Review

Diverse memory paradigms in *Drosophila* reveal diverse neural mechanisms

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In this review, we aggregated the different types of learning and memory paradigms developed in adult *Drosophila* and attempted to assess the similarities and differences in the neural mechanisms supporting diverse types of memory. The simplest association memory assays are conditioning paradigms (olfactory, visual, and gustatory). A great deal of work has been done on these memories, revealing hundreds of genes and neural circuits supporting this memory. Variations of conditioning assays (reversal learning, trace conditioning, latent inhibition, and extinction) also reveal interesting memory mechanisms, whereas mechanisms supporting spatial memory (thermal maze, orientation memory, and heat box) and the conditioned suppression of innate behaviors (phototaxis, negative geotaxis, anemotaxis, and locomotion) remain largely unexplored. In recent years, there has been an increased interest in multisensory and multicomponent memories (context-dependent and cross-modal memory) and higher-order memory (sensory preconditioning and second-order conditioning). Some of this work has revealed how the intricate mushroom body (MB) neural circuitry can support more complex memories. Finally, the most complex memories are arguably those involving social memory: courtship conditioning and social learning (mate-copying and egg-laying behaviors). Currently, very little is known about the mechanisms supporting social memories. Overall, the MBs are important for association memories of multiple sensory modalities and multisensory integration, whereas the central complex is important for place, orientation, and navigation memories. Interestingly, several different types of memory appear to use similar or variants of the olfactory conditioning neural circuitry, which are repurposed in different ways.

In the last 50 or so years, *Drosophila* has been an exceptionally successful model for memory and behavior, likely due to the ease of handling, relatively simple nervous system, and genetic expediency. Fruit flies also have a robust and broad repertoire of behaviors and cognitive abilities that are remarkably similar to mammals. In this review, we aggregated the many diverse learning and memory paradigms developed for adult *Drosophila*. We briefly characterize the behavioral assays and procedures and detail the important genes, neural circuits, and mechanisms known to support these memories. Here we focus on adult memory paradigms, but larval *Drosophila* paradigms also exist and nicely complement the neurobiological work done in adult flies (Thum and Gerber 2019). We categorized the memory paradigms into several broad groups. However, a specific paradigm can contain aspects of multiple categories (e.g., courtship memory is both multimodal and social).

We start with conditioning assays, as they are the simplest types of memories (olfactory, visual, and gustatory), followed by variants of conditioning assays (reversal learning, trace conditioning, latent inhibition, and extinction) that alter some aspect of the learning or exposure to the conditioned stimulus (CS). Most of the work on the mechanisms and neural circuitry of memory in *Drosophila* are done on these associative conditioning memories. Next, we describe paradigms developed to test spatial or place memories (thermal maze, orientation memory, and heat box) and those memory paradigms that aversely condition flies to suppress their innate behaviors (phototaxis, negative geotaxis, anemotaxis, and locomotion). Very little is currently known about the genes or neurobiology supporting these memories. Conversely, recent exciting work on multisensory and multicomponent memory assays (context-dependent long-term memory [LTM] and cross-modal memory) and higher-order memory (sensory preconditioning and second-order conditioning [SOC]) are starting to reveal the functional intricacies of the neural circuitry supporting these more complex memories. Finally, we end with what are arguably the most complex memory paradigms: those based on social behavior. Courtship conditioning is one of the oldest memory paradigms studied in *Drosophila* (Siegel and Hall 1979), second to olfactory conditioning memory (Quinn et al. 1974). Despite this, much less is known about the circuits supporting courtship memory when compared with the progress made for olfactory conditioning memory, likely reflecting the complexity of social behaviors and social memories. We then describe some interesting social learning paradigms (learning from other individuals) that were recently developed (mate-copying and egg-laying behaviors).

While we focus on *Drosophila*, we must also acknowledge that the development of these paradigms did not occur in a vacuum. Many of these paradigms are conceptually based on those developed for rodents or other arthropod species, including *Aplysia*, honeybees, moths, and crickets. For many of these paradigms, works in non-*Drosophila* insect species were the first to demonstrate evidence for different types of memory, important brain structures, or the neurotransmitters required, providing valuable experimental directions for the work conducted in *Drosophila*.

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Olfactory conditioning memory

Olfactory classical conditioning was the first learning and memory paradigm developed for *Drosophila* (Tully and Quinn 1985) and remains the most popular paradigm used to study memory in flies. Great strides have been made in identifying the genes and molecules that support and suppress memory processes, as well as the brain structures and neural circuitry that support olfactory memory. Because of this, many excellent reviews are available on the topic of olfactory conditioning in *Drosophila* (Davis 2005, 2011; Kahsai and Zars 2011; Owald and Waddell 2015; Hige 2018; Boto et al. 2020; Felsenberg 2021; Noyes et al. 2021). Here we briefly touch on some fundamental aspects of olfactory conditioning memory as it relates to the other memory paradigms in this review.

Olfactory aversive conditioning is a population assay that pairs one odor with electric shock, while a second odor is not reinforced during training (Fig. 1; Tully and Quinn 1985). During testing, flies are given a choice test in a T-maze between these two odors, and their avoidance of the shock-associated odor is taken as a measure of their memory. Although memory scores are robust immediately after training, they quickly decay by 24 h, and the memory is protein synthesis-independent. Protein synthesis-dependent LTM can be induced by training the flies using a spaced training protocol, in which flies undergo multiple training cycles that are separated by rest intervals (Tully et al. 1994) and last >14 d (Sabandal et al. 2021). Conversely, the appetitive olfactory conditioning paradigm is similar but pairs one odor with no reinforcement followed by a second odor paired with sugar, and this paradigm requires starvation (Fig. 1; Tempel et al. 1983). Unlike aversive training, one cycle of appetitive training produces a protein synthesis-dependent LTM lasting >96 h (Krashes and Waddell 2008).

