transferred to 33ºC followed by 2 min air flow and exposed to 60 s odor2 at 33ºC, instantly re-transferred to 22ºC followed by 2 min air flow and tested at 22ºC.

Test stage

Flies were loaded into the T-maze and allowed to choose between MCH and OCT for 1.5 min. The performance index was calculated as the number of flies avoiding the CS+ odors (or odors presented at 33ºC for BGAM) minus the number of flies on the other side, divided by the total number of flies. Flies were reciprocally trained with MCH or OCT and control odors (OCT or MCH) were also presented, and two performance indices for MCH or OCT were calculated. The final performance index was calculated by averaging the two performance indices for MCH or OCT.

2-4. Immunohistochemistry

Flies were dissected in a cold phosphate-buffered saline (PBS) solution, and the brains were fixed in PBT (PBS containing 0.3% TritonX-100) with 4% formaldehyde for 30 min at room temperature. After washing with PBT, the PBT was replaced by blocking solution (10 % Donkey normal serum in PBT), and the brains were blocked for 60 min at room temperature. The blocking solution was replaced by the first antibody solution and reacted for over 1 day. Subsequently, the unreacted first antibody was removed by a wash with PBT, and the PBT was replaced by the second antibody and reacted for 1 day. After the second antibody reaction, the brains were washed with PBT, and the PBT was replaced by PBS. The PBS-soaked brains were placed between a slide glass and a cover glass with medium (VECTASHIELD Mounting Medium, Vecor Laboratories).

Images were captured on a Zeiss LSM 710 (Carl-Zeiss, Jena, Germany) confocal microscope, and brightness was linearly processed by using the Fiji software (http://fiji.sc/Fiji).

Antibodies used in these experiments

mouse anti-nc82 (Developmental Studies Hybridoma Bank (DSHB)) (1:200)

anti-mouse Dylight649 (Jackson ImmunoResearch) (1:200)

2-5. Two photon *in vivo* live imaging

Live Ca^{2+} imaging in the brain was performed as described in our previous study (Hiroi et al., 2013). To visualize MBON-γ1pedc Ca^{2+}, *MB112C* flies were crossed to *UAS-myr::tdTomato; UAS-GCaMP5; UAS-GCaMP5* flies or *mb247-dsRed, UAS-GCaMP5; UAS-GCaMP5* flies. The 2-5 day old flies were transferred to a fresh food vial prior to the experiments. Only females were used in this experiment. A fly was anesthetized on ice (~3 min) and fixed in a custom-made recording chamber. A small piece of cuticle from the dorsal head capsule was removed with forceps under a dissection microscope. During the brain imaging, the chamber was perfused with Adult hemolymph-like (AHL) solution, composed of 103 NaCl, 3 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 4 $MgCl_2$, 10 D-glucose, 10 trehalose, 9 sucrose, 5 HEPES (in mM, pH 7.2-7.4, 280-290 mOsm).

Stimulation under microscope

While the fly was mounted under the microscope, a constant stream of charcoal-filtered air (1 L/min) was directed at the fly. Odorants were placed in a glass 'odor cup' (8 mm in diameter for OCT and 10 mm for MCH), which sits in the middle of the odor stream. After a trigger, a solenoid valve redirects a portion of the air stream (0.2 L/min) through the headspace of the 'odor cup' for 3 s, and the odor stream is joined with the other air stream (0.8 L/min). For delivering aversive stimuli to flies, a pair of platinum wires was attached to the abdomen and 12x electric shock pulses (~20 pA, 200 msec) were applied at 0.2 Hz.

Image acquisition and stimuli sequence

A multi-photon laser-scanning microscope (Olympus FVMPE-RS; Olympus, Tokyo, Japan) was equipped with a water-immersion objective (25 x N.A. 1.05). GCaMP5 was excited at 910 nm (Mai Tai HP DeepSee-OL; Spectra-Physics, Santa Clara, CA, USA), and the emission light was collected with a GaAsP photomultiplier detector using a dichroic mirror (DM690), an emission filter (SDM570) and two band-pass filters (BA575-645 for red and BA495-540 for green).

A dendrite of the MBON-γ1pedc in one hemisphere was randomly selected per individual fly and scanned at a resolution of 0.28 μm/pixel (256 x 256 x 1 pixels) at 410 ms/frame. At the pre-and post-training phases, flies were exposed to 3 s odor1 (OCT or MCH), 30 s clean air, followed by 3 s odor2 (MCH or OCT, which was not odor1). This odor sequence was repeated 3 times. At the training phase, flies were exposed to 60 s CS+ odor with 12 electric shocks, 30 s clean air, followed by 60 s CS- odor.

Image analysis

All of the acquired images were processed with the Fiji software (http://fiji.sc/Fiji). To stabilize the objects in the acquired images, all of the images from the same fly were aligned by template matching (template matching plugin, https://sites.google.com/site/qingzongtseng/template-matching-ij-plugin). After the stabilization, the dendrite of MBON-γ1pedc was used as the region of interest (ROI) in each animal. The fluorescence ratio (F) was calculated by averaging the fluorescence of GCaMP5 in the ROI or dividing the fluorescence of GCaMP5 by the fluorescence of myr::tdTomato. The baseline F0 was calculated as the mean of 3 sec prior to each stimulation. F0 was then used to compute the relative change in fluorescence ($\Delta F/F0 = (F(t) - F0)/F0$). To compare the odor responses in the pre-and post-training phases, the odor responses were normalized by dividing the peak of

ΔF/F0 of the post-odor response by ΔF/F0 of the pre-odor response. After the normalization, the normalized peak ΔF/F0 values of CS+ odor and CS- odor were compared.

2-6. Statistical analysis

Statistical analyses were performed using Prism6 (GraphPad, La Jolla, CA, United States). All behavior data were tested for normality and for homogeneity of variance (Bartlett's test). If the total number of data points in a set of behavior experiments was larger than 7, then the D'Agostino & Pearson omnibus normality test was performed. If the smallest number of data points in a figure was 7 or 6, then I performed the Shapiro–Wilk normality test or the Kalmogorov-Smirnov normality test, respectively.

