A Priming Circuit Controls the Olfactory Response and Memory in Drosophila

A Priming Circuit Controls the Olfactory Response and Memory in *Drosophila*

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Abstract

In implicit memory priming, exposure to one stimulus influences the subsequent experience of other stimuli. Here we found that in *Drosophila melanogaster*, a prior encounter with an aversive stimulus produces long lasting changes in the animal's neural and behavioral response to a novel odor. The encounter of a novel odor moreover activates the reward circuitry and establishes an approach memory. We identified a pair of Priming Neurons that mediate this priming effect. By recording the activity of these neurons in the priming circuit in real time, we show that Priming Neurons act as the interface of the conditioned and unconditioned pathways to collate the mushroom body output signals and distribute them selectively to the reward and punishment aversive pathways during the presentation of a novel odor. Beyond direct exposure to unconditioned stimuli, this complex memory priming system provides an animal with a larger time window to assess more sensory inputs for valence relationships during a salient learning experience.

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List of Abbreviations

AL − Antennal lobe

ARM − Anesthesia-resistant memory

ATR − All-trans retinal

BA − Benzaldehyde

CRE – Crepine

CS – Conditioned stimulus

DAN – Dopaminergic neuron

KC – Kenyon cell

LH – Lateral horn

LN – Local neuron

LTM – Long-term memory

MB – Mushroom body

MBEN − Mushroom body extrinsic neuron

MBON − Mushroom body output neuron

MCH − 4-methylcyclohexanol

MTM – Middle-term memory

OCT − 3-octanol

ORN − Olfactory receptor neuron

OSN − Olfactory sensory neuron

PAM – Protocerebral anterior medial

PI – Performance index

PN – Projection neuron

PPL1 – Paired posterior lateral 1

PrN – Priming neuron

SIP – Superior intermediate protocerebrum

SLP – Superior lateral protocerebrum

SMP – Superior medial protocerebrum

STM – Short-term memory

US – Unconditioned stimulus

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Chapter 1

Introduction

Olfactory Pavlovian conditioning in *Drosophila*

Animals receive rich sensory stimuli in the changing environment. In order to avoid danger and approach food efficiently, they must be able to discriminate different stimuli, learn and memorize the predictive outcomes and modify their behavior based on past experiences. To investigate the neural substrates underlying learning, memory and decision making in animals, the fruit fly *Drosophila melanogaster* is extensively studied as a model organism with well-characterized sensory system and reliable behavioral patterns. Its relatively short life cycle and rich genetic toolbox developed by scientists over the years have also made it an ideal model to study molecular pathways and neuronal circuits underlying learning and memory. Particularly, olfaction is one of the most important sources for fruit flies to learn about the dynamic environment and form memories that are essential for survival.

There is a long history of training flies to associate odors with electric shocks (Quinn et al., 1974). To assess olfactory learning behavior in adult flies, classical Pavlovian conditioning and behavioral analysis in a T-maze is well developed and widely used (Tully and Quinn, 1985). Typically, fifty to a hundred flies are placed in a tube in one aversive training cycle. First, one odor (conditioned stimulus +, CS+) is given to the flies for a minute. At the same time, electric shock pulses (unconditioned stimulus, US) are

delivered to fly feet. After that, flies rest in the air for 30-60 seconds before receiving a second odor (conditioned stimulus -, CS-) for a minute without electric shocks. Hydrophobic chemical odorants that are generally undesirable for flies including 3-octanol (OCT), 4-methylcyclohexanol (MCH), and benzaldehyde (BA), are most commonly used in conditioning experiments. To test their memory, the trained flies are put in a T-maze with two arms, each arm filled with one odor previously exposed in the training. A performance index (PI) is calculated as the difference between the fraction of flies that approach CS- and the fraction of flies that approach CS+, averaged for two groups of flies trained to avoid each of the two odors. This way we are able to examine how well flies with different genetic background or treatment of interest learn and memorize at the behavioral level.

Since the establishment of classical conditioning in flies, various forms of modifications have been made to the original paradigm to study different aspects and different forms of olfactory memory. For example, the T-maze test can be done at different time points after training. Short-term memory (STM) is usually tested immediately after training, while middle-term memory (MTM) is tested 3 h, and long-term memory (LTM) 24 h after training. For aversive conditioning, LTM formation requires several repetitions of the training cycle with long space in between. The unconditioned stimulus of electric shocks in aversive conditioning can be substituted with

exposure to sugar paper as in appetitive conditioning, or to bitter food as in another form of aversive conditioning. The actual US can be further replaced by thermal or optogenetic activation of specific dopaminergic neurons, with more details in later parts of this introduction. The delivery of actual odors as CS can also be replaced by artificial activation of corresponding olfactory sensory neurons for sharper onset and offset. Additionally, learning and memory of fly larvae can be tested in a plate instead of the T-maze with similar idea of conditioning.

The interstimulus interval between the presentation of CS+ and US could also be altered in different conditioning paradigms. In canonical conditioning, CS+ and US are delivered simultaneously. When an aversive US is presented later than CS+ with no temporal overlap, for example with a 1-min gap, an repulsive memory of CS+ can still be formed, though with a smaller PI (Dylla et al., 2017). This is called trace conditioning, as a memory trace of odor is kept in the sensory system before its association with the US that comes after. On the other hand, when an aversive US is presented before the onset of CS+ with no temporal overlap, an approach memory of this odor is formed in flies (Tanimoto et al., 2004; Niewalda et al., 2015). This is known as backward conditioning, or relief conditioning, where the CS+ is believed to be paired with the cessation of shocks, which is considered a reward cue instead. Similarly, backward appetitive conditioning with a reward US delivered before CS+ results in a repulsive memory. Multiple trials of

interleaving forward and backward conditioning reliably reverse the attraction and repulsion behaviors of individual flies after each trial (Handler et al., 2019), reflecting the flexibility of memories.

In all these conditioning paradigms, the CS- odor is given to animals during training solely as a neutral control to compare with CS+. For years, only the CS+ odor has been considered to be conditioned with electric shocks, while the presence of CS- has been thought to be irrelevant. However, a recent project in our laboratory that I participated in during my first few years of Ph.D. studies revealed that CS- didn't only serve as a neutral control, but also contributed significantly to the memory of flies (results described in Chapter 2). With this finding, a further modification of the training method is applied that leads to a new project, focusing a priming behavior of flies that has not been described before.

Priming behavior of *Drosophila*

Priming is a concept usually found in psychology, referring to the effect that environmental stimuli may affect subsequent responses by activating mental constructs without conscious realization or guidance (Weingarten et al., 2016). Priming has been described and studied in psychology and different aspects of social sciences, but its biological basis is not well understood. Some previous studies believe that repetition priming reflects transient changes in cortical sensory processing areas (Squire et al., 1992), and that priming leads to more efficient processing of sensory stimuli, while the neural activity in the corresponding cortex decreases with stimulus repetition (Moldakarimov et al., 2010). Similar priming effect in non-human animals has yet to be found due to the semantic nature of the typical experimental methods used in most human priming experiments.

Here we describe a behavior in *Drosophila* where fruit flies primed with electric shock experiences show an enhanced preference to a subsequently encountered novel odor. The experimental setup was modified from the canonical conditioning assay, but flies were not conditioned by pairing odor and shock in this case. Thus we describe the behavior as priming, as animals show perceptual changes to odor stimuli after receiving stimulus of electric shocks, relating to the priming concept used in psychology as well as social

sciences. My second and major project during Ph.D. studies is to try to understand the neuronal pathways underlying this behavior at a circuit level.

Drosophila olfactory memory circuits

The olfactory system of *Drosophila* resembles that of vertebrates in basic anatomical organization, but their reduced cell numbers and simpler neural networks make the circuits easier to identify and manipulate experimentally (Fiala, 2007). Odors are first detected by olfactory receptor neurons (ORN), also known as olfactory sensory neurons (OSN), in the antennae and maxillary palps. Each ORN expresses only one olfactory receptor together with a putative co-receptor or chaperone protein, Or83b (Busto et al., 2010). ORNs expressing the same membrane odor receptors project to the same glomerulus among the approximately 50 glomeruli of each of antennal lobe (AL), where they synapse with the downstream projection neurons (PN). Glomeruli are interconnected by a network of local neurons (LN). The antennal lobe is analogous to the vertebrate olfactory bulb, forming the first brain region of the fly olfactory system (Wilson, 2013). The approximately 150 PNs of each antennal lobe then transmit the olfactory information to two higher brain regions: the lateral horn (LH) that is generally considered to drive innate olfactory responses (Schultzhaus et al., 2017), and the more medially located structure, mushroom body (MB), that encodes olfactory experiences into memories of fruit flies (McGuire et al., 2001; Akalal et al., 2006; Keene and Waddell, 2007; Busto et al., 2010).

The mushroom body is the center of learning and memory for insects (De and Heisenberg, 1994; Menzel, 2014). Specifically, it's required for olfactory memory formation and retrieval in *Drosophila*. It consists of intrinsic neurons called Kenyon cells (KC), 2000 per brain hemisphere, which synapse with PN axons in the calyx and extend parallel fibers to form the MB lobes. Unlike most *Drosophila* neuroanatomy, including the olfactory sensory apparatus, significant anatomical and functional stereotypy is not found in KCs between individual flies (Murthy et al., 2008). KCs can be coarsely grouped into α/β, α'/β', and γ classes based on birth order and adult axonal projection patterns (Lee et al., 1999; Lin et al., 2007), but they are difficult to identify in the dendritic fields (Tanaka et al., 2008).

KC dendrites form a structure of MB in the posterior brain known as the calyx, which connects with MB lobes by a peduncle. Previous studies suggested that different MB lobes mediate fly memories of different phases. *Drosophila* aversive olfactory memory features several memory phases, classified by the length of temporal window between memory formation and retrieval, including STM (Tully and Quinn, 1985), MTM (Waddell et al., 2000), ARM and LTM (Tully and Quinn, 1985). For example, the γ lobe is believed to mediate STM and MTM, whereas the α / β lobes mediate LTM (Yu et al., 2006; Krashes et al., 2007; Qin et al., 2012; Bouzaiane et al., 2015). *Drosophila* olfactory LTM depends on protein-synthesis (Tully et al., 1994) and MB function, although one

study claims that MB is dispensable for LTM (Chen et al., 2012). Furthermore, each MB lobe can be segmented into 15 anatomically discrete compartments, namely α1, α2, α3, α'1, α'2, α'3, β1, β2, β'1, β'2, γ1, γ2, γ3, γ4, and γ5 lobe, each innervated by different subsets of mushroom body extrinsic neurons (MBEN), providing anatomical architecture for the formation and retrieval of different memory types (Aso et al., 2014a; Cohn et al., 2015).

Unlike in widely tuned PNs, odor is sparsely and specifically coded by a few KCs in the MB (Turner et al., 2008; Campbell et al., 2013; Caron et al., 2013; Lin AC et al., 2014), which then converge onto 34 mushroom body output neurons (MBONs) of 21 types per brain hemisphere (Aso et al., 2014b). This convergence transforms the representation of odor identity to more abstract information including the valence of an odor based on experience (Owald et al., 2015; Hige et al., 2015b; Yamazaki et al., 2018). The MBONs have dendrites in the MB lobes and project axons to neuropils outside of the MB. As the sole outputs of mushroom body lobes, their axon terminals project to five discrete neuropils in the brain: the crepine (CRE; a region surrounding the horizontal/medial lobes), the superior medial protocerebrum (SMP), the superior intermediate protocerebrum (SIP), the superior lateral protocerebrum (SLP), and the lateral horn (Aso et al., 2014a).

The plasticity of the synapse between KCs and MBONs is modified presynaptically by dopaminergic neurons (DAN). The axon terminals of approximately 130 DANs can be divided into 20 cell types (Aso et al, 2014a). DAN activity and dopamine receptors in the KCs have been shown to be required for olfactory memory formation (Schwaerzel et al, 2003; Lin S et al., 2014; Felsenberg et al., 2018). DANs are activated by unconditioned stimulus, encoding positive or negative valence. DANs activated by aversive US like electric shocks and bitter food are known as punishment DANs, and those activated by appetitive US like sugar are called reward DANs. In general, DANs of the paired posterior lateral 1 (PPL1) cluster are aversively reinforcing, while DANs of the protocerebral anterior medial (PAM) cluster are largely appetitively reinforcing (Cognigni et al., 2018). Particularly, for DANs that project to the γ lobe of MB which is necessary for short-term memory that I specifically study, γ1 and γ2-DANs are PPL1 and punishment DANs, γ4 and γ5-DANs are PAM and reward DANs, while γ3 DANs are PAM-DANs but encode punishment (Aso et al., 2012; Perisse et al., 2013; Aso et al., 2014a).

Different subsets of DANs innervate distinct, non-overlapping compartments of MB lobes in a rather robust pattern across individual flies, feeding US information onto KC fibers which code previously neutral odor identities (Aso et al., 2014a). Pairing an odor with activation of specific DANs induces odor-specific synaptic depression between KCs

and MBONs in the same MB compartments (Hige et al., 2015a). On the contrary, backward pairing of DAN activities followed by odor presentation, or strong dopamine release alone in the absence of odor, results in potentiation of KC-MBON synapses (Cohn et al., 2015; Berry et al., 2018; Handler et al., 2019). This time-dependent bidirectional synaptic modulation by DANs underlies the opposite behavioral outcomes of flies receiving forward and backward conditioning, and could potentially account for the priming behavior. In addition, artificial activation or suppression of specific subsets of DANs by optogenetic or thermal approaches is sufficient to replace the real punishment or reward, such as electric shocks or sugar delivery, and induce different forms of olfactory memories (Schroll et al., 2006; Claridge-Chang et al., 2009; Aso et al., 2010; Liu et al., 2012; Masek et al., 2015; Yamagata et al., 2015; Yamagata et al., 2016; Aso and Rubin, 2016; Waddell, 2016).

Dopamine released by DANs act on dopamine receptors (DopR) that are highly expressed in MB KCs. There are four types of DopRs found in *Drosophila*, namely Dop1R1, Dop1R2, Dop2R, and DopEcR. Dop1R1 and Dop1R2 are D1-like dopamine receptors, which activate the adenylyl cyclase / cyclic adenosine monophosphate (cAMP) pathway, while Dop2R is a D2-like receptor which inhibits the downstream cAMP pathway (Karam et al., 2019). DopEcR is a dual receptor that binds not only dopamine, but also the insect ecdysteroids ecdysone (E) and 20-hydroxyecdysone (20E) (Srivastava

et al., 2015). cAMP signaling pathway downstream of DopR activation is essential for learning and memory. Stimulation of DANs evokes consistent, compartmentalized and receptor - dependent elevation of cAMP and PKA in the MB (Boto et al., 2014). Disruptions of the G-protein α subunits Gs and Go in the cAMP pathway (Connolly et al., 1996), as well as genes encoding downstream effectors including *dunce* (Dudai et al., 1976), *rutabaga* (Goodwin et al., 1997), *PKA* (Skoulakis et al., 1993) and *CREB2* (Yin et al., 1995), all cause deficits in learning and memory formation. Of the four *Drosophila* DopRs, Dop1R1 is required for memory formation, while Dop1R2, which has a lower sensitivity to dopamine, acts in the same neurons to mediate active forgetting with slower kinetics (Berry et al., 2012). The two D1-like receptors mainly signal through different G-protein partners, with Dop1R1 coupling to Gαs to drive cAMP production (Sugamori et al., 1995) and Dop1R2 coupling to Gαq, resulting in increased intracellular calcium level (Himmelreich et al., 2017). Gαq signaling depends strictly on the temporal ordering of US and CS, making Dop1R2 essential for backward conditioning in particular (Handler et al., 2019).