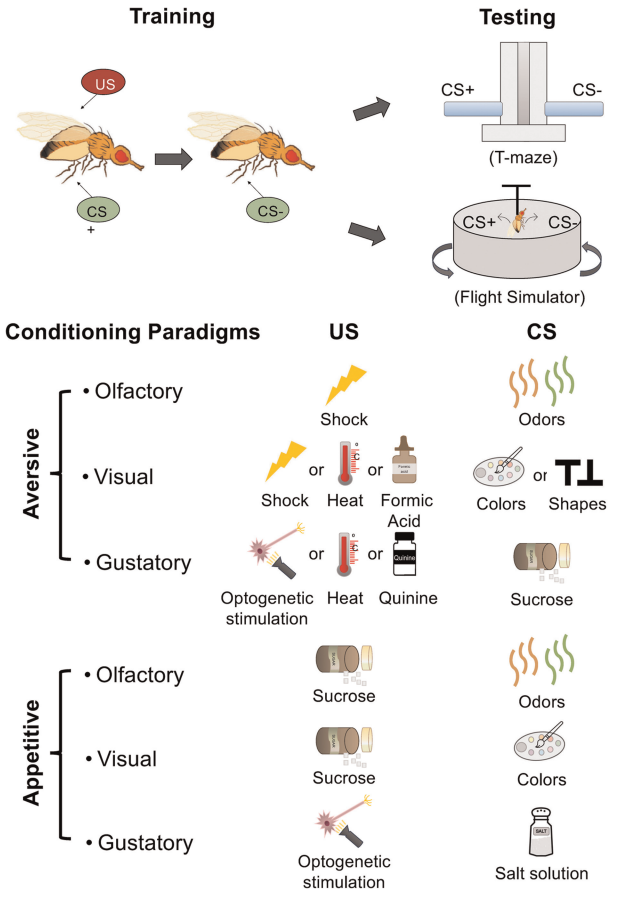

Figure 1. A variety of different stimuli can be used during aversive and appetitive classical conditioning paradigms. The typical training phase consists of subjecting the fly to a conditioned stimulus (CS) paired with an unconditioned stimulus (US; becoming the CS^+), followed by a second CS without the US (becoming the CS^-). In some paradigms, the CS^- precedes the CS^+. The testing phase typically assesses the fly's avoidance or attraction to the CS^+ compared with CS^-.

Decades of research have provided an unprecedented understanding of the genes and neural circuitry supporting olfactory conditioning memory. A trove of genes too long to detail here has been identified that support (Dudai 1988; Waddell and Quinn 2001; Davis 2005) or suppress olfactory conditioning (Noyes et al. 2021), many with conserved roles in mammals. For quite some time, it has been known that the mushroom body (MB), a distinct brain structure, is critical for this type of memory (Heisenberg 2003). Central to the MB structure are the axons of the MB neurons (MBNs) that encode olfactory information, and postsynaptic neurons (MBONs) that communicate downstream and outside of the MBs to direct behavior (Aso et al. 2014a,b). Importantly, these MBN-to-MBON synapses are regulated by dopamine (DA) neurons (DANs) that encode the aversive (PPL1 subset) or appetitive (PAM subset) reinforcer during conditioning (Schwaerzel et al. 2003; Liu et al. 2012), thus allowing conditioning to differentially alter odor-specific MBN-to-MBON synapses and behavioral response (Aso et al. 2014b; Owald and Waddell 2015). Interestingly, the MBN-to-MBON synaptic network is tiled and compartmentalized with distinct MBN, MBON, and DAN cell types. The circuit architecture, circuit modulation experiments, and in vivo functional imaging studies indicate that each compartment likely encodes different types, aspects, and dynamics of associative memories (aversive, appetitive, short term, and long term; acquisition rate; and flexibility) (Aso et al. 2014b; Hige et al. 2015; Owald and Waddell 2015; Aso and Rubin 2016; Berry et al. 2018). Overall, this work reveals that classical conditioning creates multiple parallel memories encoded in different MB compartments combined to drive the conditioned response.

Visual conditioning memory

Visual operant conditioning in the flight simulator

Wolf and Heisenberg (1991) conducted the first studies into visual operant memory in *Drosophila* using a flight simulator, whereby individual flies were tethered at the thorax to a torque meter to enable turning measurements during simulated flight (Wolf and Heisenberg 1991). The flies were centered in a cylindrical screen, onto which visual stimuli were projected to simulate movement while turning (Wolf and Heisenberg 1991, 1997), and an infrared laser provided an aversive heat stimulus as a reinforcer for turning toward a CS (Fig. 1). During the test, infrared lasers were off, and the fly's turning movements in response to visual stimuli were observed. Flies quickly learned to turn away from specific visual cues, patterns (e.g., upright or inverted "T" shape), or colors (Wolf and Heisenberg 1997; Wolf et al. 1998; Brembs and Heisenberg 2001).

Early MB ablation experiments using hydroxyurea did not affect fly performances on this task (Wolf et al. 1998). However, several recent studies implicate the MBs as important, perhaps for specific aspects of visual memory. For example, MBs are required for visual operant conditioning when the context (color of the arena) or saliency (color intensity) is changed between training and testing (Liu et al. 1999; Zhang et al. 2007). Moreover, using the optogenetic tool halorhodopsin (light-activated Cl^- pump) to hyperpolarize MBN subtypes (γ, α/β, or α′/β′) impairs visual operant conditioning (Liu et al. 2016). This effect is replicated using

Kir2.1 (an inward rectifying K^+ channel) to hyperpolarize neurons, but not by blocking the synaptic output from MBNs using *shibire^{ts}* or tetanus toxin. Thus, the investigators hypothesized that electrical synapses or gap junctions, but not chemical synapses, in the MBNs play a role in operant visual memory in the flight simulator. Liu et al. (2016) found that MBNs form many of their gap junctions with other MBNs but also with MBON-β′2mp. RNAi knockdown of gap junction subunits (*innexins* [*inx*]) identified *inx5* and *inx6* as playing an important role in visual operant conditioning in both MBNs and MBON-β′2mp. Thus, although MB ablation or blockade of chemical synaptic transmission does not affect memory in the flight simulator paradigm, hyperpolarizing MBNs or disrupting electric synapses impairs it, even under the same experimental settings (Liu et al. 2016). Some γ MBNs are known to escape hydroxyurea treatment (Armstrong et al. 1998), which may compensate for the absence of the other MBNs, or other compensatory developmental wiring or neurophysiological changes may occur following MBN ablation. Alternatively, the MBNs normally modify visual operant memory, but this may not happen in their absence. How gap junctions and electrical communication facilitate visual memory remains an intriguing question.

Visual classical conditioning

Visual classical conditioning paradigms analogous to the *Drosophila* olfactory conditioning paradigms were developed in the early 2010s (Schnaitmann et al. 2010, 2013; Vogt et al. 2014). Instead of a classical T-maze, a flat circular arena with a clear floor was used to shine different-colored LEDs (blue and green), serving as the visual stimulus. One of the colored lights would be paired with a sugar reward or an aversive acid (formic or acetic acid), each delivered to the fly on a piece of filter paper in the arena (through which the colored LED remained visible) (Schnaitmann et al. 2010). This task could also be modified to test the same color LED with different light intensities as the visual stimuli (e.g., bright blue vs. dim blue) (Schnaitmann et al. 2013). In a subsequent version, a shock grid made from a transparent material was used to deliver an electric shock as the aversive stimuli, more closely mirroring the olfactory conditioning paradigm (Vogt et al. 2014). After multiple cycles of training in these paradigms, flies were tested in the arena (four quadrants simultaneously displaying the two colors in a checkerboard pattern), and video recorded to observe their preference or avoidance of the conditioned color (Schnaitmann et al. 2010; Vogt et al. 2014). Indeed, flies can learn to associate both an appetitive and aversive US to different colors.