Gaussian distributed data were analyzed with one-way ANOVA followed by Tukey's honest significant difference (HSD) post hoc test, except for Figures 11B, C. Data in Figures 11B, C were analyzed with one-way ANOVA followed by Dunnett's test with comparison to MB112C/UAS-Shi^{ts}.

For non-Gaussian distributed data, the Kruskall-Wallis test was performed followed by Dunn's multiple comparison test.

Definition of statistical significance was set at $p < 0.05$. All bar graphs were created with the Prism 6 software.

3. Results

3-1. Functional analysis of MBON-γ1pedc in short-term memory

Previous behavioral and imaging experiments suggested that MBON-γ1pedc is involved in aversive memory (Aso et al., 2014a). Therefore, I analyzed MBON-γ1pedc by detailed behavioral experiments in order to investigate the functions of MBON-γ1pedc related to the memory processes. I first examined the role of MBON-γ1pedc in a 2 min short-term memory (STM) experiment.

MBON-γ1pedc is required for both the acquisition and retrieval of the aversive short-term memory

I used an MBON-γ1pedc-specific split-Gal4 driver, MB112C (Figure 4B, C) (Aso et al., 2014a; 2014b) to express the temperature sensitive dominant negative form of dynamin Shi^{ts} (Kitamoto, 2001; Pfeiffer et al., 2012), to block the output from MBON-γ1pedc. Flies were exposed to an odor, 4-methylcyclohexanol (MCH) or 3-octanol (OCT) paired with 12 electric shocks for 1 min (CS+), followed by OCT (or MCH) without electric shocks for 1 min (CS-) (Figure 4A). Two min later, flies were allowed to select one of the two odors to avoid. Flies were trained and tested at either the restrictive temperature (33ºC) (Figure 4D) or permissive temperature (22ºC) (Figure 4E) throughout the experiments. Blocking MBON-γ1pedc severely impaired STM, and thus the MBON-γ1pedc output is indispensable for STM, as previously reported for 2 hr memory (Aso et al., 2014a). To clarify whether this STM defect was caused by the impairment of memory acquisition or retrieval, I blocked the output from MBON-γ1pedc during the acquisition or retrieval stage. The blockade of MBON-γ1pedc only during the training stage impaired STM (Figure 4F) as well as the retrieval in the test stage

(Figure 4G). These results suggest that MBON-γ1pedc is required for the aversive memory acquisition in addition to the aversive memory retrieval.

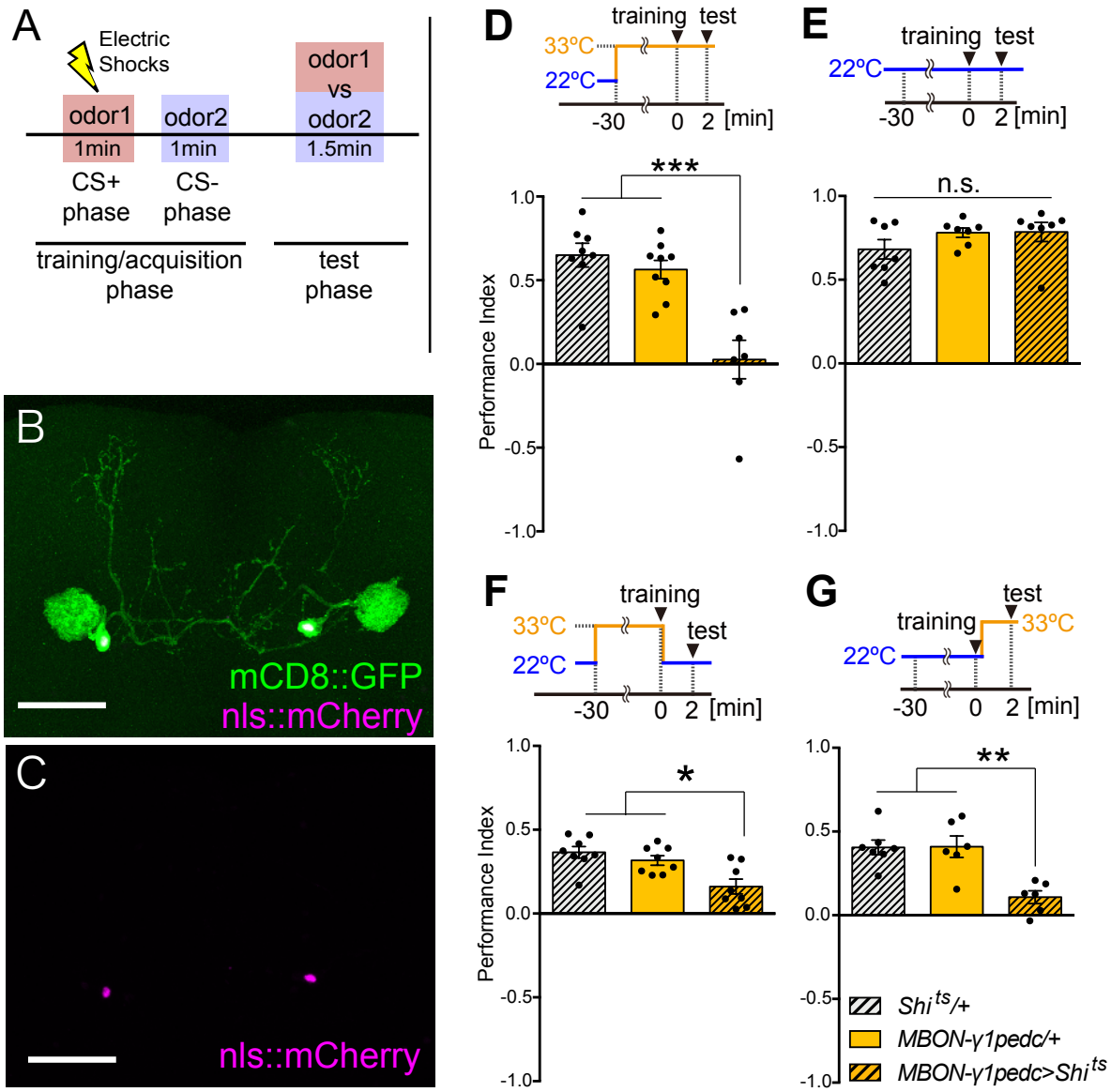


Figure 4. MBON-γ1pedc is required for both STM acquisition and retrieval

(A) Olfactory aversive memory assay scheme. At the training/acquisition stage, one odor (CS+) is delivered to flies with 12 electric shocks and another odor (CS-) is delivered without shocks for 1 min. Subsequently, the flies are allowed to choose between the CS+ odor and CS- odor at the test stage.