With DANs, US and CS signals are associated in the mushroom body, resulting in experience-dependent valence to different odors, represented by activation or inhibition of corresponding MBONs innervating the same compartments of MB lobes as DANs. Certain MBONs are presumed to activate downstream circuits, eventually motor neurons,

to guide the avoidance or approach behavior in flies. MBONs that encode valence can be divided into attractive MBONs and repulsive MBONs. Activation of attractive MBONs results in approach behavior of flies, while activation of repulsive MBONs leads to avoidance (Aso et al., 2014b). With this entire olfactory memory circuits, flies are able to associate CS and US during conditioning, and show learned behavior in the test. Presumably with the same machinery, flies can modulate their reaction to a novel odor after being electrically shocked in priming assays.

Recurrent loop between MBONs and DANs

If DANs serve solely as the input to the one-way neuronal pathway from DANs to MBONs, the activity of DANs should stay unaffected by olfactory conditioning. However, the activity of some specific DANs are found to be elevated or reduced after training (Riemensperger et al., 2005; Séjourné et al., 2011; Hige et al., 2015a; Perisse et al., 2016), suggesting a recurrent loop that feeds the valence information stored in MBONs back to DANs, either directly or indirectly (Horiuchi, 2019). Anatomical evidence shows that many DANs have their dendrites in neuropils where MBON axons project to, making it possible for recurrent circuits to form between DANs and MBONs. The recently released EM connectome of adult fly central brain also suggests that many MBONs project their axons to synapse with DANs directly.

Functionally, prior work has established that MB compartments display features of a widely interacting network, where feedback from odor-stimulated MBONs and inter-compartmental interactions coordinate DAN modulation across compartments (Cognigni et al., 2018). An example of direct feedback from γ2α’1 MBON to punishment PPL1-γ2α′1 DANs (also known as MB-MV1) and reward PAM DANs has been confirmed (Felsenberg et al., 2017; Felsenberg et al., 2018). In addition, feedback from MBONs to DANs innervating the α1 compartment is required to stabilize memory (Ichinose et al., 2015). MBON to DAN feedback loops take part in odor familiarization

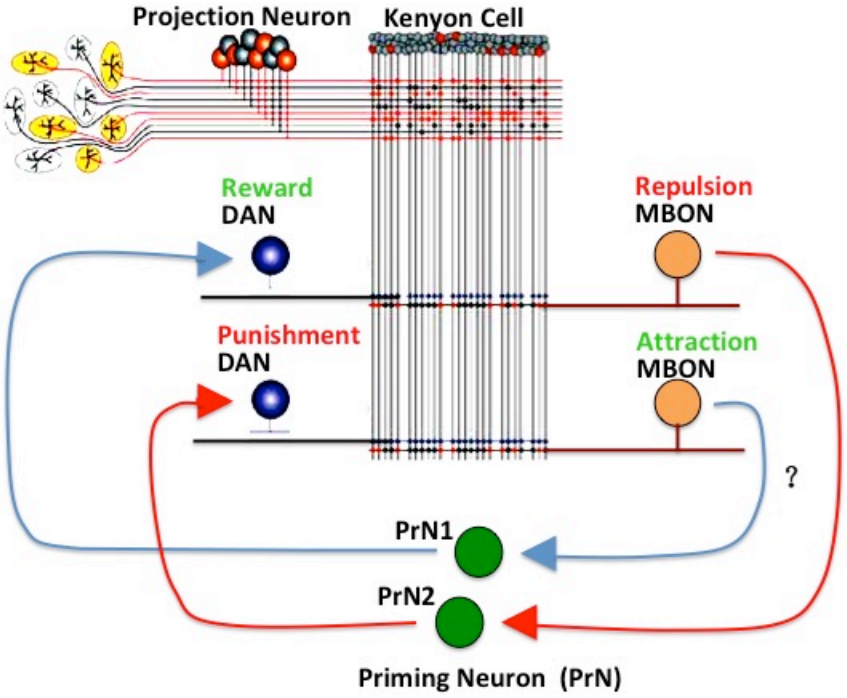
(Hattori et al., 2017) and courtship memory (Zhao et al., 2018). Cross-compartmental MBON to DAN signaling plays a role in memory extinction and reevaluation (Felsenberg et al., 2018). Local feedback from KCs to DANs has also been observed (Cervantes-Sandoval et al., 2017). Taken together, these studies suggest that extensive network interactions coordinate the activity of MBONs, DANs and KCs across multiple MB compartments.

In the meantime, an indirect recurrent neuronal network from MBON to DAN was found in the first project that I participated in. In this project, a dozen of mushroom body extrinsic neurons were screened for training-dependent plasticity by imaging intracellular calcium dynamics in flies that experience aversive olfactory conditioning while immobilized under a two-photon microscope. The screening revealed odor response plasticity in both known components of the MB circuitry, including DANs and MBONs, and uncharacterized elements.

Among these novel elements, a pair of previously uncharacterized neurons in the SMP region was identified, and they appeared to feed MBON activities back to DANs. Furthermore, these two neurons were found to be functionally necessary for priming and different aspects of conditioned memory in flies. Their activities in response to odor presentation are also modulated during priming as well as conditioning. Thus we name them Priming Neuron 1 (PrN1) and Priming Neuron 2 (PrN2) (Figure 1). Our study

shows that these two priming neurons form a bridge between MBONs and DANs with a key role in incorporating contextual information into experience-dependent olfactory response and memory.

Figure 1. Schematic diagram showing the positions of Priming Neurons relative to MBONs and DANs in the *Drosophila* olfactory system.



Chapter 2

Priming Behavior in *Drosophila*

Aversive olfactory memory in *Drosophila* is bipartite

Over decades, olfactory memory in *Drosophila* has been tested and quantified in a canonical Pavlovian conditioning paradigm using a T-maze (Tully and Quinn, 1985). Flies first receive foot shock from copper electrodes in a tube filled with a CS+ odor, then rest in the tube without shock with the presence of a CS- odor (Figure 2A). They are later transferred into a T-maze to choose to stay between the two arms, each filled with the CS+ or the CS- odor. The performance index (memory score) of flies is calculated as the difference between the fraction of flies that approach CS- and the fraction of flies that approach CS+ (Tully and Quinn, 1985).

For a long time, this aversive olfactory memory was thought to be uniform, mainly resulting from flies' repulsion to CS+ after training, with little contribution of learned behavior with respect to the CS-. However, here in a modified conditioning paradigm, we were able to specifically focus on CS- memory for the first time with the recruitment of a third, novel odor or pure air into the test (Figure 2B).

Using this method, we measured the CS- memory score independently, and found that wildtype 2u flies preferred CS- to the third odor significantly after five cycles of aversive conditioning, spaced by 15 min (Figure 2D, E). Here benzaldehyde was used as the third odor, and the use of OCT or MCH as CS- didn't affect the conclusion. Replacing the

third odor with pure air had similar results (Figure 2F), with the valence of CS- changing from slight repulsion to attraction, compared with air. Removing CS- from the training protocol, however, abolished the attraction to CS- over the third odor (Figure 2G), because the two odors were both novel in the test in this paradigm. This confirms that the attraction memory to CS- is indeed a result from the presence of CS- following the conditioning of CS+ during training.

Training paradigm with only one cycle of conditioning also results in an attraction memory to CS- (Figure 2H). We performed the conditioning cycle once, and 10 min later, we measured the CS+ and CS- memories separately by recording their preference to each CS over a third odor. Flies showed an avoidance PI score of 0.6 to 0.7 to the CS+ odor compared with the third odor, and an approach PI score of 0.1 to 0.2 to the CS- odor. The CS- memory score is small but significant and reproducible. A simple addition of the absolute values of these two scores is comparable to a typical aversive STM score of around 0.8. This implies that flies' attraction to CS- is indeed making part of the full memory, thus the aversive olfactory memory of flies is bipartite. It can be divided into two parts: a repulsion memory to avoid CS+, and an attraction memory to approach CS-.

We further tested the two components of fly memories using the 5-cycle space-training paradigm (Figure 2I-K). This paradigm is sufficient for flies to form long-term aversive memory. We tested the full memory, CS+ memory, and CS- memory separately at

different time points after training: 30 min for STM (Figure 2I), 3 h for MTM (Figure 2J), and 24 h for LTM (Figure 2K). In this case, the proportion of CS- attraction memory in the full memory is higher than that in 1-cycle STM training. At 30 min, the attraction memory scores around 0.3, contributing about 1/3 to the full memory. Interestingly, at later times when both repulsion memory and full memory have decayed largely, the attraction memory appears relatively stable, with a PI eventually as high as the repulsion memory (Figure 2C).

All together, these results show that aversive olfactory memory in flies is bipartite, consisting of a repulsion memory to CS+ and an attraction memory to CS-. These observations suggest that some elements of appetitive conditioning circuitry are recruited into the formation and maintenance of plasticity during the process of canonical olfactory conditioning.

Figure 2. Aversive olfactory memory is bipartite.

(A) Schematic describing classical aversive olfactory conditioning. CS+ and CS- can refer to odorants OCT or MCH, which were used interchangeably.

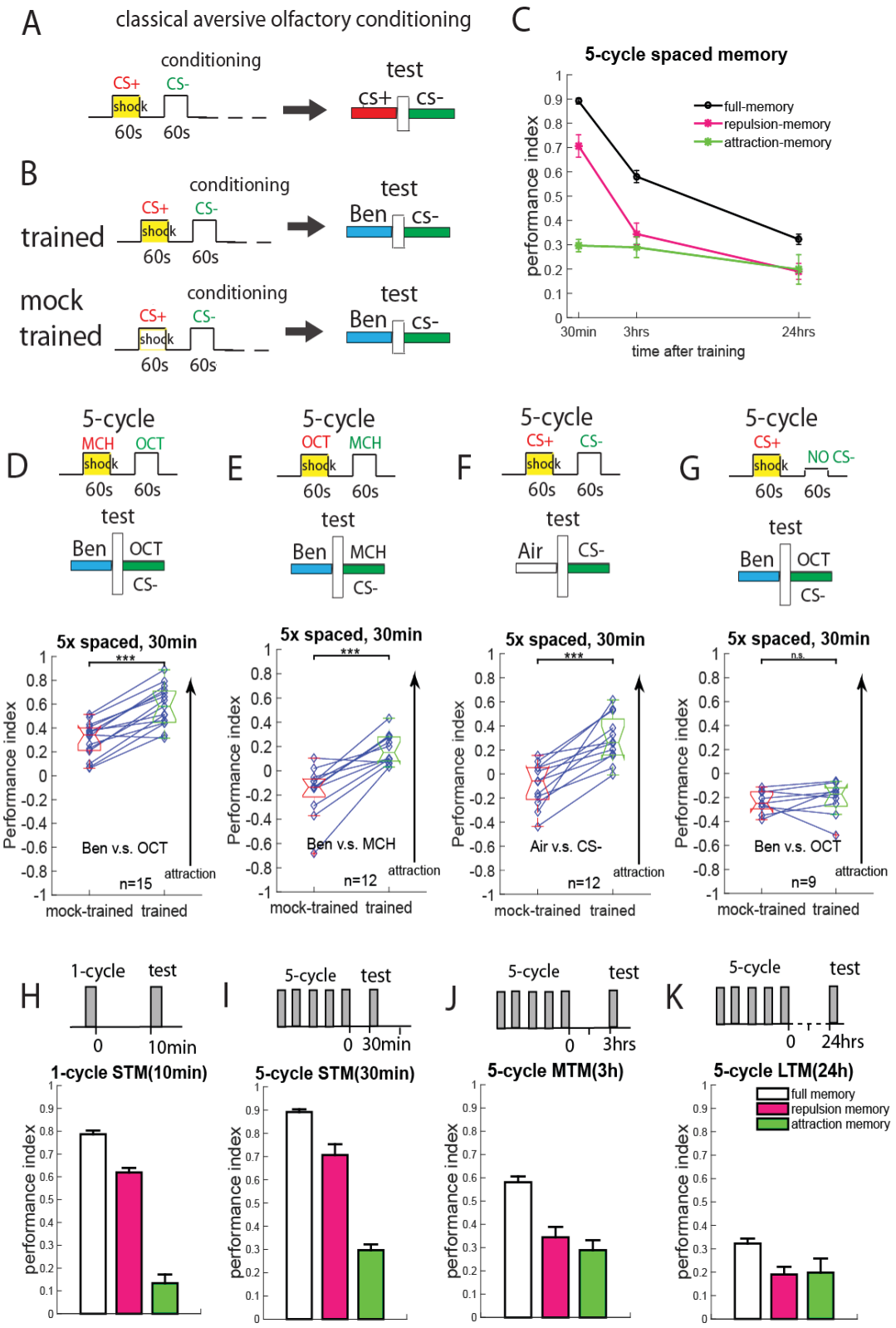
(B) Schematic of the aversive protocol modified to detect individual CS+ and CS- memories. All training was performed with animals grown in the same culture. After training, they were tested for preference between CS- and a novel odor, Ben, or pure air. The PI is calculated by subtracting the mock-trained PI score from trained PI score.

(C) Time course for the retention of approach and repulsion memories after 5-cycle spaced aversive olfactory training.

(D-G) Aversive olfactory training yields attraction to the CS- odor. In each panel, on the top is the diagram of 5x spaced training and the following test. On the bottom is corresponding PI for trained and mock-trained flies. Three asterisks denotes $p<0.001$, n.s. denotes $p>0.05$ (Paired t-test). (D) Training induced attraction to OCT used as CS- when Ben was used as reference (n=15). (E) Training induced attraction to MCH used as CS- when Ben was used as reference (n=12). (F) Training induces attraction to CS- with pure air as reference. PI scores were averaged over MCH and OCT. (G) Pairing flies with CS+ in the absence of CS- failed to induce attraction to CS-. n indicates number of fly groups.

(H-K) Phases of approach, repulsion and full memory. Data are mean ± SEM (standard error of the mean); $n>8$ for each memory type and memory phase. (H) Memories at 10min after 1-cycle training. (I-K) Memory observed at 30 min, 3 h, or 24 h after 5-cycle training. n for group number.