Interestingly, testing flies in this paradigm revealed that the neurocircuits supporting visual conditioning largely overlapped with those that support olfactory conditioning (Vogt et al. 2014). Inhibiting the PAM and PPL1 DANs that innervated the MB using *shibire^{ts}* impaired appetitive and aversive conditioning, respectively. Activating these DANs using the temperature-sensitive cation channel dTRPA1 can also replace the US, and Dop1R1 mutants (*dumb*) are impaired in visual conditioning. Thus, similar to olfactory conditioning, visual conditioning relies on the PAM and PPL1 DAN innervation of MBs to encode the reward or shock information. Furthermore, inhibiting neurotransmitter output specifically from the γ MBNs using *shibire^{ts}* impaired appetitive and aversive visual conditioning (Vogt et al. 2014, 2016). Strikingly, a small subset of γ MBNs, known as γd MBNs, whose axons make up the dorsal part of the γ lobes, is necessary for aversive visual conditioning (Vogt et al. 2016). Electrophysiological recordings from these unique γd MBNs show subsets of cells responding to blue and green light but not to odors (typical MBNs respond to odors but not visual stimuli) (Turner et al. 2008; Vogt et al. 2016). The investigators identified two types of visual projection neurons, named VPN-MB1 and VPN-MB2, whose dendrites are located in the optic lobes and synapse onto the γd MBNs (Vogt et al. 2016). Blocking the output of VPN-MB1 with *shibire^{ts}* impaired performance on visual conditioning based on color (blue vs. green), whereas inhibiting VPN-MB2 neurons impaired visual memory based on different light intensities of the same color (Schnaitmann et al. 2013; Vogt et al. 2016). Thus, color and light intensity visual conditioning is supported by dissociable circuits.

Gustatory conditioning

Gustatory or taste conditioning takes advantage of the proboscis extension reflex (PER) exhibited by *Drosophila* when presented with a sugar solution to the mouth parts or taste receptors on their legs. Flies are trained to associate a sugar solution applied to their legs with an aversive stimulus, inhibiting the PER upon subsequent presentation of the sugar solution during the test (Médioni and Vaysse 1975; DeJianne et al. 1985; Masek and Scott 2010; Keene and Masek 2012). Because sugar solutions are presented to the flies' legs, the conditioned stimuli are independent of ingestion in this paradigm. The aversive stimuli used were laser-induced heat or the application of a bitter substance (quinine) to the proboscis simultaneously with or immediately following the sugar to the legs (Fig. 1). This taste memory is relatively short-lived, with multiple training cycles forming a memory that lasts >30 min but is undetectable after 2 h (Kirkhart and Scott 2015). Inhibiting the γ MBNs throughout the assay using *shibire^{ts}* abolishes aversive taste memories in the flies (Masek and Scott 2010; Kirkhart and Scott 2015). Consistent with these behavioral findings, calcium imaging of the MB calyx reveals the MBNs respond to tastes (sugar and quinine) applied to the fly's legs or proboscis (Kirkhart and Scott 2015). Similar to olfactory and visual aversive classical conditioning, blocking the PPL1 subset of DANs abolishes taste memory (Kirkhart and Scott 2015; Masek et al. 2015). These DANs also demonstrate calcium responses to both the heat and quinine used as the aversive stimuli in taste conditioning (although they activated different subsets of the PPL1 DANs) (Kirkhart and Scott 2015), indicating that the PPL1 neurons also encoded the aversive stimuli for taste conditioning (Kirkhart and Scott 2015; Masek et al. 2015).

The brain structures for aversive taste conditioning were further explored in a recent study (Jelen et al. 2023) using a new automated appetitive version of the taste conditioning paradigm (Musso et al. 2019). For these experiments, the US was replaced with optogenetic stimulation of gustatory receptor neurons (GRNs; bitter- or sugar-sensing GRNs in the mouth and legs) or DANs (PPL1 or PAM), leading to an aversive or appetitive memory, respectively (Jelen et al. 2023). The pairing of fly feeding with optogenetic stimulation was achieved using the sip-triggered optogenetic behavioral enclosure (STROBE) that triggered red light activation in response to contact with food (Musso et al. 2019). During training, the CS used for aversive taste conditioning was a sucrose solution that has caloric value, whereas for appetitive conditioning an innately neutral salt solution was used as the CS (Fig. 1; Jelen et al. 2023). Taste memories induced by optogenetic stimulation of PAM or PPL1 DANs produce a LTM observed 24 h after training. The PAM neurons supporting short-term appetitive taste memories are dissociable from those supporting LTM. Activation of PAMs innervating β′2, γ4, and γ5 compartments produces short-term memories (STMs), whereas activation of PAMs innervating the α1 MB compartment (PAM-α1) produces LTM. These findings mirror similar functions identified for PAM neurons in olfactory appetitive conditioning (Yamagata et al. 2015). Inhibiting MBN output using tetanus toxin impaired both STM and LTM taste

conditioning. Taken together, MBNs are necessary for forming or expressing both appetitive and aversive taste memories (Kirkhart and Scott 2015; Jelen et al. 2023). Gustatory sensory neurons are known to send their axons to the subesophageal zone, but how this information is conveyed to MBNs is not currently known (Kirkhart and Scott 2015). The *Drosophila* hemibrain connectome has identified a subset of γ MBNs (γm) that receive both olfactory and gustatory information via projection neurons (Li et al. 2020), although this connection and their role for gustatory memory requires further testing.

Reversal learning

Reversal learning is frequently used as a measure of behavioral flexibility—the ability to update memories with new information. The reversal learning paradigm trains animals in a normal conditioning paradigm followed by reversal training in which the previously unpaired stimulus is now paired with a reinforcer, whereas the previously conditioned stimulus is unpaired. Reversal learning was demonstrated by Quinn et al. (1974), but the mechanisms supporting this memory were first assessed in the 2010s using an olfactory aversive reversal learning paradigm (Shuai et al. 2010; Wu et al. 2012). Flies underwent the classical aversive olfactory conditioning paradigm in which odor A was paired with an electrical shock while odor B was not, followed by reversal training 1 min later where odor B was shocked but odor A was not. When flies were tested for their avoidance between odor A and B during the test immediately after reversal training, flies displayed a stronger memory to the most recent learning event (stronger avoidance of odor B), although their avoidance of odor B (vs. odor A) was always reduced compared with flies trained in the classical conditioning paradigm only (Quinn et al. 1974; Shuai et al. 2010; Wu et al. 2012; Dong et al. 2016). Comparisons of fly responses against a third odor that was never paired with shock (i.e., odor A vs. C and odor B vs. C) were used to clarify whether experimental effects were due to changes in the odor A or odor B memory after reversal learning. These third-odor tests revealed that the reversal training caused forgetting of the initial odor A memory (Shuai et al. 2010).