(B-C) MB112C split-Gal4 expressing UAS-mCD8::GFP and UAS-nls::mCherry. nls-mCherry labeled single pair of MBON-γ1pedc. Scale bars: 50 μm.

(D) Blocking synaptic outputs from MBON-γ1pedc during the training and test stages impaired short-term memory (STM) (ANOVA, n=8,9,7). Flies were pre-heated at the restrictive temperature (33ºC) before training for 30 min, and then trained and tested at 33ºC.

(E) Flies showed no defect in STM at the permissive temperature (22ºC). Flies expressing Shi^{ts} at MBON-γ1pedc showed no significant memory deficits, as compared to the relevant Gal4 or UAS-Shi^{ts} controls (Kruskal-Wallis, n=7,7,7). Flies were trained and tested at 22ºC.

(F) Blocking synaptic outputs from MBON-γ1pedc at the training stage impaired STM (ANOVA, n=8,8,8). Flies were pre-heated at 33ºC before training for 30 min, and then trained at 33ºC. Immediately after the training, the flies were transferred to 22ºC and tested at 22ºC.

(G) Blocking synaptic outputs from MBON-γ1pedc at the test stage impaired STM (ANOVA, n=7,6,6). Flies were trained at 22ºC, transferred to 33ºC and tested at 33ºC.

(D-G) All bar-graphs are mean ± SEM, and dots represent individual trials. *:$p<0.05$, **: $p<0.01$, ***: $p<0.001$, n.s.: $p>0.05$.

MBON-γ1pedc synaptic output is necessary to inhibit aversive memory acquisition for CS-

For the further analysis of MBON-γ1pedc in memory acquisition, I blocked MBON-γ1pedc during the CS+ or CS- presentation separately. Interestingly, blocking MBON-γ1pedc during the presentation of the CS- odor impaired the memory significantly (Figure 5A), while blocking during the presentation of the CS+ odor or immediately after the CS+ presentation did not cause the memory defect (Figures 5B, C). The memory defect was observed regardless of the sequence of the odor presentation, as flies trained with the CS- presentation at 33ºC followed by the CS+ presentation at 22ºC also showed the memory defect (Figure 5D). These results indicate that MBON-γ1pedc output is needed during the presentation of the CS- odor in the acquisition (Figures 5A, D), and the aversive memory acquired during the CS+ presentation is interfered by the blockade of MBON-γ1pedc during the CS- presentation, at the level of neural circuits or behavior.

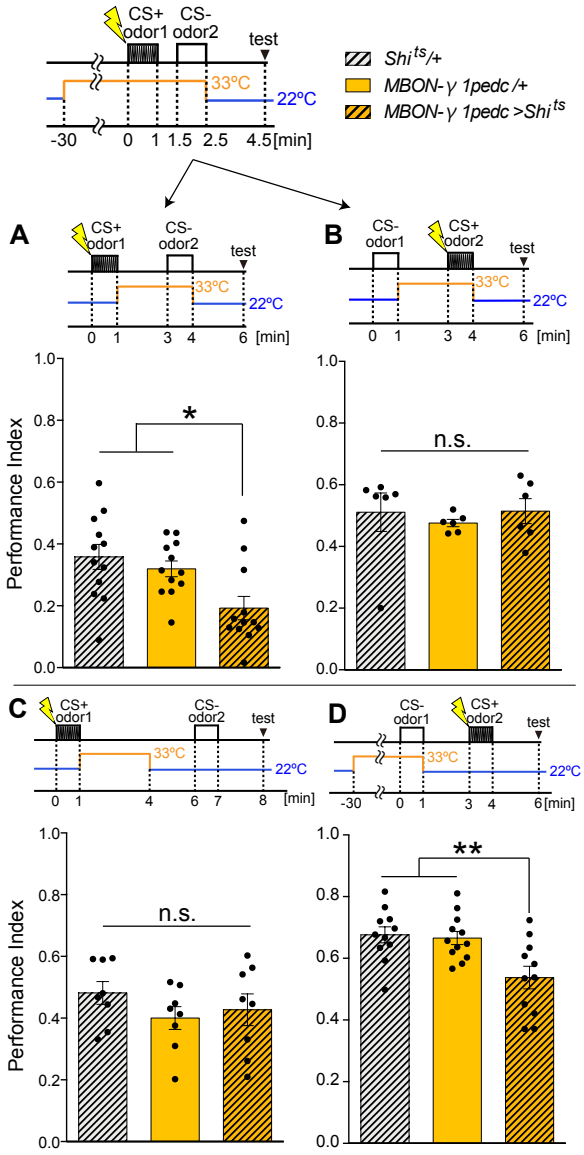


Figure 5. Blockade of MBON-γ1pedc during the CS- presentation causes memory deficits

(A) Blocking synaptic outputs from MBON-γ1pedc during the CS- presentation impaired STM (ANOVA, n=12,12,12).

(B) Blocking synaptic outputs from MBON-γ1pedc during the CS+ presentation did not impair STM (Kruskal-Wallis, n=6,6,6).

(C) Blocking synaptic outputs from MBON-γ1pedc immediately after the CS+ presentation did not impair STM (ANOVA, n=8,8,8).

(D) Blocking synaptic outputs from MBON-γ1pedc during the CS- presentation impaired STM (ANOVA, n=11,12,11). This is the sequential control to Figure 5A.

(A-D) All bar-graphs are mean ± SEM, and dots represent individual trials. *:$p<0.05$, **: $p<0.01$, n.s.: $p>0.05$.