Figure 2. (Continued)



Priming behavior in flies

We further modified the conditioning paradigm to remove CS- presentation from training (Figure 3A). Wildtype flies shocked with CS+ pairing are then tested to choose between pure air and a novel odor. Flies raised in the same bottle are randomly separated into two groups, one primed in a training tube with shocks, one mock trained in an identical tube without shock. ΔPI of each bottle was calculated as the difference in PI of shocked and unshocked groups to eliminate the bottle effect of innate odor preference. Chemical odors used in these experiments are all naturally repulsive to flies compared to air, but when comparing the preference changes between shocked and mock trained flies, we found that shocked flies demonstrated an enhanced preference for the novel odor with a ΔPI of approximately 0.15, even though this odor had not been conditioned in any way during training. We call this non-conditioning behavior priming, as shock stimulus changes the flies' reaction to a following, novel odor.

An alternative paradigm was further explored with the removal of CS+ as well. In this case, flies only receive one minute of shock pulses before they are put into the T-maze to choose between pure air and a novel odor (Figure 3B). Results show that flies developed an even more significant enhanced preference to the tested odor, with a ΔPI of ~0.4. This paradigm fits the concept of priming better, as flies are only primed but not conditioned in the process, thus is more often used in later experiments.

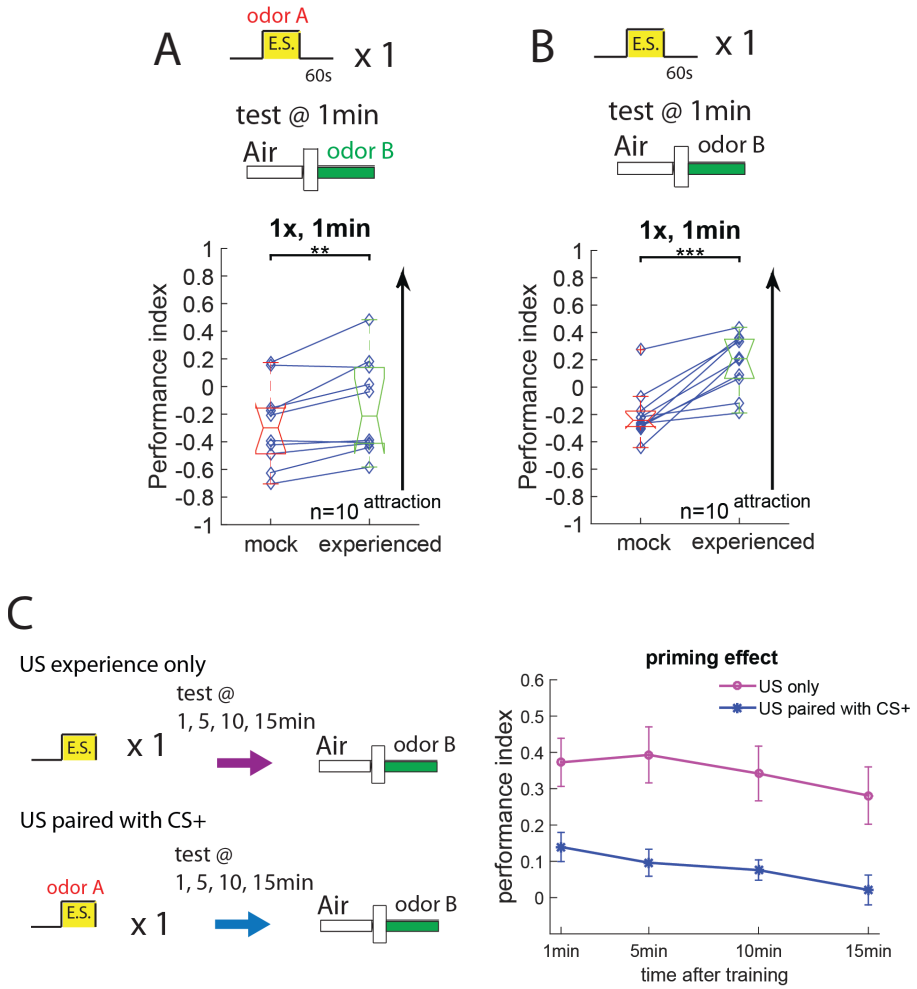
To test the time that priming effect lasts, we primed different groups of flies with either CS+ pairing or not, and tested the flies at 1, 5, 10, and 15min after shock (Figure 3C). We found out that this priming effect that decayed over time was rather long-lasting. Flies primed with CS+ presence showed an altered odor preference for about 10 min, while flies primed with shock only still had a considerably high ΔPI 15 min later. Thus this priming effect in flies is indeed robust and lasts for minutes, implying more profound changes in the neural circuit underlying this behavior.

Figure 3. Priming changes valence perception of novel odors for minutes.

(A-B) Aversive experience yields attraction to subsequent novel odors. In each panel, diagram of priming and the following test are on the top, and the performance index for primed and mock-primed flies is on the bottom. (A) Experience of electric shocks paired with CS+ induced attraction to a novel odor when pure air was used as reference ($n=10$, $p<0.01$, paired t-test). (B) Experience of shocks alone also induced attraction to a novel odor ($n=10$, $p<0.001$, paired t-test). n indicates number of fly groups.

(C) Enhanced preference to novel odors induced by aversive experience spans a relatively long time frame. Left: Schematic of imposing aversive experience on fruit flies with or without coincident odors. Right: Time course of the PI score.

Figure 3. (Continued)



Chapter 3

Dopaminergic Neurons and Mushroom Body Output Neurons Act in a Priming Circuit

Reward DANs are required for priming

To study the neural circuits underlying priming behavior, we first focused on dopaminergic neurons. In addition to directly responding to punishment and reward cues like electric shocks and sugar, DANs are also activated by odors alone, as well as by odor and shock pairing during conditioning. According to previous work in our laboratory, odor response of DANs to CS+ and CS- could change dramatically before and after training, thus DANs are considered plastic during conditioning. Since priming results in altered odor preference as well, we hypothesized that priming also affected DAN activities.

To assess the putative activity change of DANs in priming, we expressed genetically encoded calcium indicator UAS-GCaMP6s in a subset of PAM-DANs labeled by R58E02-GAL4 (Jenett et al., 2012). Specifically, we focused on DANs projecting to the γ-lobe of the mushroom body, including γ3, γ4, and γ5-DANs (Figure 4A). γ-lobe of MB is the region mostly involved in short-term memory, which shares the same timescale as priming.

To image calcium activity in vivo and prime an individual fly at the same time, we tethered the fly in a plastic chamber onto a piece of aluminum foil by wax and surgery glue when it was temporarily anesthetized on ice, and attached copper wires by agarose

gel and glue to its T3 legs to deliver electric shocks. A piece of cuticle on dorsal anterior head was removed as well as the fat tissues below to facilitate imaging. The opened part of the brain was immersed in Schneider's insect medium with calcium and sodium, separated by the aluminum foil from the antennae and abdomen, which must stay in the air. This way we were able to image the neurons in a fly brain under a water-dipping two-photon microscope while delivering shock by wires or odor by a tube to the fly at the same time.

In order to compare the calcium activity in DANs responding to an odor before and after priming, we delivered the odor to a naïve fly for 15 s first to record its baseline odor response (Figure 4B, magenta curves). Dashed lines indicate the onset and offset time point of odor delivery. We waited for at least 5 min for the odor-evoked response to fully calm down in the animal, and then delivered one minute of 12 electric shock pulses of approximately 1 mA current with 5 s intervals. Precisely one minute later, the same odor was delivered to the fly for another 15 s, and the odor response in DANs was imaged (black curves). Odor responses both before and after shocks were averaged among multiple animals.

Comparing the odor response in DANs before and after priming, we found no calcium activity change in γ3 or γ5-DANs. However, γ4-DANs showed significantly increased odor response after priming. γ4-DANs are recognized as reward DANs, and during

aversive conditioning they show increased response to CS- and decreased response to CS+. Although the odor presented in priming is not considered CS- any more, it's perceived as an odor with enhanced preference in behavioral tests, thus the property of γ4-DANs in priming is consistent with that in conditioning. This result confirms the involvement of specific DANs in priming. Notably, the odor response of γ4-DANs after priming was immediate and acute at the onset of odor delivery, indicating that this response change was due to electric shocks only, not a backward conditioning of the shocks and the first few seconds of odor delivery. In addition to PAM-DANs, calcium imaging of other PPL1 γ-DANs including γ1 and γ2-DANs will also be conducted in the future.

To test if these imaged PAM-DANs, especially γ4-DANs, are actually functional and necessary during priming, we assessed the priming behavior of flies with temperature-sensitive blocker of synaptic transmission, *shibire* (Kitamoto et al., 2001) (Figure 4C). We expressed shi^{ts} in PAM-DANs labeled by R58E02 and γ4-DANs labeled by split-GAL4 line MB312B. At the permissive temperature 21°C when shi^{ts} was not activated, the experimental flies had a similar level of priming (measured by ΔPI between primed and mock trained groups from the same bottle) with control flies, which are wildtype 2u flies crossed with shi^{ts}. At the restrictive temperature 31°C when shi^{ts} was activated and DANs were blocked, both experimental fly groups showed significantly reduced level of priming effect compared with controls. Thus PAM-DANs, especially

γ4-DANs are confirmed to be necessary in priming.

Based on these results, we conclude that the reward γ4-DANs' odor activity is changed by electric shocks, and these changes are likely to be essential for the priming effect of flies. In addition to PAM-DANs, requirement of PPL1-DAN types is going to be tested in future experiments.

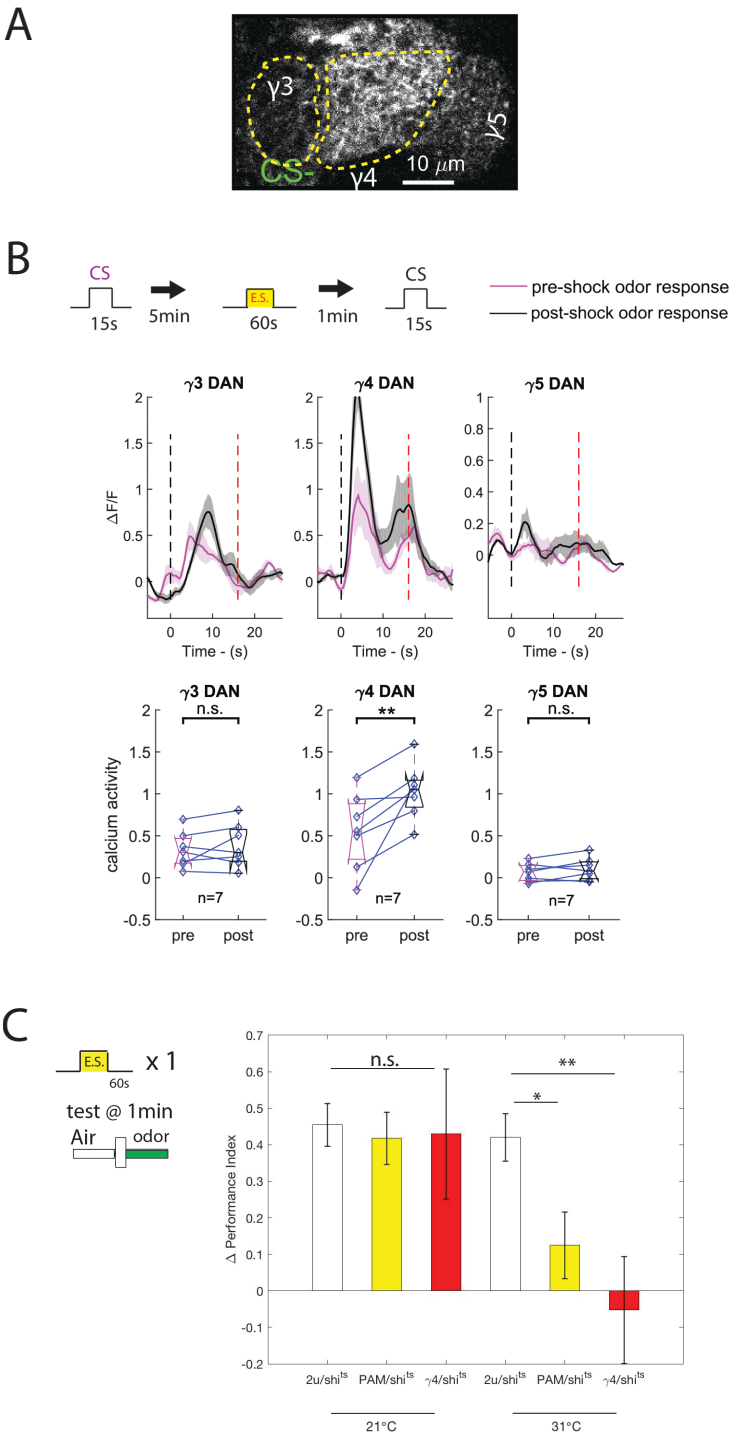
Figure 4. Reward DANs function during priming.

(A) Sample image of two-photon calcium imaging showing PAM-DANs in the γ-lobe of MB during CS- presentation in a conditioning session (genotype R58E02 / GCaMP6s). The presynaptic regions of γ3-DANs and γ4-DANs are circled out by dashed curves.

(B) Electric shocks affect DANs' odor response. Upper panel: schematic paradigm of priming. The odor OCT was first delivered to a fly for 15 s, and the activity of DANs were imaged as baseline. After at least 5 min, 60 s of 12 ~1mA shock pulses was delivered, then 1 min later another 15 s of OCT-evoked response was imaged. Middle panel: Calcium traces of γ3, γ4, and γ5-DANs. Magenta traces show pre-shock odor activity; black traces show post-shock activity. Black dashed line denotes odor onset; red dashed line denotes odor offset. Data are mean (solid curve) ± SEM (shaded area). Lower panel: Comparison of pre-shock and post-shock odor response in box plot. Calcium activity was calculated by averaging the fluorescence over the first 8 s after odor onset (n=7; n.s., $p>0.05$; **, $p<0.01$, paired t-test). n indicates number of individual flies.

(C) Silencing reward DANs by shi^{ts} disrupted priming effect. Left panel: Schematic paradigm of priming. 1 min after flies experienced 1 min of 12 60V foot shock pulses, they were made to choose between pure air and a novel odor OCT. Mock primed flies didn't experience shocks. Right panel: priming scores shown by ΔPI between primed and mock trained groups. White bars, 2u / shi^{ts}; Yellow bars, R58E02 / shi^{ts}; Red bars, MB312B/shi^{ts}. Data are mean (bar) ± SEM (error bar) (n=13, 9, 6, 15, 16, 5, respectively; n.s., $p>0.05$; *, $p<0.05$; **, $p<0.01$; unpaired t-test). n indicates number of fly groups.