Unsurprisingly, the MBs are critical for olfactory reversal learning. Expressing a constitutively active mutant for *Rac1* (critical for forgetting) (Shuai et al. 2010) in the MBNs increases forgetting to the initial memory (odor A) caused by the reversal conditioning (Shuai et al. 2010). In addition, knocking down autism risk genes *Fmr1*, *Ube3a*, *Nrx*, and *Tsc1* (known to interact with *Rac1*) in the MBNs reduces reversal-induced forgetting of the original memory (odor A) (Dong et al. 2016). Blocking output from the α′/β′ MBNs specifically during reversal training using *shibire*^{ts} results in almost an equal avoidance of odor A compared with odor B (Wu et al. 2012); thus, the MBNs play an important role for olfactory reversal learning. Reducing GABA synthesis in the inhibitory APL neuron that broadly innervates the MBs (using a GAD RNAi) also improves the original memory and impairs its forgetting (assessed using a third odor), suggesting that the APL neuron is important for forgetting of the original memory upon reversal learning (Wu et al. 2012). The APL neuron and α/β MBN function are also necessary for visual reversal learning in the flight simulator but not for the initial memory itself (Ren et al. 2012). Calcium responses to odors during reversal learning show that the original learning-induced synaptic plasticity to odor A (i.e., memory trace) is eroded upon reversal training in the MBN-to-MBON-γ2α′1 synapses that are important for STM (Berry et al. 2018). The calcium imaging and the above experiments implicate forgetting mechanisms as playing an important role in reversal learning, particularly for the first memory.

Functional imaging also reveals that the absence of the shock upon odor A presentation during reversal training elicits an increased calcium response specifically in the reward PAM-β′2a neurons (McCurdy et al. 2021). This activity in the PAM-β′2a neurons encoding omission of punishment is important for reducing the aversive response to the originally trained odor A and results from the reduced depression of MBON-γ2α′1 upon presentation of odor A during reversal training. One important distinction between the paradigms used by McCurdy et al. (2021) and those discussed above is that repeated training cycles of the initial memory training are followed by two cycles of reversal training, and these changes in calcium responses were generally observed after the first reversal training cycle. A second distinction is that McCurdy et al. (2021) presented flies with odor A (no shock) and then odor B (shock) during reversal training, which is an altered sequence compared with the standard odor presentation (odor B + shock followed by odor A no shock). The PPL1 DA release upon shock administration causes forgetting of a previously conditioned odor and increases MBON-γ2α′1 responses to that odor (Berry et al. 2018). Because odor B + shock was presented second, this may explain why changes in responses to odor A were seen in the second cycle of reversal training (McCurdy et al. 2021).

Trace conditioning

Trace conditioning memory is formed when a CS and then a US are separated by a gap in time. This presents interesting questions about the mechanisms supporting this memory, as it necessitates that the CS information be held in some form across this gap in time. This question was explored using an aversive olfactory trace conditioning protocol in which odor was delivered to a fly followed by a 5-sec gap and then electric shocks, after which the flies were immediately tested (Galili et al. 2011; Lüdke et al. 2018). Behavioral testing reveals the odor information can be retained for up to 15 sec after the offset of the odor delivery to form trace memory (Galili et al. 2011). Calcium imaging of odor responses in the olfactory receptor neurons (ORNs) that synapse onto antennal lobe glomeruli reveals odor-specific calcium responses in glomeruli 15 sec after odor offset. Despite these offset responses being odor-specific, the particular set of glomeruli or their calcium response magnitude and direction cannot be predicted based on the calcium responses of glomeruli to the odor itself. Upon odor offset, calcium responses in the projection neuron and MBN cell bodies can also be observed (Lüdke et al. 2018). However, unlike the pattern seen for antennal lobe glomeruli, a similar set of MBN cell bodies maintains a calcium response to the odor and after odor offset. In addition, classical olfactory conditioning and trace conditioning seem to rely on partly independent cellular mechanisms, as *rutabaga* mutants are impaired in classical aversive olfactory conditioning but perform normally in trace conditioning (Shuai et al. 2011).

Interestingly, the time gap between the CS and the reinforcer for successful trace memory formation can be manipulated. Expression of dominant-negative *Rac1* in MBNs enhances trace memory and results in trace conditioning after a temporal gap of 60 sec (Shuai et al. 2011). Additionally, increasing serotonergic release from the DPM neurons that innervate the MBs lengthens the temporal gap for successful trace memory formation, whereas decreasing serotonergic release shortens the temporal gap for trace association (Zeng et al. 2023). Serotonergic manipulations of temporal windows for behavioral associations were mirrored physiologically in cholinergic decreases to the conditioned odor in γ MBNs. It remains to be seen whether serotonin from the DPM neurons regulates trace conditioning via interacting with the Rac1 pathway or is an independent mechanism.

A visual trace conditioning paradigm was also developed using the flight simulator, in which a visual stimulus is paired with aversive heat (Grover et al. 2022). In this paradigm, *Drosophila* were able to encode a trace memory with time gaps of up to 20 sec. The investigators identified the R2 and R4 ring neurons of the ellipsoid body as likely to be encoding the visual stimulus, and the PPM3 subset of DANs innervating the central complex and ellipsoid body as encoding the unconditioned heat stimulus. Reduction of Dop2R DA receptors in the R2 and R4m ring neurons specifically impaired trace conditioning (but not delay conditioning in which the end of the visual stimulus overlapped with the start of the reinforcer), suggesting that Dop2R facilitates the maintenance of visual stimulus memory during the temporal gap for trace conditioning. These data support the idea that trace conditioning has different mechanisms than classical or operant conditioning (or delay conditioning).