Considering the possibility that blocking MBON-γ1pedc during the CS- presentation alone forms an aversive memory for the CS- odor, and the competition between the aversive memory for the CS+ and CS- odors might cause the memory defect, flies were exposed to two odors in sequence, followed by the test stage. One odor was presented at the permissive temperature, and the other was at the restrictive temperature to block MBON-γ1pedc (Figure 6A). I found that the flies formed an aversive memory toward the odors presented without synaptic output from MBON-γ1pedc (Figure 6B). I named this BGAM, for blockade-of-MBON-γ1pedc-induced aversive memory. These results indicate that the memory defect evoked by blocking MBON-γ1pedc at the acquisition stage (Figure 4F) is at least partially caused by the competition between the aversive memory for CS+ and the BGAM for CS-. Thus, the output from MBON-γ1pedc is needed to prevent the aversive memory for CS-.

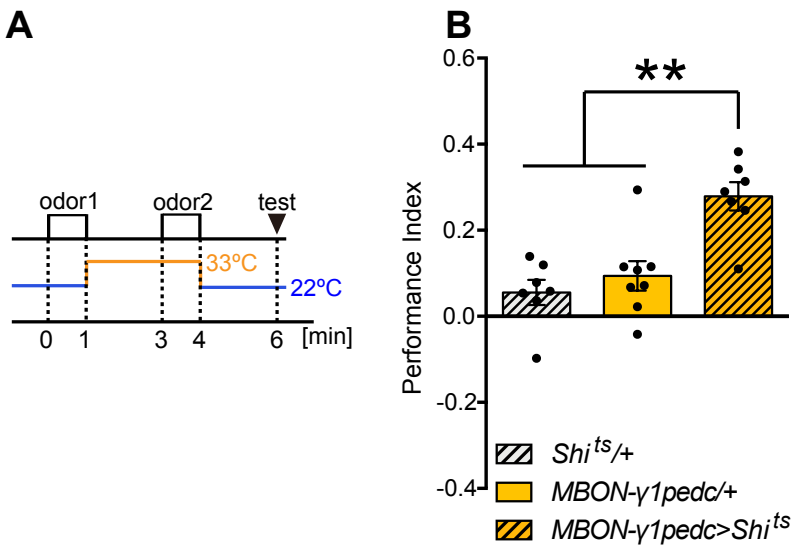


Figure 6. Flies acquire the aversive memory for odors sensed without output from MBON-γ1pedc

(A) Blockade-of-MBON-γ1pedc-induced aversive memory (BGAM) scheme. At the training/acquisition stage, one odor is delivered to flies at the permissive temperature for one min followed by temperature shifting to the restrictive temperature, and another odor is delivered at the restrictive temperature for one min. Subsequently, the flies are allowed to choose between the odors at the test stage.

(B) Blocking synaptic outputs from MBON-γ1pedc during odor presentation caused aversive STM, as compared to controls (ANOVA, n=7,8,7). The Performance Index in this figure was calculated for odor2, and the positive index indicates that flies avoid odor2 over odor1. Bar-graphs are mean ± SEM, and dots represent individual trials. **: $p<0.01$.

BGAM is acquired without odor presentation at the permissive temperature

In BGAM acquisition, a control odor is presented before the temperature shift. I investigated the possibility that some type of memory could be formed for the control odor by temperature shifting immediately after the presentation of the odor, since the timing-dependent behavioral plasticity was reported (Tanimoto et al., 2004). To test this possibility, only a single odor was presented at the restricted temperature in the training session, and the control odor was not presented. As a result, BGAM was also observed in the training session, regardless of the presentation of the control odor at the permissive temperature (Figure 7).

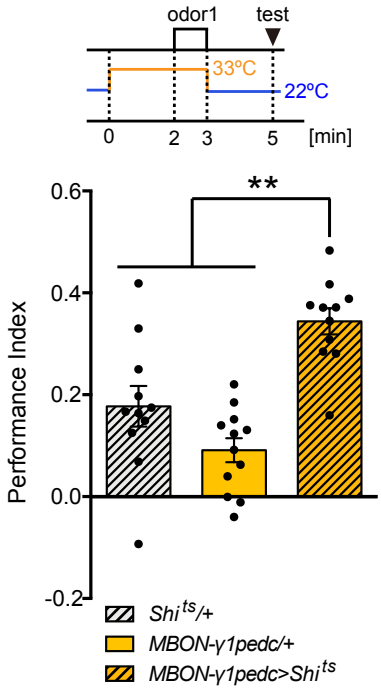


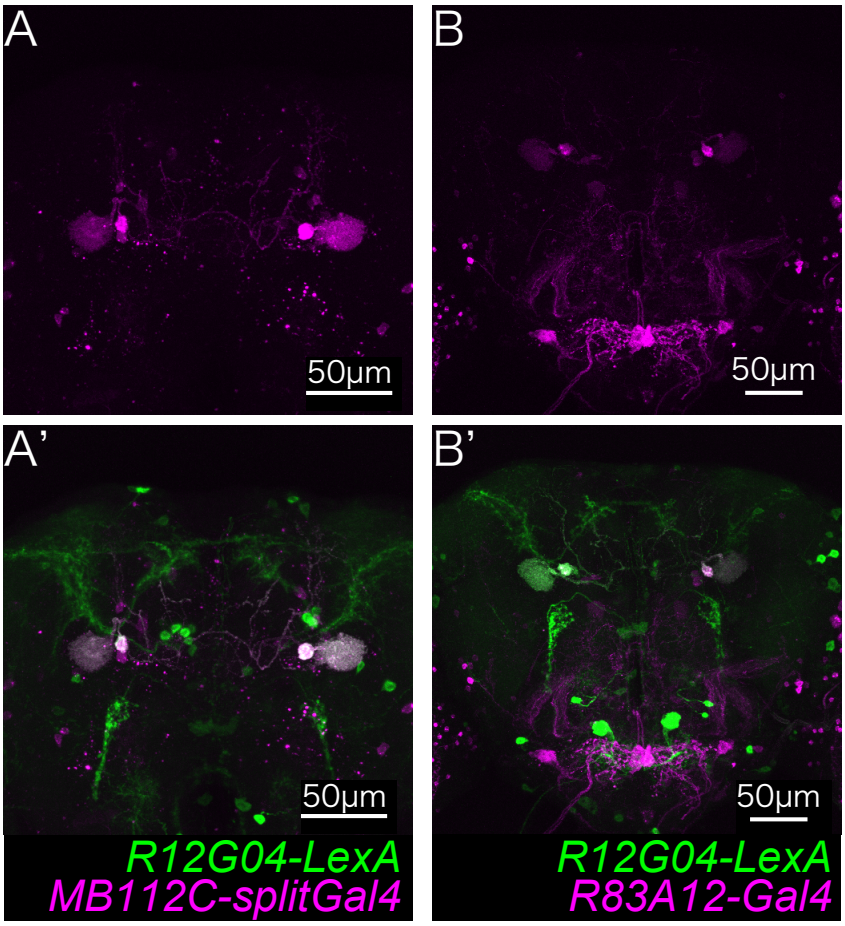
Figure 7. Presentation of control odor is not necessary for BGAM