Figure 4. (Continued)



Dopamine receptor Dop1R1 in KCs is required for priming

Dopaminergic neurons function in learning and memory by releasing dopamine onto MB to modulate the synaptic strength between KCs and downstream MBONs. Previous studies showed that this dopamine-dependent plasticity happened in the presynaptic terminals of KCs.

To study how DANs modulate KCs to facilitate priming, we first asked whether MB KCs are required for priming. Although the necessity of KCs has been long confirmed in learning, its requirement for priming is less obvious because animals could develop an enhanced preference to any novel odor after being shocked, implying that the specific identity of an odor, which is coded by KCs, might not be essential. To test the requirement of KCs in priming, we expressed shi^{ts} in KCs labeled by OK107, which covered all the subdivisions of all the lobes of MB (Aso et al., 2009). The results showed that flies with KC outputs blocked under higher temperature showed significant defects in priming, examined by the ΔPI between shocked and unshocked groups, compared with controls (Figure 5A). Flies under lower temperature had statistically similar level of priming compared to controls. Thus the outputs of MB KCs are indeed necessary for priming.

We then asked if dopamine receptors in KCs underlie the DAN-dependent priming

effect in flies. There are four types of dopamine receptors in *Drosophila*, which are Dop1R1, Dop1R2, Dop2R, and DopEcR (Yamamoto and Seto, 2014). D1-like dopamine receptors including Dop1R1 and Dop1R2 have been proposed to play opposing roles in olfactory memory regulation at the behavioral level, with Dop1R1 necessary for memory formation and Dop1R2 for memory erosion (Kim et al., 2007; Berry et al., 2012; Qin et al., 2012). Behavioral and neural plasticity depend on the balance of Dop1R1 and Dop1R2, which use different signaling cascades (Handler et al., 2019).

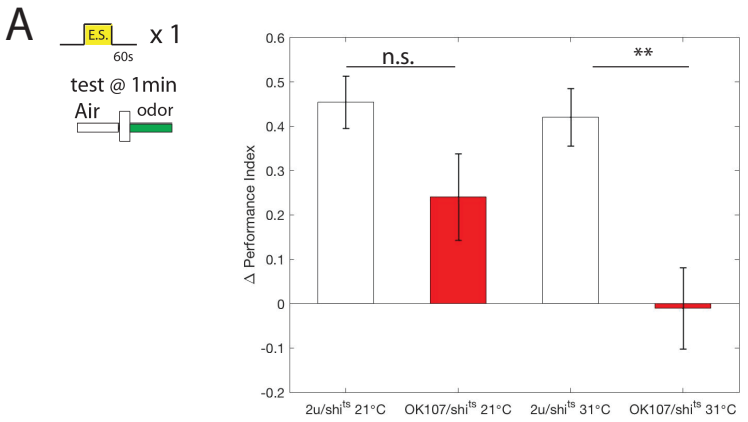
We crossed OK107 flies with RNAi lines of different dopamine receptors to knockdown each receptor in KCs and tested their priming behavior. Among the four receptors in *Drosophila*, only RNAi knockdown of Dop1R1 disrupted priming behavior (Figure 5B). Thus we conclude that Dop1R1 is required in KCs for priming, consistent with its function in memory formation during conditioning. Dop1R1 is known to mediate memory formation by cAMP signaling pathway, providing us a starting point to study the subcellular biochemical mechanisms underlying priming in the future.

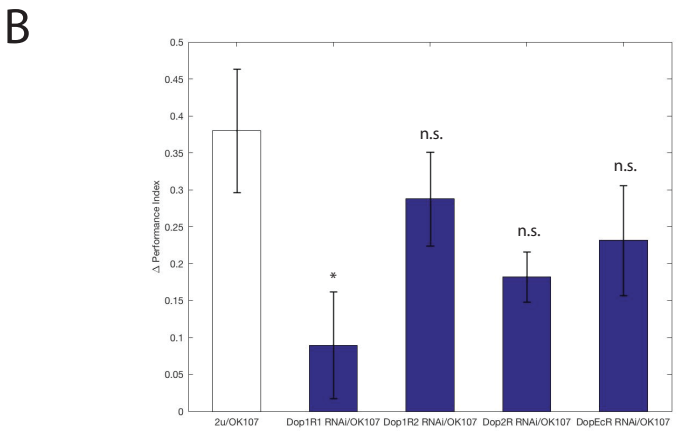
Figure 5. Dop1R1 in KCs is required for priming.

(A) Silencing KCs by *shi*^{ts} disrupted priming effect. Left panel: Schematic paradigm of priming. Right panel: priming scores shown by ΔPI between primed and mock trained groups. White bars, 2u / *shi*^{ts}; Red bars, OK107 / *shi*^{ts}. Data are mean (bar) ± SEM (error bar) (n=13, 10, 15, 7, respectively; n.s., $p>0.05$; **, $p<0.01$; unpaired t-test). n indicates number of fly groups.

(B) Knockdown of different dopamine receptors by RNAi. Only Dop1R1 knockdown resulted in defects in priming. Behavioral paradigm same as in (A). Priming scores shown by ΔPI between primed and mock trained groups. RNAi lines used: Bloomington #31765 for Dop1R1, #51423 for Dop1R2, #26001 for Dop2R, #31981 for DopEcR. Data are mean (bar) ± SEM (error bar) (n=12, 11, 6, 9, 14, respectively; n.s., $p>0.05$; *, $p<0.05$; unpaired t-test). n indicates number of fly groups.

Figure 5. (Continued)





MBON-γ4γ5 functions in priming

Flies need to recruit mushroom body output neurons to summarize the neural computations in higher order brain regions and guide the eventual behavioral valence. To identify the MBONs responsible for priming, we imaged several γ-MBONs using GCaMP6s, including attractive MBON-γ2α'1 and MBON-γ3, and repulsive MBON-γ4>γ1γ2 (Aso et al., 2014b), which were all known to be plastic during conditioning experiments according to previous work in our laboratory. Particularly, we hypothesized that MBON-γ4>γ1γ2 could be plastic during priming as well, considering that this MBON projects its dendrites into the γ4 lobe of mushroom body, which is innervated by γ4-DANs that are functional during priming. The imaging setup and paradigm were similar to that used in DAN imaging experiments, with 1 min shock, 1 min temporal gap after, and 15 s odor delivery (Figure 6A). However, in those neurons, we found no significant odor response change before and after priming, suggesting the involvement of other γ-MBONs that we less frequently studied in earlier conditioning work.

To explore the activities of other γ-MBONs, we then imaged MBON-γ1γ2 and MBON-γ4γ5, labeled by a single GAL4 line VT999036 (Aso et al., 2014b; Shuai et al., 2015) (Figure 6B). MBON-γ1γ2 had no activity change after priming (data not shown), but the odor response of MBON-γ4γ5 was significantly reduced after the animal being

shocked (Figure 6C). In fact, this MBON reacts to neither odor nor shock presented alone, but odor delivered 1 min after shock was sufficient to inhibit its basal calcium activity. The down regulation recorded in MBON-γ4γ5 is consistent with the activation of γ4-DANs we found during priming, because dopamine release from DANs results in depression of the synaptic strength between KC and MBON in the same MB compartment.

To further confirm the requirement of MBON-γ4γ5 in priming, a more restricted split-GAL4 line that labels only MBON-γ4γ5 is needed. Recently we were provided with such a split-GAL4 line by Ito and Rubin at Janelia Research Campus, with which we are setting up to silence MBON-γ4γ5 by *shi*^{ts} to test the priming effect in flies. Before that, we tried to silence MBON-γ4γ5 with the more broadly labeling VT999036, and confirmed that priming effect was indeed disrupted in experimental flies (Figure 6D). Since MBON-γ4γ5 is down regulated during priming, we hypothesized that hyperactivation of this neuron might also lead to defects in priming. We expressed TrpA1, a temperature sensitive cation channel, in VT999036 to activate MBON-γ4γ5 at high temperature, and confirmed that priming was disrupted as well compared with controls (Figure 6E). These silencing and activation experiments using VT999036 are known to have caveats because this GAL4 line labels more than just MBON-γ4γ5 of interest. Nevertheless, MBON-γ4γ5 and MBON-γ1γ2 were the only visible neurons around the MB region during my imaging with GCaMP6s, and MBON-γ1γ2 is known to have

calcium change to neither odor nor priming, so the behavioral results can still imply the possible requirement of MBON-γ4γ5 during priming.

Overall, MBON-γ4γ5 is the only MBON discovered so far to function in priming. Little was known about this MBON because of its relatively late identification in the field. The only paper that mentioned this neuron after its discovery concluded that it was not required for three-hour olfactory memory based on their screening (Shuai et al. 2015). To understand its function in priming, more properties of MBON-γ4γ5 need to be characterized.

Figure 6. MBON-γ4γ5 functions in priming.

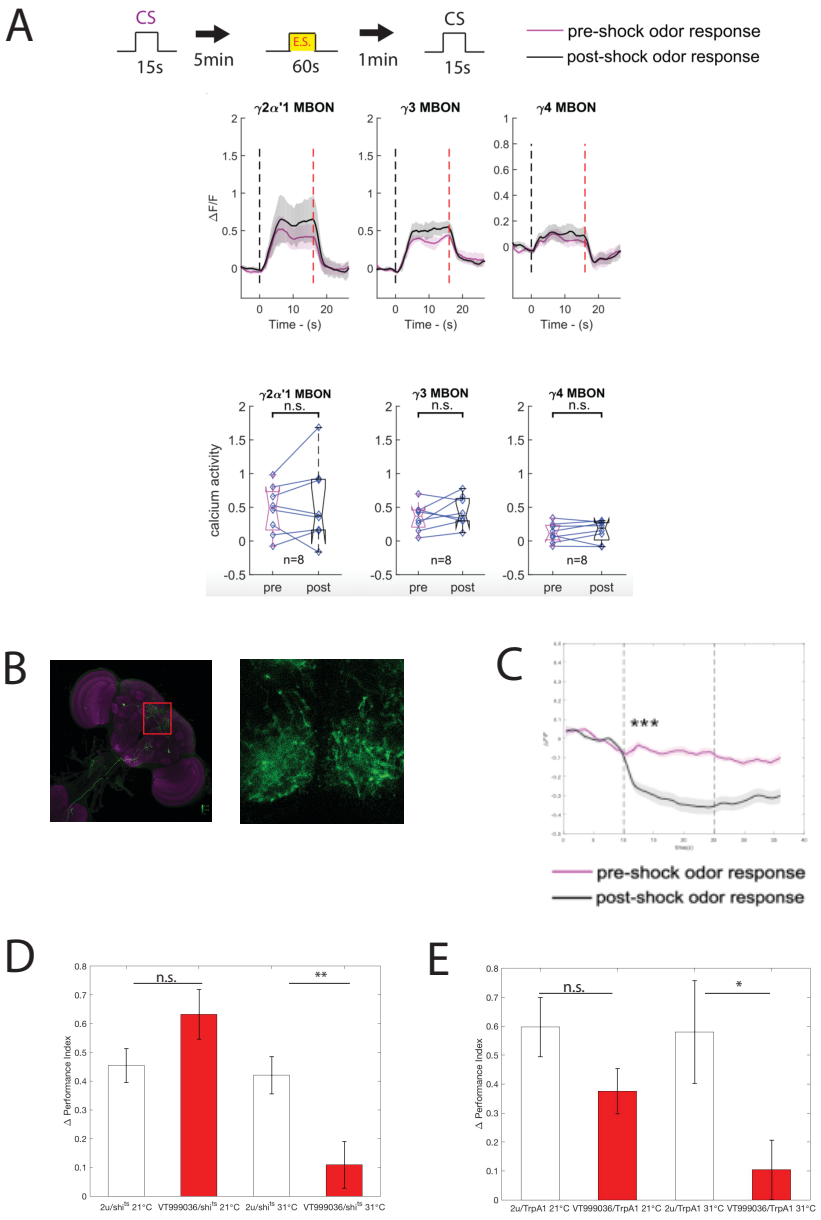
(A) Electric shocks don't affect the odor response of MBON-γ2α'1, MBON-γ3, and MBON-γ4>γ1γ2. Upper panel: schematic paradigm of priming, same as in prior figures. Middle panel: Calcium traces of MBON-γ2α'1, MBON-γ3, and MBON-γ4>γ1γ2. Magenta traces show pre-shock odor activity; black traces show post-shock activity. Black dashed line denotes odor onset; red dashed line denotes odor offset. Data are mean (solid line) ± SEM (shaded area) curves. Lower panel: Comparison of pre-shock and post-shock odor response in box plot. Calcium activity was calculated by averaging the fluorescence over the first 8 seconds after odor onset (n=8 flies; n.s., $p>0.05$; paired t-test).

(B) Anatomical morphology of MBON-γ4γ5. Left panel: immunostaining of MBON-γ4γ5 provided by Ito and Rubin from Janelia. Red square highlights the region for imaging. Right panel: sample image of calcium imaging showing MBON-γ4γ5.

(C) Electric shocks affect the odor response of MBON-γ4γ5. Paradigm same as in (A). Calcium traces of MBON-γ4γ5 during 15 s odor presentation shown in magenta (pre-shock) and black (post-shock) curves (n=8 flies; ***, $p<0.001$; paired t-test).

(D-E) Both silencing with shi^{ts} and activation with TrpA1 of MBON-γ4γ5 by broader-labeling GAL4 line VT999036 results in reduced priming. Behavioral paradigm same as in Fig. 4A. White bars, 2u / shi^{ts} (or TrpA1); Red bars, VT999036 / shi^{ts} (or TrpA1). Data are mean (bar) ± SEM (error bar). (D) n=13, 9, 15, 11, respectively. (E) n= 8, 8, 5, 12 groups, respectively. n.s., $p>0.05$; *, $p<0.05$; **, $p<0.01$; unpaired t-test.

Figure 6. (Continued)



MBON-γ4γ5 has repulsive features

In order to characterize MBON-γ4γ5 that has not been well studied before, we imaged this neuron during a 5-cycle space conditioning and test paradigm, which was normally used to generate LTM in flies (Figure 7A). In this paradigm, an individual fly was mounted in the imaging chamber and first delivered with 60 s of CS+ odor with the pairing of 10 electric shock pulses, then a minute later with 60 s of CS- odor without shock. This training trial was repeated for five times with 12 min intervals in between, making the entire cycle around 15 minutes in total. After the completion of 5 cycles and another 12-min space, the fly went through a test cycle where it encountered the two odors sequentially without shock. The order of odor delivery in the test cycle was reversed to prevent the fly from memory extinction resulting from omission of the expected shock. Calcium traces of imaged flies show that MBON-γ4γ5 had decreased odor response to both odors in the initial cycle (Figure 7A). After that, it began to discriminate the two odors and develop a significantly reduced response to CS- compared to CS+ in training cycle 2 to 5 as well as in the test cycle. Calcium intensity changes (ΔF/F) during the entire 60 s of odor presentation in response to CS+ and CS- were compared using paired t-test. Note that the odor off response in test cycle was different from those in training cycles, possibly due to the omission of shock during CS+ presentation.