Latent inhibition

Latent inhibition is the phenomenon whereby prior familiarity with a stimulus before conditioning reduces the strength of the conditioned response. A latent inhibition paradigm for appetitive and aversive olfactory conditioning is performed by pre-exposing flies to a soon-to-be-conditioned odor before training and testing on the olfactory conditioning paradigms (Jacob et al. 2021). However, although the expected reduced memory scores were observed for appetitive conditioning, an enhancement of memory was observed for aversive conditioning (latent facilitation). The investigators show that the odor pre-exposure produces a temporary aversive memory in the flies, leading to reduced appetitive memory scores and adding to the aversive memory score. Imaging of the PPL1-α3 and PPL1-γ2α′1 DANs shows that repeated exposure to an odor reduces their calcium responses to the odor, which is also observed in the calcium responses of MBON-α3 and MBON-γ2α′1. These alterations would promote avoidance behavior in the fly (Aso et al. 2014a). Jacob et al. (2021) showed that familiarity has a negative valence, likely due to innate avoidance of the concentration of odors used. It would be interesting to see whether pre-exposure to innately appetitive odors would impart a positive value that latently inhibits aversive conditioning and facilitates appetitive conditioning.

Extinction

Extinction is observed when the CS is (repeatedly) presented following conditioning in the absence of the reinforcer, resulting in a reduction in the magnitude of the conditioned response. Evidence in mammals and *Drosophila* indicates that extinction itself is a new memory. This is one mechanism by which memories can be updated; for example, in a scenario in which the original pairing of stimulus and reinforcer was erroneous. Early flight simulator experiments demonstrated that extinction of the aversive visual memory occurs across repeated presentations of the visual CS (without heat), but significant memory still remains after many cycles of extinction training (Xia et al. 1997). However, the neural mechanisms contributing to the extinction of visual memories have not yet been explored.

Extinction of single-cycle olfactory aversive memories is also observed after multiple presentations of the conditioned odor (without shock) immediately after training (Schwaerzel et al. 2002). Blocking MBN output using *shibire*^{ts} during extinction does not affect extinction, but blocking the olfactory projection neuron input into the MBNs inhibits extinction. Aversive olfactory memories produced through both spaced conditioning (protein synthesis-dependent) and massed conditioning (protein synthesis-independent) can be extinguished following repeated exposure to the conditioned odor at 24 h after initial conditioning and then tested 24 h following extinction (Lagasse et al. 2009; Hirano et al. 2016). Interestingly, blocking protein synthesis using cycloheximide solely during extinction training blocks the extinction of memory formed by both spaced and massed training (Lagasse et al. 2009). This supports the idea that extinction is a separate learning event occurring after the original learning and therefore may be supported by independent mechanisms. Furthermore, a prolonged (>4 d) extinction of the protein synthesis-dependent memory can be obtained if flies undergo extinction training 2 d, but not 4 d, after spaced training, indicating a critical early window after the original memory is consolidated, during which it is more susceptible to extinction (Hirano et al. 2016). Expressing constitutively active CRTC in MBNs extends the window for successful extinction to >4 d after initial conditioning. Hirano et al. (2016) identified several genes whose expression was regulated by CREB/CRTC, and after RNAi screening, they showed that knockdown of β-*spectrin* in MBNs abolishes extinction memory without affecting the original conditioned memory.

Recent work on the neural circuitry supporting olfactory extinction revealed that behavioral extinction is due to the creation of a parallel memory of opposite valence during extinction training (Felsenberg et al. 2017, 2018). Flies trained in an appetitive olfactory extinction paradigm show no preference for the conditioned odor and thus successful extinction of the memory (Felsenberg et al. 2017). Surprisingly, using *shibire*^{ts} to block the PAM subset of DANs that encode reward memories during extinction training does not affect extinction, but blocking the PPL1 subset of DANs (particularly PPL1-γ2α′1) impairs extinction. In a symmetrical set of findings for aversive olfactory conditioning, blocking PAM, but not PPL1, output during extinction training impairs extinction (Felsenberg et al. 2018). Thus, for both appetitive and aversive memory, extinction exposure stimulates opposite valence DANs. Calcium imaging in aversively conditioned flies shows that two parallel memory traces exist for the conditioned odor after extinction training: The original aversive memory is observed after extinction as a learning-induced depression in MBON-γ2α′1 (also present after the initial aversive training), whereas a depression in MBON-γ5 can be observed only after extinction training. Interestingly, this extinction memory circuit appears to share similarities with the aversive conditioning reversal learning neural circuit (McCurdy et al. 2021), although there are fundamental differences between these two paradigms. If we consider that odors alone delivered to flies before conditioning cause a temporary aversive memory (Jacob et al. 2021), it follows that some change occurs after aversive conditioning that allows for the aversive odor to signal to reward memory compartments (Felsenberg et al. 2018).

Extinction of appetitive olfactory conditioning was found to be transient, persisting for <24 h after extinction training (Wang et al. 2019; Yang et al. 2023). Although *Rac1* plays an important role in forgetting of aversive olfactory memories, it does not affect appetitive memories (Yang et al. 2023). However, *Rac1* expression in the MBNs does affect the transient nature of appetitive olfactory extinction memory. Dominant-negative *Rac1* expression in MBNs prolongs the extinction memory beyond 48 h, whereas constitutively active *Rac1* rapidly degrades the extinction memory in <6 h. That *Rac1* affects extinction memory but not the original appetitive memory supports the idea that behavioral extinction of appetitive memories results from an extinction-induced parallel aversive memory (Felsenberg et al. 2017). Yang et al. (2023) also show that the *Rac1* downstream effector *diaphanous*, a cytoskeletal regulator, mediates this effect.

Spatial memory

Thermal maze: the *Drosophila* Morris water maze

Place memory in rodents has long been studied using the Morris water maze. *Drosophila* are also capable of forming place memories in a "thermal maze" version of the Morris water maze (Foucaud et al. 2010; Ofstad et al. 2011). This task uses a circular arena heated to aversive temperatures, with one location within the arena designated as a "safe zone" with a cooler temperature, analogous to the platform in the Morris water maze. Distinct visual cues are placed on arena walls that are used by the animal to locate the safe zone. Populations of flies or single flies can be trained and tested on this task, and *Drosophila* learn the location of the safe zone across multiple training trials. This is followed by a memory test with no safe zone to test their place memory, during which flies spend a disproportionate amount of time searching the area where they remember the safe zone to be. The olfactory memory mutant *rutabaga* is impaired in place learning using this heat maze (Melnattur et al. 2021), but silencing MBNs by expressing *Kir2.1* or ablating the MBNs using hydroxyurea does not affect place memories in *Drosophila* (Ofstad et al. 2011), indicating that the MBNs are not involved in encoding this place memory. However, expressing *Kir2.1* in subsets of neurons projecting to the ellipsoid body or in DANs abolishes place memories in the flies (Ofstad et al. 2011; Melnattur et al. 2021). Place memory can also be improved or impaired with pharmacological manipulations that increase or decrease DA neurotransmitter levels, respectively (Melnattur et al. 2021). Thus, although the MBNs do not seem to play a role in thermal maze place memories, DANs and the ellipsoid body appear important for encoding these memories. The ellipsoid body's role in the thermal maze likely relates to its ability to encode retinotopic representations of visual features in the fly's environment (Seelig and Jayaraman 2013).