Single odor presentation during the blockade of MBON-γ1pedc caused significant aversive STM, as compared to control strains (ANOVA, n=11,12,11). The Performance Index in this figure was calculated for the odor presented at the restrictive temperature, and the positive index indicates that flies avoid the odor over another control odor that was not presented during training.

All bar-graphs are mean ± SEM, and dots represent individual trials. **: $p<0.01$.

MBON-γ1pedc, but not the other neurons that could also be labeled by the MB112C driver, is responsible for aversive memory acquisition and BGAM

In the above experiments, MB112C was used as the specific driver to label MBON-γ1pedc. Although MBON-γ1pedc seemed to be the only neurons labeled by the MB112C driver, according to the confocal images, a few neurons may be labeled by MB112C (Figures 8A, A'). To test if MBON-γ1pedc, but not the other neurons, is responsible for aversive memory acquisition and BGAM, R83A12 was used as another driver to examine the role of MBON-γ1pedc (Figures 8B, B'). The blockade of the R83A12-positive neurons by Shi^{ts} impaired STM acquisition (Figure 8C). Output from the R83A12-positive neurons was necessary during the CS- presentation (Figure 8D), but not during the CS+ presentation (Figure 8E). Furthermore, BGAM was also observed by blocking the R83A12-positive neurons during odor presentation (Figure 8F). These results indicate that the neurons responsible for STM acquisition and BGAM formation were likely to be MBON-γ1pedc, but not the other neurons that could potentially be labeled by the drivers.



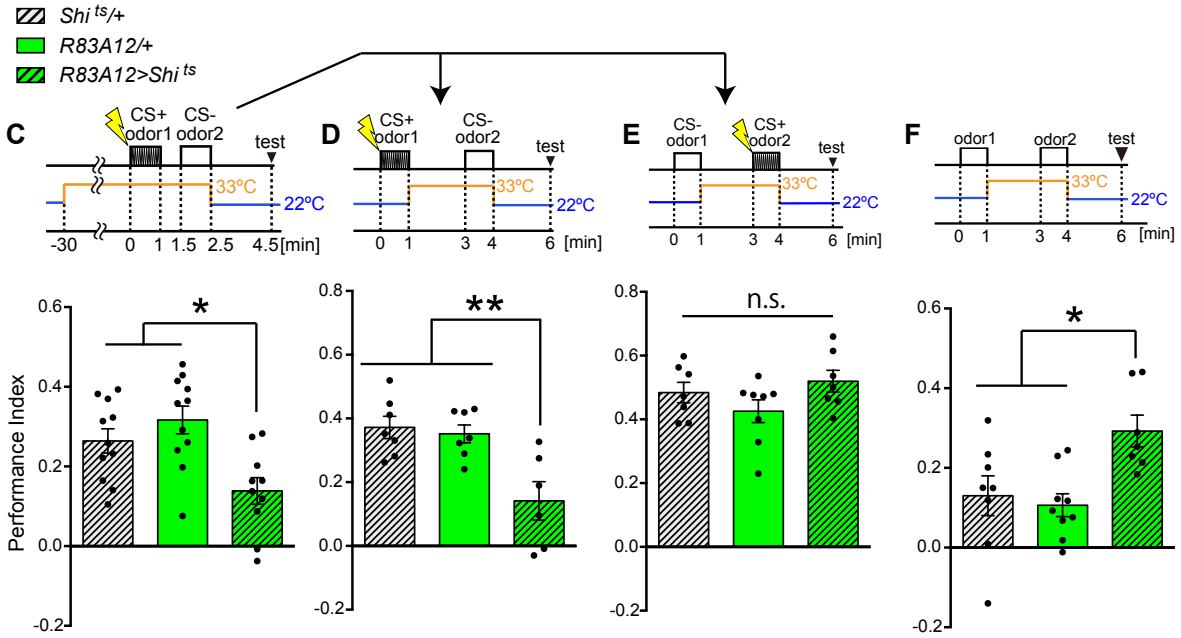


Figure 8. R83A12 driver also induced STM acquisition impairment and BGAM formation

(A, A') *MB112C-splitGal4* and *R12G04-LexA* expressed mCD8::RFP and mCD8::GFP, respectively. Both drivers label MBON-γ1pedc. Scale bars: 50 μm.

(B, B') *R83A12-Gal4* and *R12G04-LexA* expressed mCD8::RFP and mCD8::GFP, respectively. Both drivers label MBON-γ1pedc. Scale bars: 50 μm.

(C) Blocking synaptic outputs from R83A12-positive neurons at the training stage impaired STM (ANOVA, n=11,11,10). The flies were pre-heated at 33ºC before training for 30 min at 33ºC. Immediately after the training, the flies were transferred to 22ºC and tested at 22ºC.

(D) Blocking synaptic outputs from R83A12-positive neurons during the CS- presentation impaired STM (ANOVA, n=7,7,6).

(E) Blocking synaptic outputs from R83A12-positive neurons during the CS+ presentation did not impair STM (ANOVA, n=7,8,7).