These results are similar to those of other well studied MBONs, which also developed discriminating responses to CS+ and CS- during 5-cycle conditioning, demonstrating neural plasticities consistent with their valence coding. The relatively decreased odor response to CS- of MBON-γ4γ5 in conditioning is also consistent with its decreased post-shock odor response in priming imaging, indicating a valence of repulsion for this MBON.

To test this valence, we crossed the split-GAL4 line of MBON-γ4γ5 with light-activated CsChrimson, a red-shifted channelrhodopsin which can excite the labeled neurons with red light when flies are fed with all-trans retinal (ATR) several days before experiment (Klapoetke et al., 2014). The crossed flies were placed in an arena with two quadrants lit and two quadrants dark to choose their preferred area (Figure 7B). For each group of flies, the number of flies in each quadrant was counted at the end of a 1-min lighting session, and counted again at the end of another 1-min session followed immediately, this time with red / dark regions switched. The ratio of flies staying in the red light was averaged between the two sessions, and then averaged among groups of samples. To our surprise, experimental flies fed with ATR showed similar prference of the red light compared with flies that were not fed with ATR, as well as genetic control groups which were crossed between wildtype 2u flies and CsChrimson, fed with ATR or not.

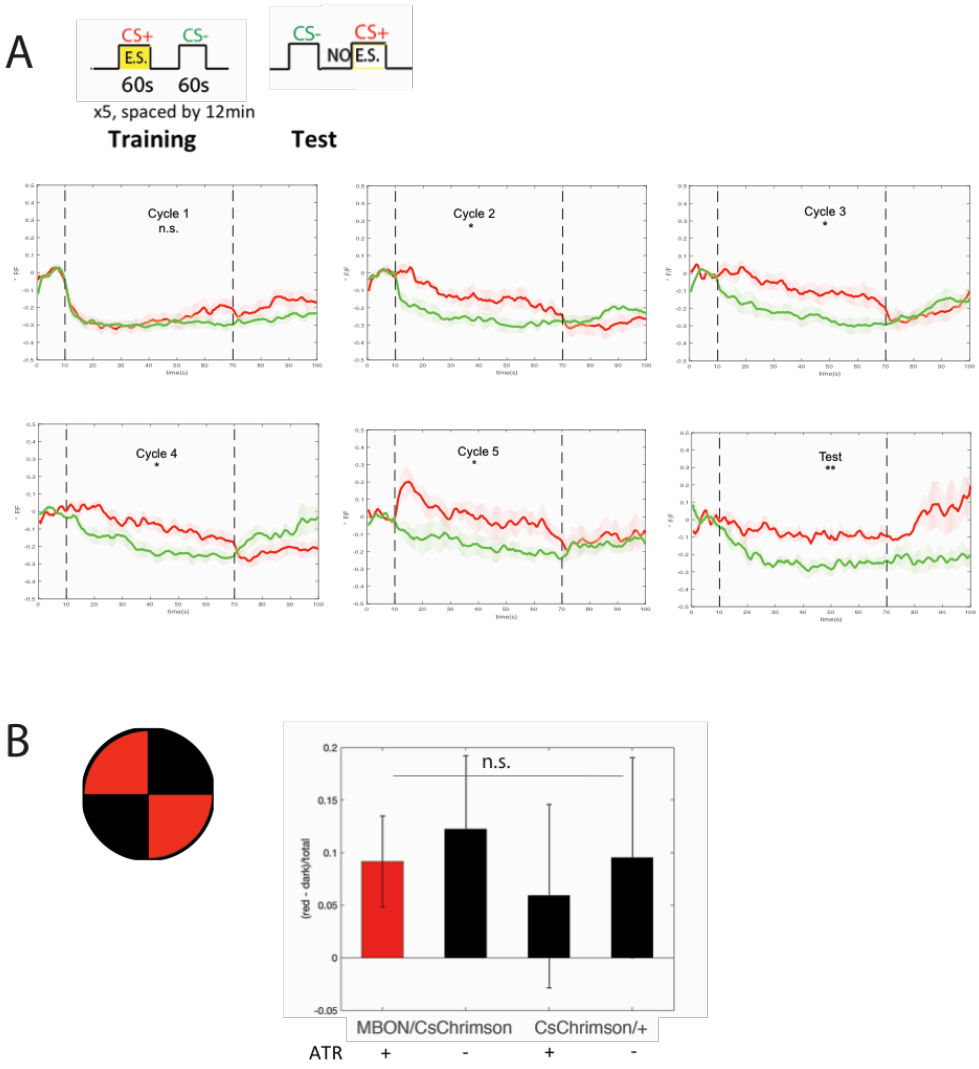
These results suggest that although MBON-γ4γ5 demonstrates some repulsive features in priming and conditioning, its activation alone is insufficient to drive repulsion behaviors directly, unlike MBON-γ4>γ1γ2 which also projects dendrites to the γ4 lobe of mushroom body (Aso et al., 2014b). Nevertheless, not all MBONs encode valence in such a straightforward way. The general function of MBON-γ4γ5 could be more complicated and requires further studies.

Figure 7. MBON-γ4γ5 has repulsive features.

(A) MBON-γ4γ5 is plastic during 5-cycle conditioning. Upper panel: experimental paradigm of 5-cycle conditioning and test. Lower panels: Calcium traces of MBON-γ4γ5 in response to CS+ (red) and CS- (green) in training cycle 1-5 and test cycle. Odor onset and offset time indicated by dashed lines. Data are mean (solid line) ± SEM (shaded area) (n=7,6,6,6,5,5 flies, respectively; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$, n.s., $p>0.05$; paired t-test,).

(B) Activation of MBON-γ4γ5 doesn't drive behavioral valence. Left panel: Flies were put in an arena with two quadrants lit by red light and two quadrants dark. Right panel: Flies carrying CsChrimson in MBON-γ4γ5 fed with ATR showed no significant difference in preference of red or dark regions, compared with MBON-γ4γ5 / CsChrimson flies not fed with ATR, or 2u / CsChrimson flies, ATR fed or not. Height of bar marks the ratio of flies staying in the lit quadrants subtracted by the ratio staying in the dark quadrants at the end of each session. Data are mean (bar) ± SEM (error bar). n=7, 4, 3, 3 groups, respectively; n.s., $p>0.05$; unpaired t-test.

Figure 7. (Continued)



Chapter 4

A Pair of Priming Neurons

Control Olfactory Response and Memory

Two newly identified neurons are plastic during priming

In a previous project in my laboratory, neurons with potential input to DANs were screened. Among the neurons projecting into SMP and CRE neuropils (Figure 8A), which are occupied by DAN dendrites, two novel neurons were identified and found to be plastic to CS+ and CS- during conditioning. These neurons are glutamatergic, and they don't belong to any known neural clusters in the central brain. They reside close to the MB, but don't synapse with KCs directly.

Here we found that these two neurons were also plastic during priming, tested by the calcium imaging paradigm same as that used for DANs and MBONs (Figure 8B). Because of their plasticity in priming, the two neurons were named Priming Neuron 1 (PrN1) and Priming Neuron 2 (PrN2). Calcium traces during priming show that PrN1 has increased odor response after shock, while PrN2 has decreased odor response after shock, indicating their potential functions in priming behavior.

Both PrN1 and PrN2 are odor responsive. During electric shocks alone with the absence of odor, however, only PrN2 is strongly activated by shock pulses (Figure 8C). The shock response curve of PrN1 is basically flat, with slight rebounds after the conclusion of each shock pulse. The shock response traces of PrN1 and PrN2 resemble those of γ4-DANs and γ3-DANs, respectively. This indicates that PrNs, like DANs,

encode the values of unconditioned stimulus as well. Similar to DANs, PrN1 can be regard as a reward neuron, and PrN2 a punishment neuron. The value coding of PrNs is consistent with their odor activity changes during priming, as the reward PrN1 reacts more strongly to the novel odor which becomes more attractive after shock, and the punishment PrN2 reacts more weakly to this preferred odor.

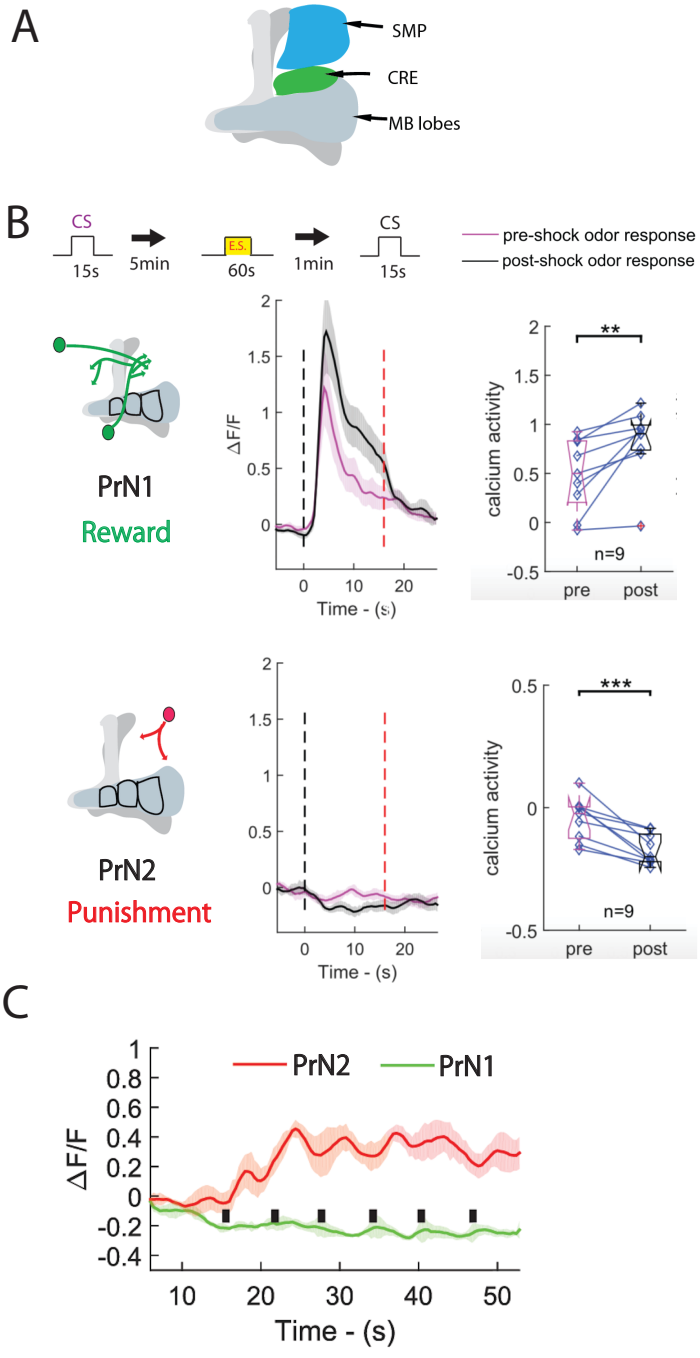
Figure 8. Electric shocks alter the odor response of PrNs.

(A) Schematic of Superior Medial Protocerebrum (SMP) and Crepine (CRE) neuropils, adjacent to the mushroom body lobes.

(B) Electric shocks affect the odor response of PrNs. Upper panel: Paradigm same as in prior figures. Left panel: schematic representation of the locations of PrN1 and PrN2 relative to the MB. Middle panel: Calcium traces of PrNs during 15 s odor presentation shown in magenta (pre-shock) and black (post-shock) curves. Right panel: Comparison of pre-shock and post-shock odor response in box plot. Calcium activity was calculated by averaging the fluorescence over the first 8 seconds after odor onset (n=9 flies; **, $p<0.01$; ***, $p<0.001$; paired t-test).

(C) Electric shock stimulation of GCaMP6s fluorescence in PrNs in the absence of odor stimuli. PrN2 responds strongly to shocks while PrN1 doesn't. Black bars denote presentation of electric shock pulses.

Figure 8. (Continued)



PrN1 is required for priming

To test if Priming Neurons are required for priming, we silenced each neuron by *shi*^{ts} and measured the priming cores by behavioral assays. At the permissive temperature 21 °C, the experimental flies had similar priming effect as controls. At the restrictive temperature 31 °C, PrN2 silenced flies still had normal level of priming, while PrN1 silenced flies showed significant defects in priming, measured by the ΔPI between primed and mock primed flies raised in the same bottle (Figure 9A). Thus PrN1 is required for priming and PrN2 is not.

This result is consistent with previous studies of PrNs in conditioning. In a behavioral test of CS+ and CS- memories with 5-cycle conditioning, silencing of PrN1 with *shi*^{ts} during either test (Figure 9B, C) or training (Figure 9D, E) period resulted in defects in attraction memory of CS- but not repulsion memory of CS+, while silencing of PrN2 during either test (Figure 9B, C) or training (Figure 9D, E) period resulted in defects in repulsion memory but not attraction memory. As a positive control, silencing PAM-DANs during either period by temperature raise disrupted all forms of memories (Figure 9B-E).