Orientation memory

Orientation memory is tested in flies by exploiting their fixation and movement toward two visual stimuli in Buridan's paradigm (Götz 1980). Flies presented with two inaccessible vertical lines on the walls of a circular arena will spontaneously and repeatedly walk from one line toward the other for a prolonged period of time. If the visual lines are removed after their presentation, flies will persist in walking toward or between the now invisible lines for a short period of time (~24 sec), which is dependent on spatial working memory and orientation memory (Strauss and Pichler 1998; Neuser et al. 2008; Yen et al. 2019; Han et al. 2021). Furthermore, in a "detour paradigm," if a distracter visual cue appears at a new position for a few seconds after the disappearance of the two target vertical lines, the fly will briefly orient toward the distracter before reorienting and navigating toward the original, still invisible, targets (Neuser et al. 2008). Flies with MB ablation using hydroxyurea maintain their orientation memories, whereas the *ellipsoid body open* (*ebo*) mutants with structural defects in their central complex are impaired in their orientation memories (in both the original and the detour paradigm). Inhibiting the GABAergic R3 and R4 ring neurons projecting to the ellipsoid body by expressing tetanus toxin or *Kir2.1* also impairs orientation memory (Neuser et al. 2008; Han et al. 2021). Neuser et al. (2008) identified *ignorant*, a null mutant for a ribosomal serine kinase (S6KII), that is unable to orient to the original target vertical lines after they have disappeared (*dunce* mutants performed normally). S6KII is a regulator of the MAP kinase pathway (Kim et al. 2006), and restoring S6KII to the R3 and R4 ring neurons in *ignorant* mutants fully rescues their orientation memory on the detour paradigm (Neuser et al. 2008).

Consistent with the role of ring neurons for orientation memory, it was recently reported that the release of the diffusible gaseous signaling molecules nitric oxide (NO) and hydrogen sulfide (H_2S) from R3 ring neurons is important for orientation memory in the detour paradigm (Kuntz et al. 2017). Mutants for NO synthase (NOS; which produces NO) and cystathionine β-synthase (CBS; which produces H_2S) are impaired on the detour paradigm. Their expression in R3 or neighboring R2 neurons rescues their respective mutant behavioral defects and rescues orientation memory deficits in *ebo* mutants.

Heat box

The heat box consists of a small chamber with one half that can be heated and is used for testing simple operant conditioning (Wustmann et al. 1996; Wustmann and Heisenberg 1997; Putz and Heisenberg 2002; Baggett et al. 2018). When a fly enters one half of the chamber, the temperature is increased to an aversive level, whereas entry into the other half of the chamber is associated with a preferable temperature during training. In the testing phase, the chamber is not heated, and the location of the fly within the chamber is quantified. Whereas wild-type flies avoid the heated chamber, avoidance behavior in the classical olfactory memory mutants *dunce* and *rutabaga* is significantly reduced despite avoiding the heated arm during training (Wustmann et al. 1996; Baggett et al. 2018). However, the contribution of *dunce* and *rutabaga* to place memory may be outside of the MB, as flies with ablated MBs (using hydroxyurea) remember the heat-associated chamber, similar to wild-type flies (Wolf et al. 1998; Putz and Heisenberg 2002). Heat box place memory is also dependent on serotonergic but not DANs (Sitaraman et al. 2008). It remains to be seen whether the ellipsoid body regulates heat box memory like other types of spatial memory. This heat box apparatus can theoretically be modified to study classical conditioned place memory analogous to place conditioning in rodents.

Aversive conditioning to suppress innate behaviors

Several memory paradigms use aversive conditioning to suppress innate behaviors in *Drosophila*. These paradigms are operant conditioning paradigms and are analogous to the passive avoidance paradigm used in rodents (Bartus et al. 1980).

Aversive phototaxis suppression

Drosophila innately move toward light, a behavior known as positive phototaxis (Hirsch and Boudreau 1958), and will naturally prefer a lighted maze arm to a darkened arm (Le Bourg and Badia 1995). If the lighted arm contains filter paper with an aversive bitter substance (quinine) (Le Bourg and Buecher 2002) or light is paired with heat (Baggett et al. 2018), flies will avoid the lighted arm for a short time during the test phase (<1 h), thus demonstrating aversive phototaxis suppression (APS) (Le Bourg and Buecher 2002; Seugnet et al. 2009). This paradigm can also be performed in the heat box chambers (Baggett et al. 2018). APS is dependent on DA signaling (Seugnet et al. 2008), and blocking neurotransmitter output from the α/β and γ MBs using *shibire*^{ts} during testing impairs APS (Seugnet et al. 2009). Multiple olfactory memory mutants are also impaired in this task, with some mutants demonstrating sex differences (mutants *linotte*, *latheo*, *pastrel*, *dunce*, *rutabaga*, and *dumb*) (Seugnet et al. 2009; Baggett et al. 2018).

Aversive negative geotaxis suppression

Drosophila have an innate preference to move upward in a vertical chamber, a behavior known as negative geotaxis (Hirsch and Tryon 1956). Negative geotaxis has long been used to test locomotor

abilities in flies, but similar to other innate behaviors in this section, negative geotaxis can be suppressed if it is associated with an aversive stimulus (Baggett et al. 2018; Pak and Murashov 2021). This paradigm uses a vertical two-chamber apparatus (Pak and Murashov 2021) or the heat box apparatus offset from the horizon (Baggett et al. 2018). The training and testing procedures are similar to those described above, whereby flies entering the upper or higher chamber encounter an aversive shock or heat (Baggett et al. 2018; Pak and Murashov 2021). During testing 24 h later, the flies avoid the upper compartment. The neurocircuits supporting this memory have not yet been queried, but as DANs are known to deliver the aversive electric shock stimuli in the other paradigms, they may serve a similar function for aversive negative geotaxis suppression. Olfactory memory mutants *dunce* and *rutabaga* are impaired in this paradigm (Baggett et al. 2018).

Aversive anemotaxis suppression

When a low airflow is provided to one end of the heat box, wild-type flies display a strong preference for the upwind compartment (Baggett et al. 2018). However, if this upwind compartment is paired with an aversive heat stimulus, flies will learn to avoid it. Testing olfactory memory mutants *dunce* and *rutabaga* on this paradigm yielded mixed findings that were difficult to interpret due to their altered responses to the airflow itself and low locomotor activity compared with wild-type flies. The neural circuits supporting this memory are unclear.