(F) Blocking synaptic outputs from R83A12-positive neurons during odor presentation caused aversive STM, as compared to controls (ANOVA, n=8,9,7). The Performance Index in this figure was calculated for odor2, and the positive index indicates that flies avoid odor 2 over odor1.

(C-F) All bar-graphs are mean ± SEM, and dots represent individual trials. *:$p<0.05$, **: $p<0.01$, n.s.: $p>0.05$.

BGAM is retrieved without synaptic output from MBON-γ1pedc

To understand the pathway of the BGAM retrieval, I blocked MBON-γ1pedc during the BGAM retrieval. After BGAM formation, the flies were kept at 33ºC and tested at 33ºC. Flies with MBON-γ1pedc blocked at the retrieval stage still retained BGAM as compared to the control flies (Figure 9), although the performance index was smaller than that of the usual BGAM (Figure 6B). This indicates that BGAM is retrieved through MBONs other than MBON-γ1pedc, assuming that MBONs are the sole output of KCs.

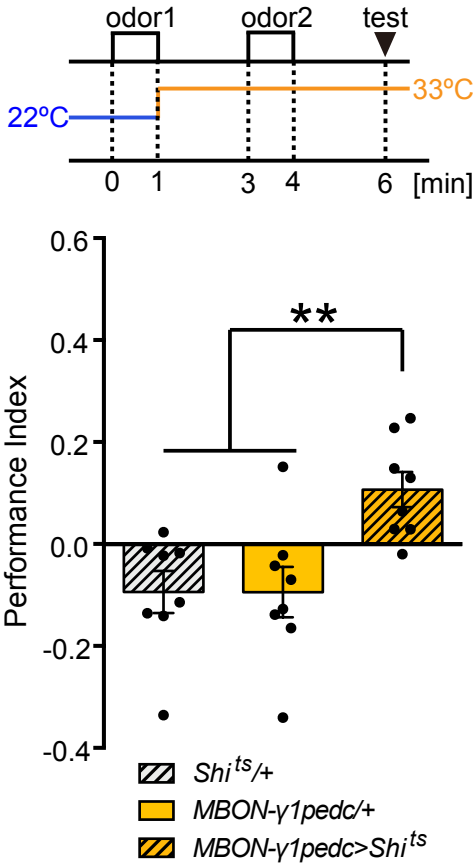


Figure 9. BGAM is retrieved without synaptic output from MBON-γ1pedc

Flies with blocked synaptic outputs from MBON-γ1pedc during odor presentation and test phase also retained BGAM, as compared to controls (ANOVA, n=8,8,8). All bar-graphs are mean ± SEM, and dots represent individual trials. **: $p<0.01$.

DANs are required for the BGAM acquisition

MBONs and DANs constitute microcircuits (Aso et al., 2014b), and DANs transmit various kinds of aversive information (Galili et al., 2014; Vogt et al., 2014; Masek et al., 2015). Therefore, I tested if DANs are involved in the BGAM acquisition. I used tyrosine-hydroxylase (TH) Gal4 (TH-Gal4) (Friggi-Grelin et al., 2003) to label the DANs, as it is thought to cover most of the DANs that convey the aversive information (Claridge-Chang et al., 2009; Aso et al., 2012). Flies without synaptic outputs from both MBON-γ1pedc and DANs showed severe impairment of the BGAM (Figure 10A), suggesting that the BGAM is acquired only when MBON-γ1pedc is inactive and DANs are active. The activation of DANs during the CS- presentation might be the underlying cause of the memory defect observed by blocking MBON-γ1pedc during the acquisition stage (Figure 4F). To test this, I expressed Shi^{ts} in MBON-γ1pedc and DANs, and performed the same experiments as in Figure 5A and 5B. Blocking MBON-γ1pedc alone during the CS- presentation impaired the memory, while blocking both MBON-γ1pedc and DANs during the CS- presentation did not cause significant memory impairments (Figure 10B), indicating that blocking MBON-γ1pedc during the CS- presentation caused memory defects via DANs output. However, blocking DANs during the CS+ presentation caused memory defects regardless of the blockade of MBON-γ1pedc. Blocking MBON-γ1pedc during the CS+ presentation did not exert any significant effects on the memory, as compared to the control Gal4 strain, and did not rescue the memory defect caused by the blockade of DANs (Figure 10C).