Since the odor presented in priming experiments becomes an attractive odor after the animal being shocked, it's valence coding should be similar to that of CS- odor in

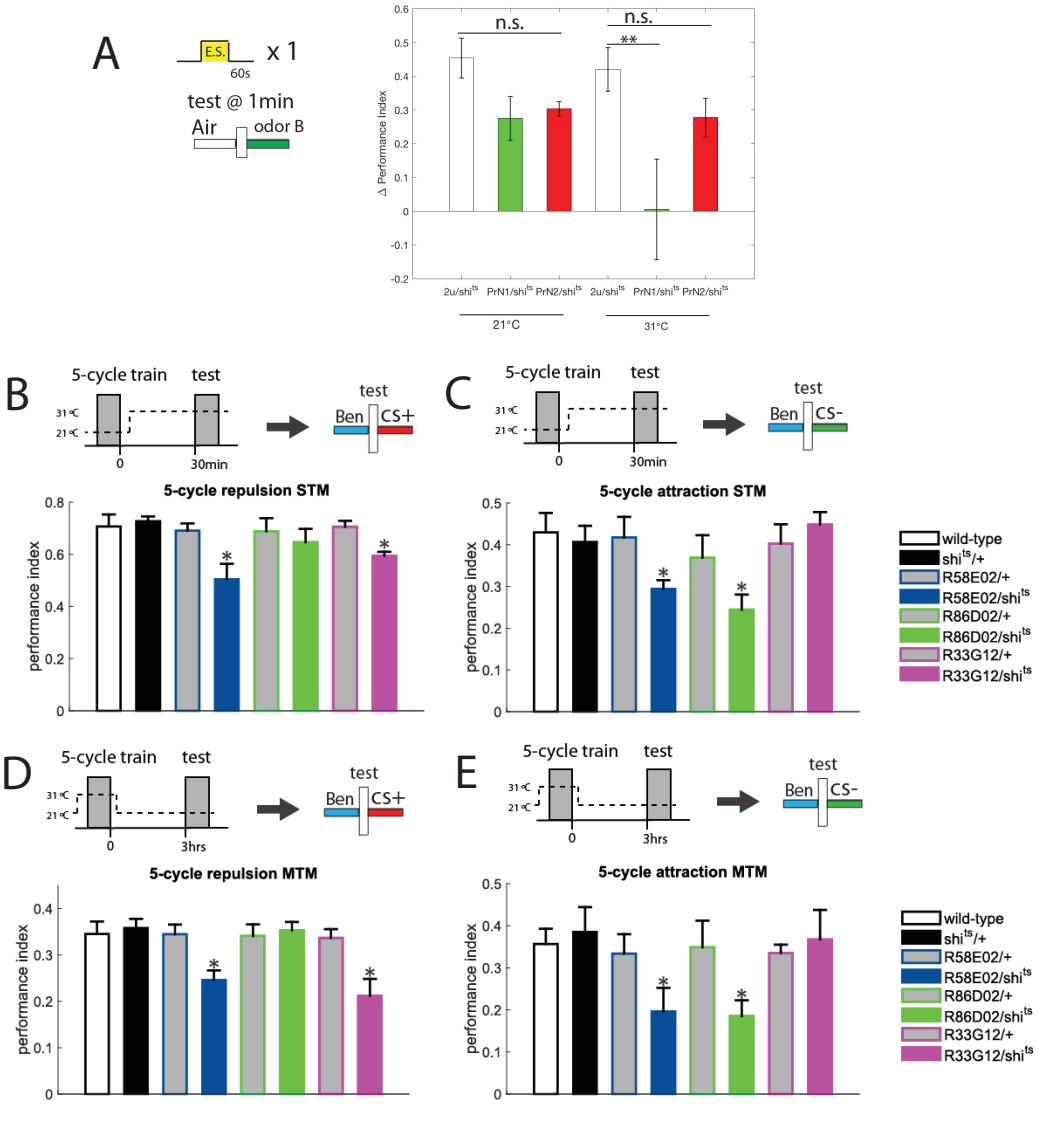
conditioning experiments, thus the necessity of PrN1, which is considered a reward neuron, for both priming and attraction memory is consistent. Similarly, the requirement of PrN2 for neither priming nor attraction memory is also expected, and consistent with its punishment nature.

Figure 9. Silencing PrN1 disrupts priming.

(A) Silencing PrN1, but not PrN2, by *shi*^{ts} disrupted priming effect. Left panel: Schematic paradigm of priming, same as in prior experiments. Right panel: priming scores shown by ΔPI between primed and mock trained groups. White bars, 2u / *shi*^{ts}; green bars, R86D02 (PrN1) / *shi*^{ts}; red bars, R33E06 (PrN2) / *shi*^{ts}. Data are mean (bar) ± SEM (error bar) (n=13, 8, 4, 15, 8, 7 groups, respectively; n.s., $p>0.05$; **, $p<0.01$; unpaired t-test).

(B-E) Blocking output from PrNs or DANs by *shi*^{ts} leads to defect in repulsion or attraction memory. (E): Blocking output from R58E02 (PAM-DANs) or R33G12 (PrN2) after 5-cycle spaced training impairs 30min repulsion memory. Blocking output from R86D02 (PrN1) doesn't cause the defect (Kruskal-Wallis, n>10 for each genotype). (F): Blocking output from R58E02 (PAM-DANs) or R86D02 (PrN1) after 5-cycle spaced training impairs 30 min attraction memory. Blocking output from R33G12 (PrN2) has no effect (Kruskal-Wallis, n>10 for each genotype). (G): Blocking output from R58E02 (PAM-DANs) or R33G12 (PrN2) during 5-cycle spaced training impairs 3 h repulsion memory. Blocking output from R86D02 (PrN1) has no influence. 3 h memory was chosen to allow for full neuronal function recovery after *shi*^{ts} inactivation for 1.5 h (Kruskal-Wallis, n>10 for each genotype). (H): Blocking output from R58E02 (PAM-DANs) or R86D02 (PrN1) during 5-cycle spaced training impairs 3 h attraction memory. Blocking output from R33G12 (PrN2) doesn't result in defect (Kruskal-Wallis, n>10 groups for each genotype).

Figure 9. (Continued)



PrNs and MBONs are functionally upstream of DANs

Calcium imaging results show that PrN1 and PrN2 share similar activity patterns during priming, conditioning, and shock presentation with γ4-DANs and γ3-DANs, respectively. PrNs also project their presynaptic axonal terminals to the SMP region of the brain where dendrites of DANs reside, suggesting potential synaptic connections between these neuronal types.

To test their functional connectivity, we expressed optogenetic neuronal activator CsChrimson to drive neural activity in PrN1 or PrN2 while imaging calcium dynamics with GCaMP6s expressed in DANs. We found that light pulse activation of CsChrimson in reward neuron PrN1 resulted in strong activation of the reward γ4-DANs and much weaker activation of the punishment γ3-DANs and the reward β'2-DANs (Figure 10A). In contrast, activation of CsChrimson in punishment neuron PrN2 strongly activated γ3-DANs and inhibited γ4-DAN and β'2-DAN activities, compared with controls (Figure 10B). Thus PrN1 and PrN2 mainly activate the DANs with same value coding, and in some cases inhibit DANs that reinforce the opposing valences. This way PrNs are confirmed to act as upstream neurons of DANs, possibly conveying the value information of unconditioned stimulus to DANs. More importantly, considering the function of PrNs in priming and conditioning, they could mediate the behavioral effects by feeding the information of valence change back onto DANs, acting as the interface of the conditioned

and unconditioned pathways to relay the experience of an aversive stimulus and novel odor to the reward dopaminergic circuitry.

Nevertheless, PrNs alone are not sufficient to mediate the plasticity of DANs during priming and conditioning. Because mushroom body is the olfactory learning center of flies, all the experience dependent neural plasticity requires input from the output of MB, which are, by definition, MBONs. Thus MBONs are hypothesized to feed back onto DANs as well, either directly or indirectly.

To test the functional connectivity between MBONs and DANs, CsChrimson was expressed by GAL4 drivers in selected MBONs while DANs were imaged using GCaMP6s by LexA drivers. Activation of the repulsive MBON-γ4 and MBON-β’2 with pulses of 630nm laser light reduced calcium fluorescence in the reward γ4-DANs and β′2-DANs (Figure 10C), and strongly increased fluorescence in the punishment γ3-DANs. In contrast, the approach MBON-γ2α′1 strongly activated the reward β′2-DANs, and moderately activated the punishment γ3-DANs. This sampling of the interactions indicates that there are direct synaptic connections between MBONs and DANs, establishing a recurrent network allowing MBONs to mediate the activities of reward and punishment DANs by relatively specific feedback pathways. These results suggest that in general, repulsion MBONs activate punishment DANs and inhibit reward DANs, while approach MBONs most strongly activate reward DANs, reinforcing the experience

dependent valence.

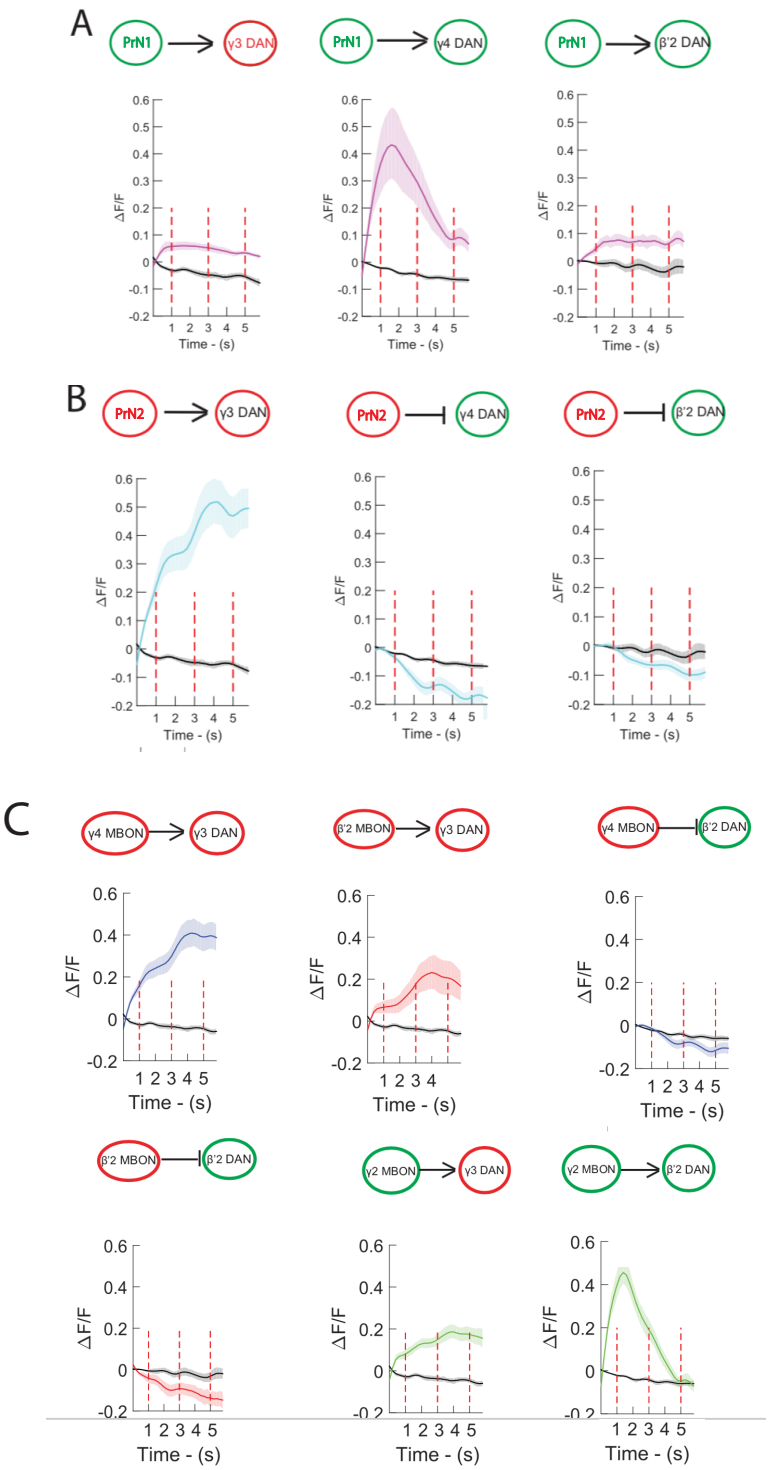
Overall, both PrNs and MBONs function upstream of DANs, forming recurrent feedback loops between the output and input of mushroom body. Considering the requirement of DANs, PrNs, and MBONs in priming, more functional pathways, especially the potential connections between MBONs and PrNs are still being studied. In particular, functional mapping of the downstream neurons of MBON-γ4γ5, the only MBON known to be functional during priming, is part of the future work to be carried out with our recent obtaining of its split-GAL4 line. Our results suggest that PrNs serve as entry points for higher order network activity that assigns valence to a contextual odor, and thus contribute to priming, reflecting a multi-faceted learning experience.

Figure 10. Activation of PrNs or MBONs excites or silences specific DANs.

(A-B) UAS-CsChrimson was expressed in PrN1 (R86D02-GAL4) or PrN2 (R33E06-GAL4) and lexAop-GCaMP6s was expressed in DANs with R58E02-lexA. Punishment neurons are circled by red and reward neurons are circled by green. GCaMP6s fluorescence was recorded when ~10μw/mm^2 630nm laser light pulses were used to activate CsChrimson. In each plot, vertical red dashed lines indicate the time of ~80ms light pulses (3 pulses with an interval of 2 seconds, for a total of 5.5 seconds). Negative fluorescence changes (ΔF/F) are considered to indicate inhibition. Colored curves: experimental groups of flies with genotypes stated. Black curves: control groups with no GAL4 expression. All fed with ATR before experiments. Scale bar: 20μm.

(C) UAS-CsChrimson was expressed in specific MBONs and lexAop-GCaMP6s was expressed in DANs with R58E02-lexA. Punishment / repulsion neurons are circled by red and reward / approach neurons are circled by green. GCaMP6s fluorescence was recorded when ~10μw/mm^2 630nm laser light pulses of 80 ms were used to activate CsChrimson. Negative fluorescence changes (ΔF/F) are considered to indicate inhibition. Colored curves: experimental groups of flies with genotypes stated. Black curves: control groups with no GAL4 expression. All fed with ATR before experiments. Scale bar: 20μm.

Figure 10. (Continued)



Chapter 5

Discussion

Priming differs from conditioning

The priming effect newly identified in this work has not been described in *Drosophila*, nor in other model organisms. Notably, the priming experiment is different from another traditional learning paradigm known as backward conditioning, or sometimes called relief conditioning, where animals receive CS+ after US with a small temporal gap in between during training, and show opposite valence to CS+ in test (Tanimoto et al., 2004; Gerber et al., 2014; Sanderson et al., 2014; Niewalda et al., 2015; Handler et al., 2019). For example, flies backward-conditioned with an odor coming after the conclusion of electric shocks would be attracted to this odor in the T-maze test instead of avoiding it, compared with a control odor. The backward conditioning and priming paradigms are different in nature, because the conditioned odor used for backward pairing, either being called CS+ or CS-, is not present in the priming setup (the paradigm without CS+, Figure 3B). After being shocked in air, flies tracked towards the normally undesired odor immediately when they smelled it in the T-maze. The calcium changes in the γ4-DAN and MBON-γ4γ5 curves are also immediately visible after odor onset, implying that the odor preference change doesn't depend on a backward conditioning of the shock and the odor that only come to present at the beginning of the test. Thus there is no conditioning of any kind happening during priming, but the perception of a naïve odor is changed by shock experience alone.

There was another study describing an effect called blocking odor response by electric shock (BOBE) (Song et al., 2018), where flies staying in a T-maze containing odor and pure air in each arm for the whole time were first shocked in the maze and then free to choose between the two arms. An enhanced preference to the chemical odor was also found, and was explained by the interruptive effect of electric shock on odor response, which required the functions of specific parts of mushroom body. Our results are consistent, but the difference in experimental paradigms is critical to make the two behaviors distinct, because BOBE involves coincident pairing of odor and shock while priming doesn't.

In addition, the priming effect is not merely due to flies conditioned to associate shock with pure air. First, air isn't known to be encoded by specific neurons in the olfactory sensory system, as flies are raised in the air constantly. Even during odor delivery, the diluted odor stream consists of 99.99% of air. Secondly, even if conditioning with air could be contributing partly to the reduced preference to air in the priming paradigm without CS+ (Figure 3B), the paradigm recruiting CS+ still allows the pairing of shocks and a non-air odor (Figure 3A), so air is not conditioned with electric shocks in this case, and the priming effect is still seen in the results. Thus we conclude that the change of odor preference in flies results from the unconditioned aversive experience instead of merely another form of conditioning.