Aversive locomotor suppression

Spontaneous locomotor activity in flies can be suppressed if paired with an aversive stimulus (Sun et al. 2020). Flies were placed into a glass tube, and their baseline locomotor movement was recorded, followed by training and testing. During training, movement by the fly triggered a mild heat stress delivered via infrared laser. The locomotor activity of flies during testing sessions (no heat applied) was significantly reduced. However, Dop1R1 and Dop2R mutant flies did not suppress locomotor activity after training, while Dop1R2 and DopEcR mutants successfully learned the association. The neural circuits underlying this learning are unknown.

Multisensory and multicomponent memory

Context-dependent aversive olfactory conditioning

The classical aversive olfactory conditioning assay delivers a foot shock to flies during training via a copper grid, which is absent from the T-maze during testing. Thus, classical olfactory memory is tested in a different context than training. However, if the major contextual stimuli (copper grid, temperature, and lighting) are kept consistent between the training and testing phases, a context-dependent olfactory memory can be observed that is dissociable from the classical olfactory conditioning memory in several interesting ways (Zhao et al. 2019). Context-dependent olfactory memory is a LTM persisting for at least 14 d following single-cycle training but is independent of protein synthesis, is normal in classical olfactory memory mutant *rutabaga*, is independent of cAMP and CREB activity, and is independent of the MBNs but is dependent on non-MB innervating DANs captured in the TH-GAL4 driver. Through inhibiting neuronal communication using *shibire*^{ts}, the investigators determined that context-dependent olfactory LTM results from the integration of multisensory information in the lateral horn (LH). Blocking synaptic inputs from the olfactory projection neurons (excitatory and inhibitory), antennal mechanosensory motor center (AMMC) neurons, the visual system (GMR and optic lobe neurons), and LH output neurons abolishes context-dependent LTM. By incorporating context into their olfactory memory paradigm, Zhao et al. (2019) identified the existence of a previously unknown and unusual form of LTM. It is unexpected that such a persistent memory (lasting weeks) can do so without de novo protein synthesis and be independent of classical cAMP and CREB signaling mechanisms. Moreover, this paradigm and novel LTM expand the scope for learning and memory research, as they allow the identification of novel neurological and molecular mechanisms that may underlie these unorthodox memories.

Cross-modal memory

A cross-modal memory paradigm was recently developed using the olfactory T-maze to examine how combining an odor and visual cue (blue or green LED) as the CS affected classical conditioning memory (Okray et al. 2023). During training, flies were presented with both an odor and colored light paired with a sugar reward. If these flies were given both odor and visual stimuli during testing, their memory performance was higher than if they were trained to either odor or color alone. If the odor and color pairs were inconsistent between training and testing, the flies produced memory scores comparable with those of unimodal training. Remarkably, if flies were trained using both sensory modalities, flies had enhanced memory scores regardless of whether they were tested with only one or both modalities. Thus, multimodal classical conditioning improves memory and suggests that two memories might support or facilitate each other's expression.

To assess how the olfactory and visual information come together to enhance memory, the investigators examined the γd and αβp MBNs that receive visual input from optic lobe projection neurons (Li et al. 2020). When flies were trained multimodally but were presented with only odor during the test, silencing γd MBNs inhibited memory retrieval, but silencing αβp MBNs did not have an effect. Thus, the γd MBNs appear to facilitate the cross-modal memory effects (Okray et al. 2023). While γd MBNs do not typically respond to odors, imaging of the γd MBN axons using voltage sensor ASAP2f showed that conditioned odors elicit an increase in membrane potential following cross-modal training, but that the same odors elicit a hyperpolarization following unimodal training. This is specific to the γ5 compartment known to be important for reward memories, as this does not occur in the γ1 compartment important for aversive memories (although aversive cross-modal training produced a similar effect in γ1). Conversely, the γm MBNs known to respond to odors but not visual stimuli exhibit color-induced membrane potential increases in the γ5 compartment after cross-modal training. Taken together, the cross-modal training appears to functionally link the γd and γm MBN axons carrying the visual and odor stimulus information in the γ5 reward memory compartment. Okray et al. (2023) further demonstrated that this functional link occurs through serotonergic release from the DPM neurons, as *shibire*^{ts} inhibition of DPM neuron output or RNAi knockdown against the $5HT_{2A}$ serotonergic receptor in γ MBNs disrupts cross-modal memory enhancement. DPM neurons have highly branched axons synaptically connected to both γm MBNs and γd MBNs in the γ5 compartment. Voltage sensor imaging in the DPM neuron γ5 compartment indicates increased excitatory responses to the odor after cross-modal training. Given that the DPM neurons highly innervate the whole of the MB structure, it will be interesting to see whether this circuit mechanism functions in other compartments to bridge other multisensory memories.

Higher-order learning

In higher-order learning, an animal learns about the predictive value of one stimulus from its association with another stimulus, without the first stimulus being directly paired with a reinforcer itself. Thus, the value of the nonconditioned stimulus is inferred

based on its relationship to the CS in an "inference-based" or "model-based" learning paradigm. Two paradigms used to assess higher-order learning are sensory preconditioning and SOC, which differ in the timing of pairing events. For sensory preconditioning, the pairing of the two neutral stimuli occurs before one of them is associated with a US, whereas for SOC, the pairing of the two stimuli occurs after one of them is paired with the US.

Sensory preconditioning

The first reports of sensory preconditioning in *Drosophila* occurred in the early to mid-2000s, in which the flight simulator was used to pair two visual stimuli (colors + patterns) or a visual (pattern) and odor stimulus together, which we refer to here as S1 and S2 (Brembs and Heisenberg 2001; Guo and Guo 2005). S1 + S2 were presented simultaneously for pairing, and then flight toward one of these stimuli was associated with heat serving as the aversive US (S1 + US). When flies were presented with the nonreinforced stimulus S2, they flew away from it, despite S2 never being paired with the heat. Either of the stimuli can serve as the S1 or S2, and the sensory preconditioned response to S2 is smaller than the conditioned response to S1. Thus, these early reports established that *Drosophila* can undergo sensory preconditioning.

The cross-modal sensory preconditioning paradigm (visual + odor) developed by Guo and Guo (2005) was subsequently used to identify the neural circuits supporting this learning. For these experiments, neural communication was blocked in specific neurons using *shibire*^{ts} only during the sensory test phase S2 presentation (Zhang et al. 2013). If the visual cues were reinforced and the response to odor was tested, blocking MBN communication (specifically the α/β MBNs) prevents the conditioned response to S2. However, if the olfactory cues were reinforced and then the conditioned response to the visual cues was tested, blocking MBNs did not have an effect. Interestingly, blocking neurotransmission in layer 5 fan-shaped body neurons (F5) did not affect the conditioned response to either odor or visual cues in the sensory preconditioning test, but simultaneously blocking the MBNs and F5 neurons during S2 presentation resulted in no conditioned response, regardless of the S2 sensory modality. Taken together, these seem to indicate that parallel memory traces are established to support sensory preconditioning.