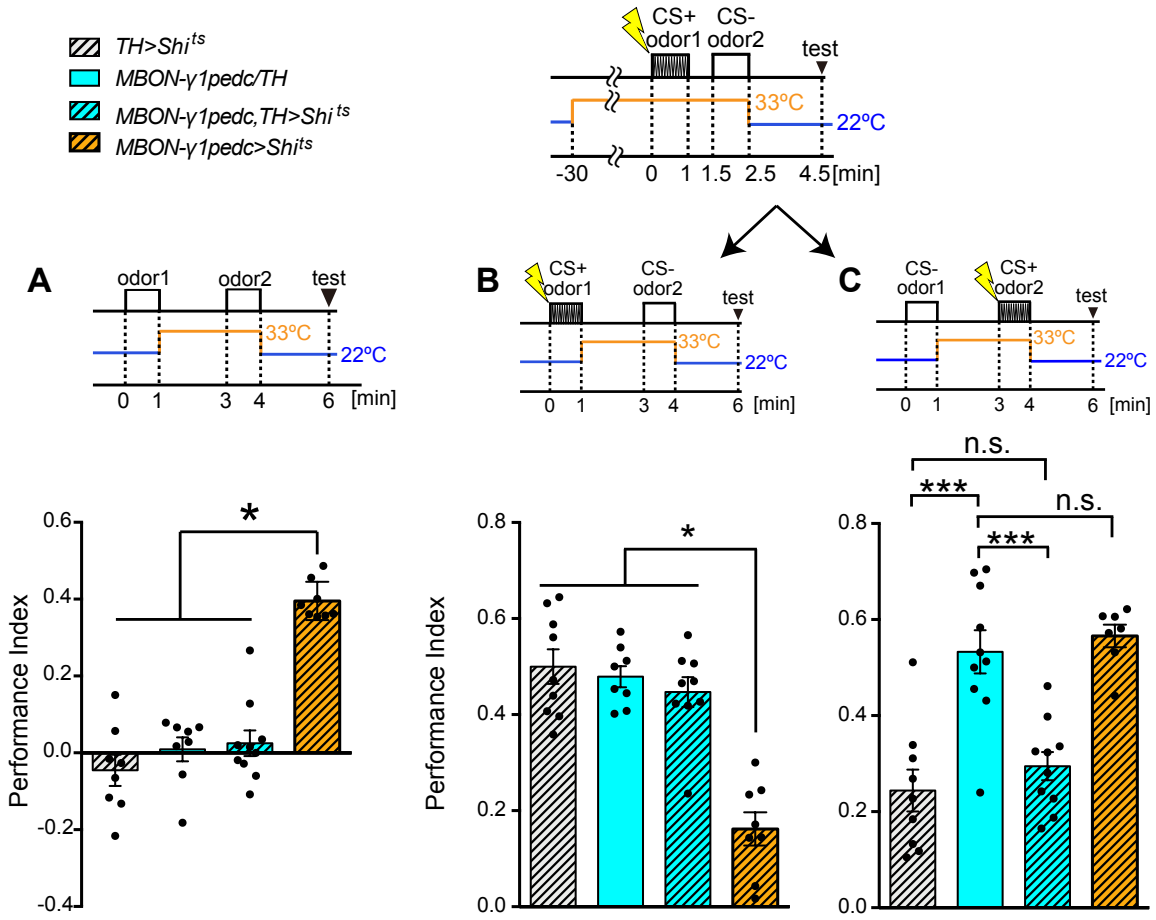


Figure 10. BGAM is acquired through DANs, and blocking DANs rescued the memory deficits caused by BGAM

(A) BGAM was completely diminished by blocking the synaptic output from DANs during the odor2 presentation (Kruskal-Wallis, n=8,8,10,8). Blocking DANs did not cause any difference as compared to controls.

(B) Blocking synaptic outputs from DANs during the CS- presentation rescued the memory impairment caused by BGAM (Kruskal-Wallis, n=9,8,9,8).

(C) Blocking synaptic outputs from DANs during the CS+ presentation impaired STM, regardless of the MBON-γ1pedc output (ANOVA, n=9,10,10,7).

(A-B) All bar-graphs are mean ± SEM, and dots represent individual trials. *:$p<0.05$, ***: $p<0.001$, n.s.: $p>0.05$.

In memory acquisition, DANs are effectively dominant to MBON-γ1pedc

Taken together, in classical conditioning, the DANs output was ineffective in the CS- presentation and needed during the CS+ presentation, while the MBON-γ1pedc output was needed during the CS- presentation, but not the CS+ presentation. In addition, the aversive memory induced by the DANs output in classical conditioning was not affected by blocking MBON-γ1pedc during the CS+ presentation (Figure 10C), while BGAM induced by blocking MBON-γ1pedc was affected by blocking DANs (Figure 10A). Thus, DANs are effectively dominant to MBON-γ1pedc in the aversive memory acquisition stage. In addition, MBON-γ1pedc and DANs negatively modify each other's functions, since DANs inhibit the input from KCs to MBON-γ1pedc (Hige et al., 2015a), and this study suggests that MBON-γ1pedc inhibits the function of DANs.

For the further dissection of the DANs involved in BGAM, a panel of split-Gal4 drivers (Aso et al., 2014b) was used to manipulate subsets of TH-Gal4 positive neurons. I first used drivers labeling a large population of TH-Gal4 positive neurons along with MB112C (Figure 11B), to express Shi^{ts} in the subsets of DANs and MBON-γ1pedc. As compared to the MBON-γ1pedc blocked flies, the flies without synaptic output from MBON-γ1pedc and TH or MB504B positive DANs showed significantly lower BGAM. The blockade of DANs labeled by MB060B did not cause a significant decrease of BGAM. These results indicate that the DANs labeled by TH or MB504B, but not by MB060B, are important for BGAM formation. Note that MB060B and MB504B label a similar subset of DANs, but only MB504 labels PPL1-γ1pedc DANs. I next used an MB438B split-Gal4 driver to manipulate PPL1-γ1pedc neurons (Figure 11C), and tested whether the BGAM was impaired by the expression of Shi^{ts} in PPL1-γ1pedc and MBON-γ1pedc. I found that the inactivation of PPL1-γ1pedc did not impair the BGAM. This indicates that the BGAM is acquired through

the combination of PPL1-γ1pedc and some of the other MB504B positive neurons (PPL1-γ2α'1, PPL1-α'2α2 and PPL1-α3), consistent with the notion that some DANs work coordinately (Aso et al., 2012; Plaçais et al., 2012; Cohn et al., 2015; Aso and Rubin, 2016).