The concept of priming has only been used for studies of human behaviors before. As we borrow the term to describe a novel behavior in *Drosophila*, it would be reasonable to hypothesize that similar priming effect exists in other organisms as well, especially in species more closely related to human. Like learning and memory, the logic of neural networks underlying priming-like behaviors could also be conservative among species, and fruit fly would be a simple enough model organism to start with.

Toward a more complete priming circuit

In this project, we described a priming circuit involving DANs, MBONs, and the newly identified PrNs that are functionally interconnected. To fully explain the priming behavior, more properties about these neurons need to be characterized, and their connections, especially between PrNs and MBONs, need to be better illustrated. In addition, more neural components are likely to be added into this priming circuit in later work.

Because priming results in a form of approach behavior, we focused more on the circuits of PAM-DANs, which are largely rewarding. However, recent observations suggest that both reward and punishment DANs are integrated into circuits that assess a range of cues and are subject to network-wide plasticity. Thus the function of punishment PPL1-DANs projecting to γ-MB is also worth studying. Moreover, an EM connectome of a fly central brain that was released only very recently suggests that MBON-γ4γ5 seems to feed back directly onto punishment DANs including γ3-DANs and γ1-DANs. Our results suggest that PAM-γ3-DANs don't show odor response change after priming. However, considering the essential role of MBON-γ4γ5 in priming, it's possible that the punishment PPL1-γ1-DANs, as well as the known local connections between γ1 and γ4 lobes of mushroom body carried out by neurons PAM-DAN-γ4<γ1γ2 and MBON-γ4>γ1γ2, can be recruited into the reward priming circuit downstream of

MBON-γ4γ5 and connect with γ4-DANs that are known to function during priming, forming a complete priming circuitry that involves neurons encoding opposing values and valences.

MBON-γ4γ5 is the only MBON found so far to show an altered odor response after shock. Its plasticity is consistent with its putative valence of repulsion, which is the same as the other MBON that innervates the γ4 lobe of MB, but a simple activation of MBON-γ4γ5 doesn't directly lead to avoidance behavior in flies. It's also different from the other neighboring MBONs as it doesn't react to odor stimulus in absence of shock. Its reduced calcium activity observed during priming is also very rare among the MBONs, as most MBONs regulate their downstream neurons by different levels of activation (Hige et al., 2015b). These all suggest a potentially unique role that MBON-γ4γ5 plays in not only priming, but also other fly behaviors. In fact, EM segmentation data of the fly brain connectome project suggests that this MBON forms multiple connections with fan-shaped body (FB) neurons. Other MBONs including those with projections into the γ1 and γ5 lobes of the mushroom body may also be tested in the future for their possible functions in priming.

In addition, we're not clear about the functions of the two newly identified PrNs apart from priming and conditioning. We are currently trying to identify these neurons by anatomical structures in the fly EM connectome to get an understanding of their full

connections with other neurons. These PrNs don't belong to any known neural clusters, and they reside in SMP and CRE, which are brain regions that contain a lot of neuropils of well-studied important neurons, but the regions themselves are not well characterized before. Future work about these brain regions might help us understand the functions of PrNs, as well as a large number of unknown neurons in the fly brain in a more systematic way.

Materials and Methods

Fly stocks

Non-temperature sensitive flies were raised on standard cornmeal-agar-molasses medium at 25 °C and approximately 70% humidity, under 12-hour light / 12-hour dark cycles. Flies used for *shi*^{ts} silencing and TrpA1 activation experiments were grown at 21 °C until use.

VT999036 and γ4-splitGAL4 were gifts from Y. Shuai (Shuai et al., 2015). MBON-γ4γ5 splitGAL4 was obtained from Ito and Rubin with collaboration. All other fly stocks were bought from Bloomington *Drosophila* Stock Center.

Genotypes used in each figure

Figure 2: 2u.

Figure 3: 2u.

Figure 4: (A-B) R58E02/GCaMP6s. (C) 2u/*shi*^{ts}. R58E02/*shi*^{ts}. MB312B / *shi*^{ts}.

Figure 5: (A) 2u/*shi*^{ts}. OK107/*shi*^{ts}. (B) 2u/OK107. Bloomington #31765/OK107. 51423/OK107. 26001/OK107. 31981/OK107.

Figure 6: (A) R74B04-Gal4/UAS-GCaMP6s. R52G04-Gal4/UAS-GCaMP6s. R74B04-Gal4/UAS-GCaMP6s.

(B) VT999036/GCaMP6s. (C) 2u/*shi*^{ts}. VT999036/*shi*^{ts}.

(D) 2u/TrpA1. VT999036/TrpA1.

Figure 7: (A) VT999036/GCaMP6s. (B) 2u/CsChrimson::tdTomato.

MBON-γ4γ5 splitGAL4/CsChrimson::tdTomato.

Figure 8: R86D02/GCaMP6s. R33E06/GCaMP6s.

Figure 9: (A) 2u/*shi*^{ts}. R86D02/ *shi*^{ts}. R33E06/ *shi*^{ts}. (B) 2u. 2u/*shi*^{ts}. R58E02/2u. R58E02/ *shi*^{ts}. R86D02/2u. R86D02/ *shi*^{ts}. R33G12/2u. R33G12/ *shi*^{ts}.

Figure 10: (A) UAS-CsChrimson::tdTomato/R58E02-lexA, lexAop-GCaMP6s; R86D02.

(B) UAS-CsChrimson::tdTomato/R58E02-lexA, lexAop-GCaMP6s; R33E06.

(C) UAS-CsChrimson::tdTomato/R58E02-lexA, lexAop-GCaMP6s; γ4-splitGAL4.

UAS-CsChrimson::tdTomato/R58E02-lexA, lexAop-GCaMP6s; MB011B.

UAS-CsChrimson::tdTomato/R58E02-lexA, lexAop-GCaMP6s;MB077B.

Functional calcium imaging

All functional calcium imaging experiments were performed on a Zeiss two-photon laser-scanning microscope (LSM780 NLO). Images were taken on a single focal plane with a frequency of 2.5Hz. The Ca^{2+} reporter GCaMP6s was expressed in all relevant neurons. Fly heads with the brain exposed were submerged in Schneider's medium (Sigma) supplemented with 2mM Ca^{2+} and 4mM NaHCO3. Odor stimulation was achieved by a custom-built odor delivery system with a switch design to ensure maintenance of a constant airflow rate when valves were switched. A continuous stream (2000 ml/min) of air, directed at fly antenna, was maintained with mass flow controllers (Alicat Scientific). At a trigger, a 10% of air stream was diverted through a

vial containing an odorant diluted in paraffin oil (Sigma), and the air stream was humidified by passing through a water tube. Chemical odors of 0.3% 3-octanol (OCT) and 0.15% 4-methylcyclohexanol (MCH; Sigma) diluted in paraffin oil were used in all imaging experiments.

Odor stimulation

Odor stimulation was achieved by directing a continuous stream (2000ml/min) of clean air through a 1/8 inch inner diameter Teflon tubing directed at the fly's antenna (carrier stream). 10% of the total air stream was diverted through a glass vial containing 5mL paraffin oil (odor stream). A third compensating air stream (200ml/min) was directed out of the system. At a trigger, a custom-built solenoid valve controller system redirected the third compensating air stream to a vial containing odorants diluted in 5mL paraffin oil (Sigma) and simultaneously switched the compensating air stream with the second air stream. This was designed to reduce air stream disturbance during valve switches. Final odorant dilutions were usually around 1:1000, depending on the identity of odorants. Odorants used in imaging were 3-octanol (CAS #589- 98-0) and 4-methancyclohexanol (CAS #589-91-3).

Tethered fly conditioning

For all in vivo imaging experiments, brains were dissected in 0.9x Schneider Insect Medium (Sigma) supplemented with 2mM $CaCl_2$ and 4mM $NaHCO_3$. A special chamber

was designed for robust imaging of mushroom body, SMP and CRE regions with high resolution. Flies were prepared as below. 5-10 day old flies were anaesthetized on ice and glued to a hole cut out on a small piece of aluminum foil. Bio-compatible adhesive Kwik-Sil Adhesive (World Precision Instrument) was used as glue. The piece of aluminum foil with one fly tethered was then attached onto imaging chamber. The chamber was then filled with Schneider medium and the head capsule was opened by carefully cutting the cuticle covering the dorsal portion of the head. Obstructing trachea was removed with forceps. Care was taken to keep antenna, antennal nerves and other brain tissues intact. The animal's legs were secured in paraffin wax with tips exposed to allow for wire attachment. Copper wires were placed against the legs and surrounded with agarose gel dissolved in saline for conduction. Kwik Sil silicone glue (World Precision Instruments) was then applied around the agarose gel to prevent drying out. A stimulator (S48 Stimulator, Grass Technology) was used to apply electric current to the wires to deliver shock.

Functional calcium imaging with optogenetic stimulation

Larvae were grown on standard cornmeal food and kept for another 1-5 days after eclosion. Adult flies were kept on standard food supplemented with 1mM all-trans-retinal (Sigma) for 7-10 days. A UAS-CsChrimson transgene was expressed in MBONs or PrNs with corresponding split-GAL4 or GAL4 lines, as indicated. 633 nm red light illumination was achieved by focused laser scanning in Zeiss LSM 780 NLO

Multi-Photon Microscope. A photo-bleaching option available in Zeiss system was used to provide red light stimulation as neuronal activity was simultaneously recorded by two-photon laser scanning. An external light source, such as a mounted LED, was found to be too strong for use as it triggered calcium flux in multiple DANs, even in control animals lacking CsChrimson expression. Laser scanning allowed us to focus light onto a relatively tiny spot on fly brain (roughly estimate <400 μm x 400 μm) to minimize light intensity, thus reducing red light activated DANs' response to negligible level. 100 ms light pulses were used in each experiment.

Behavior in arena with optogenetic stimulation

Split-GAL4 line of MBON-γ4γ5 (or 2u in genetic control groups) was crossed with light-activated CsChrimson. Experimental flies were fed with 1 mM ATR food for 5-7 days before the test, while no-ATR controls were fed with regular food. The crossed flies were placed in a round shallow plastic dish on top of a larger round plate, which had two opposing quadrants translucent and two quadrants covered by black tape. For each group of flies, the number of flies in each quadrant was recorded by camera at the end of a 1-min red light lighting session and counted later, and photo recorded and counted again at the end of another 1-min lighting session followed immediately, this time with red / dark regions switched by rotating the containing dish. The ratio of flies staying in the red light was averaged between the two sessions, and then averaged among groups of samples.

Image processing and data analysis

Most image processing was done in FIJI/ImageJ (NIH). Further analysis was done in Matlab. When necessary, to correct for motion during in vivo imaging, time series images were aligned using the TurboReg FIJI plugin.

Calcium intensity plots

For imaging of DANs and MBONs, ROIs were manually drawn based on clear anatomical segregation of the innervation patterns in different compartments. For imaging of PrNs, ROIs were manually drawn covering the entire axonal branches in the field of view, while avoiding inclusion of branches from irrelevant neurons. For DANs, we can clearly observe fluorescence punctae in each compartment. For MBONs and PrNs, we can clearly observe neuronal branches. In order to extract those punctae and branches while excluding background noise, a difference of Gaussian (DoG) filter for blob detection was applied to each frame to extract punctae and branch features. Two Gaussian kernels were used for DoG filter and the standard deviation (σ) for each kernel was 1.8 and 2.4 pixels respectively. An image mask was calculated from DoG treated raw image. Then image mask was applied to each raw image to obtain punctae or branch blobs. Fluorescence intensity was finally calculated by averaging the fluorescence over the entire image. For calcium traces, $\Delta F/F$ was calculated by subtracting the difference between the pre-stimulus values, an average of 20 frames (~ 7 seconds) ending 1 frame

before stimulus onset, and the post-stimulus values (for each frame) divided by pre-stimulus value. For plotting ΔF/F over training cycles, calculation was done by averaging ΔF/F values over the first 8 seconds after stimulus onset. Note that in the case of DAN imaging, DAN exhibit relatively strong fluctuations in their basal activity, making it more difficult to obtain an accurate estimate of basal activity. However, we observed that that 7 seconds before stimulus onset is long enough for an estimate of the basal activity of DANs. We used the average of the 7 s fluorescence values as the basal line.

Statistical analysis

Statistical analysis was performed using custom scripts in Matlab. Paired t-test was used for all paired comparison of DAN, PrN and MBON response to odor before and after priming, as well as comparison of primed and mock trained T-maze test results. Two-sample t-test was used for data analysis in comparing priming scores between different groups of flies.

Classical aversive olfactory conditioning

Conditioning experiments were done in a dark room with only red light on, where the humidity of the room was kept at 60% and the temperature of the room was kept at 21°C or 23°C unless heat shock (31°C) was required. An automated training apparatus was built with help from the Harvard Center for Brain Science (HCBS) machine shop.

Training experiments were performed as previously described (Tully et al., 1994). Briefly, 12 pulses of 1-second electric shock (60V) were presented to the animal's legs during a 1-min CS+ presentation. After a 45 s of fresh air flush, the CS- odor was presented for 1 min. In spaced training, a 12-min interval occurred between training cycles. A custom-made T-maze was used to measure the performance index at the indicated time post-training. The performance index was calculated as PI=(n1–n2)/(n1+n2), where n1 and n2 are the numbers of flies that chose odor 1 or odor 2 in the T-maze, respectively. Odorant concentrations were 0.3% OCT and 0.15% MCH in all conditioning experiments.

Priming

Behavioral assays of priming were conducted using the same apparatus and behavioral room for conditioning. In the paradigm with CS+ presentation, flies receive 1 min of 12 60V electric shock pulses with CS+ odor delivered at the same time. 1 min (or other interval time as indicated in the figures) after the cessation of shocks, flies were transferred into the T-maze to choose between air and a novel odor (0.01% OCT was usually used unless specified) for 1 min. The performance index was calculated as PI=(n1–n2)/(n1+n2), where n1 and n2 are the numbers of flies that chose novel odor or air in the T-maze, respectively. Priming level was represented by ΔPI. It was calculated as the difference between the PI of primed and mock primed group of flies which had been raised in the same bottle of food to eliminate the bottle effect of innate preference of

odors. The priming paradigm without CS+ presentation was similar, with the only difference in removing CS+ presentation during shocks. The priming paradigm without CS+ was used in all the behavioral experiments testing the requirement of different neurons during priming, including silencing by shi^{ts}, activation by TrpA1, and knockdown by RNAi.

References

Akalal DB, Wilson CF, Zong L, Tanaka NK, Ito K, Davis RL. Roles for Drosophila mushroom body neurons in olfactory learning and memory. Learn Mem. 2006;13(5):659-68.

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.

Aso Y, Hattori D, Yu Y, et al. The neuronal architecture of the mushroom body provides a logic for associative learning. Elife. 2014a;3:e04577.

Aso Y, Herb A, Ogueta M, et al. Three dopamine pathways induce aversive odor memories with different stability. PLoS Genet. 2012;8(7):e1002768.

Aso Y, Rubin GM. Dopaminergic neurons write and update memories with cell-type-specific rules. Elife. 2016;5.

Aso Y, Sitaraman D, Ichinose T, et al. Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. Elife. 2014b;3:e04580.

Aso Y, Siwanowicz I, Bräcker L, Ito K, Kitamoto T, Tanimoto H. Specific dopaminergic neurons for the formation of labile aversive memory. Curr Biol. 2010;20(16):1445-51.

Berry JA, Cervantes-sandoval I, Nicholas EP, Davis RL. Dopamine is required for learning and forgetting in Drosophila. Neuron. 2012;74(3):530-42.

Berry JA, Phan A, Davis RL. Dopamine Neurons Mediate Learning and Forgetting through Bidirectional Modulation of a Memory Trace. Cell Rep. 2018;25(3):651-662.e5.

Boto T, Louis T, Jindachomthong K, Jalink K, Tomchik SM. Dopaminergic modulation of cAMP drives nonlinear plasticity across the Drosophila mushroom body lobes. Curr Biol. 2014;24(8):822-31.

Bouzaiane E, Trannoy S, Scheunemann L, Plaçais PY, Preat T. Two independent mushroom body output circuits retrieve the six discrete components of Drosophila

aversive memory. Cell Rep. 2015;11(8):1280-92.

Busto GU, Cervantes-sandoval I, Davis RL. Olfactory learning in Drosophila. Physiology (Bethesda). 2010;25(6):338-46.

Campbell RA, Honegger KS, Qin H, Li W, Demir E, Turner GC. Imaging a population code for odor identity in the Drosophila mushroom body. J Neurosci. 2013;33(25):10568-81.

Caron SJ, Ruta V, Abbott LF, Axel R. Random convergence of olfactory inputs in the Drosophila mushroom body. Nature. 2013;497(7447):113-7.

Cervantes-sandoval I, Phan A, Chakraborty M, Davis RL. Reciprocal synapses between mushroom body and dopamine neurons form a positive feedback loop required for learning. Elife. 2017;6.

Chen CC, Wu JK, Lin HW, et al. Visualizing long-term memory formation in two neurons of the Drosophila brain. Science. 2012;335(6069):678-85.

Claridge-chang A, Roorda RD, Vrontou E, et al. Writing memories with light-addressable reinforcement circuitry. Cell. 2009;139(2):405-15.

Cognigni P, Felsenberg J, Waddell S. Do the right thing: neural network mechanisms of memory formation, expression and update in Drosophila. Curr Opin Neurobiol. 2018;49:51-58.

Cohn R, Morantte I, Ruta V. Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in Drosophila. Cell. 2015;163(7):1742-55.

Connolly JB, Roberts IJ, Armstrong JD, et al. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science. 1996;274(5295):2104-7.

De belle JS, Heisenberg M. Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science. 1994;263(5147):692-5.

Dudai Y, Jan YN, Byers D, Quinn WG, Benzer S. dunce, a mutant of Drosophila deficient in learning. Proc Natl Acad Sci USA. 1976;73(5):1684-8.

Dylla KV, Raiser G, Galizia CG, Szyszka P. Trace Conditioning in Induces Associative Plasticity in Mushroom Body Kenyon Cells and Dopaminergic Neurons. Front Neural Circuits. 2017;11:42.

Feinberg EH, Vanhoven MK, Bendesky A, et al. GFP Reconstitution Across Synaptic Partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron. 2008;57(3):353-63.

Felsenberg J, Barnstedt O, Cognigni P, Lin S, Waddell S. Re-evaluation of learned information in Drosophila. Nature. 2017;544(7649):240-244.

Felsenberg J, Jacob PF, Walker T, et al. Integration of Parallel Opposing Memories Underlies Memory Extinction. Cell. 2018;175(3):709-722.e15.

Fiala A. Olfaction and olfactory learning in Drosophila: recent progress. Curr Opin Neurobiol. 2007;17(6):720-6.

Gerber B, Yarali A, Diegelmann S, Wotjak CT, Pauli P, Fendt M. Pain-relief learning in flies, rats, and man: basic research and applied perspectives. Learn Mem. 2014;21(4):232-52.

Goodwin SF, Del vecchio M, Velinzon K, et al. Defective learning in mutants of the Drosophila gene for a regulatory subunit of cAMP-dependent protein kinase. J Neurosci. 1997;17(22):8817-27.

Handler A, Graham TGW, Cohn R, et al. Distinct Dopamine Receptor Pathways Underlie the Temporal Sensitivity of Associative Learning. Cell. 2019;178(1):60-75.e19.

Hattori D, Aso Y, Swartz KJ, Rubin GM, Abbott LF, Axel R. Representations of Novelty and Familiarity in a Mushroom Body Compartment. Cell. 2017;169(5):956-969.e17.

Hige T, Aso Y, Modi MN, Rubin GM, Turner GC. Heterosynaptic Plasticity Underlies Aversive Olfactory Learning in Drosophila. Neuron. 2015a;88(5):985-998.

Hige T, Aso Y, Rubin GM, Turner GC. Plasticity-driven individualization of olfactory coding in mushroom body output neurons. Nature. 2015b;526(7572):258-62.

Himmelreich S, Masuho I, Berry JA, et al. Dopamine Receptor DAMB Signals via Gq to Mediate Forgetting in Drosophila. Cell Rep. 2017;21(8):2074-2081.

Horiuchi J. Recurrent loops: Incorporating prediction error and semantic/episodic theories into Drosophila associative memory models. Genes Brain Behav. 2019;18(8):e12567.

Ichinose T, Aso Y, Yamagata N, Abe A, Rubin GM, Tanimoto H. Reward signal in a

recurrent circuit drives appetitive long-term memory formation. Elife. 2015;4:e10719.

Jenett A, Rubin GM, Ngo TT, et al. A GAL4-driver line resource for Drosophila neurobiology. Cell Rep. 2012;2(4):991-1001.

Karam CS, Jones SK, Javitch JA. Come Fly with Me: An overview of dopamine receptors in Drosophila melanogaster. Basic Clin Pharmacol Toxicol. 2019;

Keene AC, Waddell S. Drosophila olfactory memory: single genes to complex neural circuits. Nat Rev Neurosci. 2007;8(5):341-54.

Kim YC, Lee HG, Han KA. D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in Drosophila. J Neurosci. 2007;27(29):7640-7.

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

Klapoetke NC, Murata Y, Kim SS, et al. Independent optical excitation of distinct neural populations. Nat Methods. 2014;11(3):338-46.

Krashes MJ, Keene AC, Leung B, Armstrong JD, Waddell S. Sequential use of mushroom body neuron subsets during drosophila odor memory processing. Neuron. 2007;53(1):103-15.

Lee T, Lee A, Luo L. Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development. 1999;126(18):4065-76.

Lin AC, Bygrave AM, De calignon A, Lee T, Miesenböck G. Sparse, decorrelated odor coding in the mushroom body enhances learned odor discrimination. Nat Neurosci. 2014;17(4):559-68.

Lin HH, Lai JS, Chin AL, Chen YC, Chiang AS. A map of olfactory representation in the Drosophila mushroom body. Cell. 2007;128(6):1205-17.

Lin S, Owald D, Chandra V, Talbot C, Huetteroth W, Waddell S. Neural correlates of water reward in thirsty Drosophila. Nat Neurosci. 2014;17(11):1536-42.

Liu C, Plaçais PY, Yamagata N, et al. A subset of dopamine neurons signals reward for odour memory in Drosophila. Nature. 2012;488(7412):512-6.

Livingstone MS, Sziber PP, Quinn WG. Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. Cell. 1984;37(1):205-15.

Masek P, Worden K, Aso Y, Rubin GM, Keene AC. A dopamine-modulated neural circuit regulating aversive taste memory in Drosophila. Curr Biol. 2015;25(11):1535-41.

Mcguire SE, Le PT, Davis RL. The role of Drosophila mushroom body signaling in olfactory memory. Science. 2001;293(5533):1330-3.

Menzel R. The insect mushroom body, an experience-dependent recoding device. J Physiol Paris. 2014;108(2-3):84-95.

Mohammad F, Stewart JC, Ott S, et al. Optogenetic inhibition of behavior with anion channelrhodopsins. Nat Methods. 2017;14(3):271-274.

Moldakarimov S, Bazhenov M, Sejnowski TJ. Perceptual priming leads to reduction of gamma frequency oscillations. Proc Natl Acad Sci USA. 2010;107(12):5640-5.

Murthy M, Fiete I, Laurent G. Testing odor response stereotypy in the Drosophila mushroom body. Neuron. 2008;59(6):1009-23.

Niewalda T, Michels B, Jungnickel R, et al. Synapsin determines memory strength after punishment- and relief-learning. J Neurosci. 2015;35(19):7487-502.

Owald D, Felsenberg J, Talbot CB, et al. Activity of defined mushroom body output neurons underlies learned olfactory behavior in Drosophila. Neuron. 2015;86(2):417-27.

Perisse E, Burke C, Huetteroth W, Waddell S. Shocking revelations and saccharin sweetness in the study of Drosophila olfactory memory. Curr Biol. 2013;23(17):R752-63.

Perisse E, Owald D, Barnstedt O, Talbot CB, Huetteroth W, Waddell S. Aversive Learning and Appetitive Motivation Toggle Feed-Forward Inhibition in the Drosophila Mushroom Body. Neuron. 2016;90(5):1086-99.

Qin H, Cressy M, Li W, Coravos JS, Izzi SA, Dubnau J. Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in Drosophila. Curr Biol. 2012;22(7):608-14.

Quinn WG, Harris WA, Benzer S. Conditioned behavior in Drosophila melanogaster. Proc Natl Acad Sci USA. 1974;71(3):708-12.

Riemensperger T, Völler T, Stock P, Buchner E, Fiala A. Punishment prediction by dopaminergic neurons in Drosophila. Curr Biol. 2005;15(21):1953-60.

Sanderson DJ, Cuell SF, Bannerman DM. The effect of US signalling and the US-CS interval on backward conditioning in mice. Learn Motiv. 2014;48:22-32.

Scholz-kornehl S, Schwärzel M. Circuit Analysis of a Drosophila Dopamine Type 2 Receptor That Supports Anesthesia-Resistant Memory. J Neurosci. 2016;36(30):7936-45.

Schroll C, Riemensperger T, Bucher D, et al. Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. Curr Biol. 2006;16(17):1741-7.

Schultzhaus JN, Saleem S, Iftikhar H, Carney GE. The role of the Drosophila lateral horn in olfactory information processing and behavioral response. J Insect Physiol. 2017;98:29-37.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-grelin F, Birman S, Heisenberg M. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci. 2003;23(33):10495-502.

Séjourné J, Plaçais PY, Aso Y, et al. Mushroom body efferent neurons responsible for aversive olfactory memory retrieval in Drosophila. Nat Neurosci. 2011;14(7):903-10.

Shuai Y, Hirokawa A, Ai Y, Zhang M, Li W, Zhong Y. Dissecting neural pathways for forgetting in Drosophila olfactory aversive memory. Proc Natl Acad Sci USA. 2015;112(48):E6663-72.

Skoulakis EM, Kalderon D, Davis RL. Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron. 1993;11(2):197-208.

Song W, Zhao L, Tao Y, et al. The interruptive effect of electric shock on odor response requires mushroom bodies in Drosophila melanogaster. Genes Brain Behav. 2019;18(2):e12488.

Squire LR, Ojemann JG, Miezin FM, Petersen SE, Videen TO, Raichle ME. Activation of the hippocampus in normal humans: a functional anatomical study of memory. Proc Natl Acad Sci USA. 1992;89(5):1837-41.

Srivastava DP, Yu EJ, Kennedy K, et al. Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel Drosophila G-protein-coupled receptor. J Neurosci. 2005;25(26):6145-55.

Sugamori KS, Demchyshyn LL, Mcconkey F, Forte MA, Niznik HB. A primordial dopamine D1-like adenylyl cyclase-linked receptor from Drosophila melanogaster displaying poor affinity for benzazepines. FEBS Lett. 1995;362(2):131-8.

Tanaka NK, Tanimoto H, Ito K. Neuronal assemblies of the Drosophila mushroom body. J Comp Neurol. 2008;508(5):711-55.

Tanimoto H, Heisenberg M, Gerber B. Experimental psychology: event timing turns punishment to reward. Nature. 2004;430(7003):983.

Tully T, Preat T, Boynton SC, Del vecchio M. Genetic dissection of consolidated memory in Drosophila. Cell. 1994;79(1):35-47.

Tully T, Quinn WG. Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol A. 1985;157(2):263-77.

Turner GC, Bazhenov M, Laurent G. Olfactory representations by Drosophila mushroom body neurons. J Neurophysiol. 2008;99(2):734-46.

Waddell S, Armstrong JD, Kitamoto T, Kaiser K, Quinn WG. The amnesiac gene product is expressed in two neurons in the Drosophila brain that are critical for memory. Cell. 2000;103(5):805-13.

Waddell S. Neural Plasticity: Dopamine Tunes the Mushroom Body Output Network. Curr Biol. 2016;26(3):R109-12.

Weingarten E, Chen Q, Mcadams M, Yi J, Hepler J, Albarracín D. From primed concepts to action: A meta-analysis of the behavioral effects of incidentally presented words. Psychol Bull. 2016;142(5):472-97.

Wilson RI. Early olfactory processing in Drosophila: mechanisms and principles. Annu Rev Neurosci. 2013;36:217-41.

Yamagata N, Hiroi M, Kondo S, Abe A, Tanimoto H. Suppression of Dopamine Neurons Mediates Reward. PLoS Biol. 2016;14(12):e1002586.

Yamagata N, Ichinose T, Aso Y, et al. Distinct dopamine neurons mediate reward signals for short- and long-term memories. Proc Natl Acad Sci USA. 2015;112(2):578-83.

Yamamoto S, Seto ES. Dopamine dynamics and signaling in Drosophila: an overview of genes, drugs and behavioral paradigms. Exp Anim. 2014;63(2):107-19.

Yamazaki D, Hiroi M, Abe T, et al. Two Parallel Pathways Assign Opposing Odor Valences during Drosophila Memory Formation. Cell Rep. 2018;22(9):2346-2358.

Yin JC, Del vecchio M, Zhou H, Tully T. CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in Drosophila. Cell. 1995;81(1):107-15.

Yu D, Akalal DB, Davis RL. Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. Neuron. 2006;52(5):845-55.

Zhao X, Lenek D, Dag U, Dickson BJ, Keleman K. Persistent activity in a recurrent circuit underlies courtship memory in . Elife. 2018;7