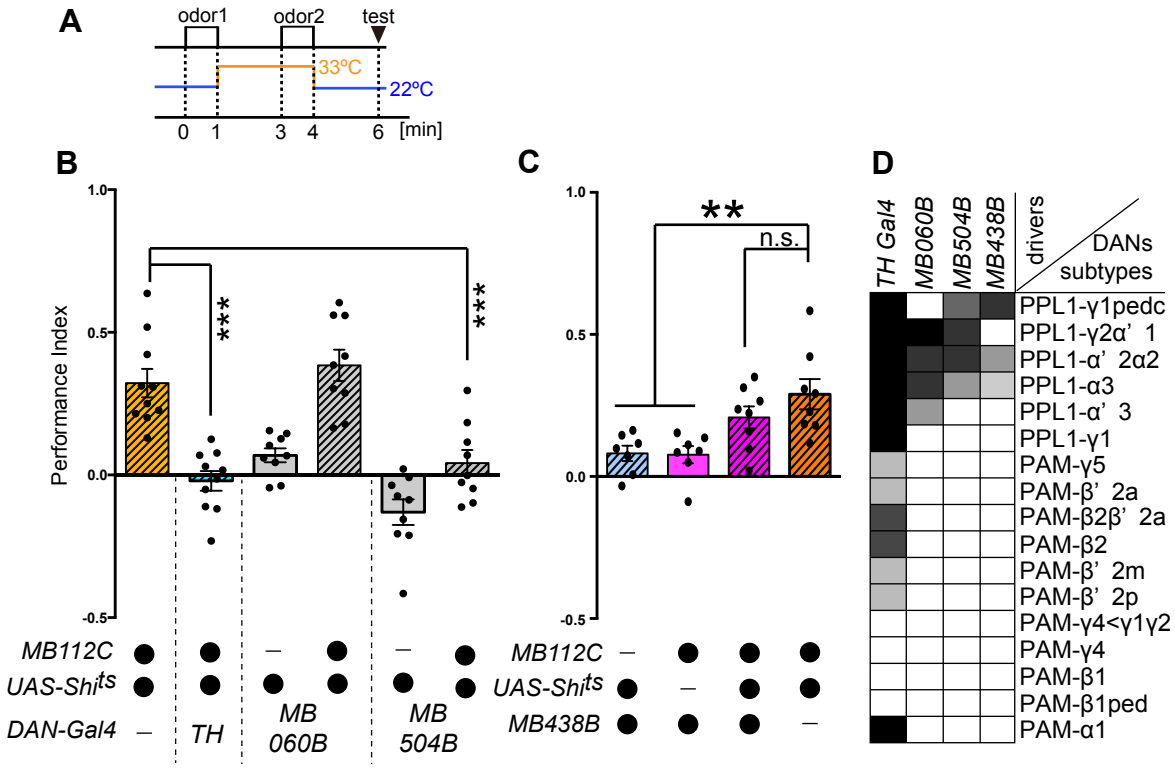


Figure 11. Through DANs, flies acquire aversive memory for odors sensed without output from MBON-γ1pedc

(A) Blockade-of-MBON-γ1pedc-induced aversive memory (BGAM) scheme.

(B) Blocking synaptic outputs from a subset of DANs during odor presentation affected BGAM (ANOVA followed by Dunnett's test comparing with *MB112C/UAS-Shi^{ts}*, n=8-10).

(C) Blocking synaptic outputs from PPL1-γ1pedc during odor presentation did not affect BGAM (ANOVA followed by Dunnett's test comparing with *MB112C/UAS-Shi^{ts}*, n=7,7,8,8)

(D) Expression patterns of specific drivers. The gray scale represents subjectively determined intensities of termini in the MB. Partially modified from (Aso et al., 2012; Masek et al., 2015).

(B, C) All bar-graphs are mean ± SEM, and dots represent individual trials. **: $p<0.01$, ***: $p<0.001$, n.s.: $p>0.05$.

Acquisition of aversive STM for CS+ is not impaired by the activation of MBON-γ1pedc

Assuming that DANs activation is necessary at the CS+ presentation and MBON-γ1pedc inhibits the effect of DANs, I investigated whether the activation of MBON-γ1pedc at the CS+ presentation impaired the STM. To activate MBON-γ1pedc artificially, dTrpA1, a temperature-sensitive cation channel (Hamada et al., 2008), was expressed by using the MB112C driver. Neurons expressing dTrpA1 are transiently activated at the restrictive temperature (33ºC), and not at the permissive temperature (22ºC). The flies were transferred to the restrictive temperature and immediately the CS+ odor and ESs were presented for 1 min. The flies were then re-transferred to the permissive temperature, exposed to the CS- odor and tested. This manipulation of MBON-γ1pedc did not impair the aversive STM significantly (Figure 12). The dTrpA1 inducing the artificial activation of MBON-γ1pedc might be too weak to suppress the effect of DANs induced by ESs sufficiently. Thus, MBON-γ1pedc might suppress the weak effect of DANs.

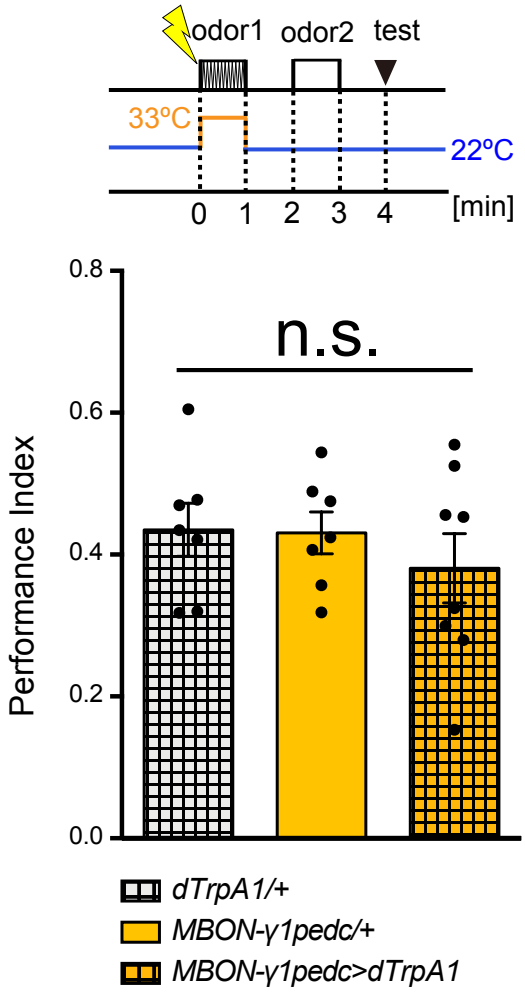


Figure 12. Activation of MBON-γ1pedc did not impair the association of odor and ESs

Activating MBON-γ1pedc during CS+ and ESs presentation did not impair STM (ANOVA, n=7,7,8). All bar-graphs are mean ± SEM, and dots represent individual trials. n.s. : p>0.05.

3-2. Functional analysis of MBON-γ1pedc in mid-term memory

本章は、5 年以内に学術雑誌などで刊行予定のため、非公開。

3-3. Functional analysis of MBON-γ1pedc by *in vivo* calcium imaging

本章は、5 年以内に学術雑誌などで刊行予定のため、非公開。

4. Discussion

本章は、5 年以内に学術雑誌などで刊行予定のため、非公開。

5. Conclusion

本章は、5 年以内に学術雑誌などで刊行予定のため、非公開。

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