Recently, a unimodal olfactory version of the sensory preconditioning paradigm was developed, revealing the neural traces of olfactory sensory preconditioning and the involvement of the small GTPase *Rac1* (Martinez-Cervantes et al. 2022). For this paradigm, the pairing of olfactory stimuli (S1 + S2) occurred sequentially over multiple cycles with a 1-sec interval between. This was followed by training of the S1 to shock. When presented with the S2 odor, the flies showed a significant avoidance, thus demonstrating sensory preconditioning. Calcium imaging of the S1 and S2 odors after sensory preconditioning in MBON-γ1 peduncle > α/β showed a strong depression to S1 (associative memory) and a moderate depression to S2 (sensory preconditioning memory). Of note is that the investigators observed sensory preconditioning calcium traces after a single pairing of S1 and S2 odors during the preconditioning step, but 10 cycles of pairing were necessary to observe behavioral effects. This likely indicates that synaptic changes in multiple MB compartments are required to observe the behavioral response. In control flies, a short interval between S1 and S2 (1 sec) was necessary to observe both calcium and behavioral sensory preconditioning. However, when a dominant-negative form of *Rac1* was expressed in the MBNs, sensory preconditioning was observed after a long interval (30 sec, but not with intervals of 5 min). Thus, Rac1 plays an important role in maintaining a precise window for associations in sensory preconditioning (Martinez-Cervantes et al. 2022), similar to *Rac1*'s role in MBNs for trace conditioning (Shuai et al. 2011) mentioned above. How exactly S1 and S2 become associated such that valence information for one odor can be "transferred" to another is unknown. It is possible that the sustained calcium responses seen after odor offset under the trace conditioning paradigm (Galili et al. 2011; Lüdke et al. 2018) may play a role in associating S1 and S2 odors.

Second-order conditioning

Unimodal visual SOC was first demonstrated in the flight simulator. Color was used as the first stimulus (S1), which was paired with heat (aversive US) from an infrared laser (Brembs and Heisenberg 2001). Immediately after training, S1 was presented simultaneously with a visual pattern that served as the second stimulus (S2). During the test, flies strongly avoided S1 and moderately avoided S2, which was never originally paired with the heat, thus demonstrating visual SOC in *Drosophila*. This was followed by the development of a unimodal olfactory SOC paradigm (Tabone and de Belle 2011). Odor S1 was paired with shock, and then odors S1 and S2 were paired by simultaneous presentation. Flies again displayed a strong avoidance of S1 and a weaker but robust avoidance of S2 (second-order memory) tested against a third odor not paired with any stimuli. Thus, flies can undergo both visual and olfactory SOC.

Recent exciting work has unraveled much of the neural framework underlying olfactory SOC in *Drosophila* (Yamada et al. 2023). Yamada et al. (2023) first established an appetitive olfactory SOC paradigm, in which the first odor (S1) was paired with a sugar reward (appetitive US), followed by S2 + S1 sequential odor pairing. Behavioral responses to S1 and S2 were tested against a third odor not paired with any of the stimuli. Appetitive conditioning produces a strong preference for S1 that is long-lasting (>24 h) and stable, as expected, whereas SOC produces a moderate preference for S2 that is transient (<24 h) and sensitive to extinction. Optogenetic stimulation of PAM-α1 is sufficient to replicate the behavioral response to S2, whereas inhibition of PAM-α1 using tetanus toxin impairs appetitive conditioning to S1 and SOC to S2. In contrast, simultaneous inhibition of combinations of other PAM neurons (γ4, γ5, and β′2a) does not affect the first conditioning to S1 but impairs the SOC to S2. These optogenetic findings on behavioral memory are corroborated with the electrophysiological responses in the corresponding MBONs. Thus, the MBN α1 compartment appears to instruct the MBN γ5,β′2a compartments to establish SOC.

Yamada et al. (2023) then determined how the output from MBN compartment α1 after reward conditioning can activate PAM-γ5,β′2a neurons to encode second-order memory. For this, they used information from the fly brain connectome (Li et al. 2020; Scheffer et al. 2020) in addition to a machine-learning algorithm to predict the neurotransmitters released by interneurons to identify neurons connecting MBON-α1 to PAM-γ5,β′2a excitation. This approach revealed a potential circuit as MBON-α1 → SMP353/354 → SMP108 → PAM reward neurons (including PAM-γ5,β′2a) (Yamada et al. 2023). Optogenetic activation of SMP353/354 or SMP108 leads to DA release (measured using the DA sensor GRAB DA2m) in reward compartments β′2, γ4, and γ5, as well as, to a lesser extent, β1 and β2 but not α1, functionally confirming the circuit predicted from the connectome. Silencing SMP108 using tetanus toxin does not impair the initial S1 appetitive conditioning but impairs the SOC of S2. Thus, Yamada et al. (2023) revealed for the first time a circuit mechanism for how SOC can occur. The initial olfactory appetitive memory is known to be encoded in the MBNs as a reduced synaptic response to the odor, observable in the neural activity response of MBON-α1 to the S1 odor. The MBONs of reward memory compartments are glutamatergic, which is inhibitory; thus, reduced MBON activity results

in reduced inhibition in downstream neurons. Following the formation of reward memory to S1, the pairing of S2 + S1 then results in activation of SMP353/354 → SMP108 → PAM-γ4,γ5,β′2a shortly after the presentation of S2, presumably allowing S2 to acquire a rewarding valence.

Courtship conditioning

Courtship conditioning is the second most widely studied form of learning and memory in *Drosophila* after olfactory conditioning. Here we briefly summarize some aspects of courtship memory, but we invite the reader to refer to more in-depth reviews on courtship conditioning and neural circuitry (for reviews, see Griffith and Ejima 2009; Yamamoto and Koganezawa 2013; Raun et al. 2021). In comparison with olfactory conditioning, courtship memory is multimodal (olfactory, visual, gustatory, auditory, and somatosensory) and complex but has clear ethological relevance (Kamyshev et al. 1999; Montague and Baker 2016). Likely due to the complexity of courtship conditioning, the neural circuits supporting this memory are less clear. One disadvantage is that courtship memory can only be tested in males.

In this paradigm, during training, a naive male (no prior mating experience) is paired with a female fly that is not receptive to mating (typically mated females or immature females), resulting in a suppression of their subsequent courtship attempts upon pairing with a receptive female during the test phase (Fig. 2A; Siegel and Hall 1979). Interestingly, there are many parallels between courtship suppression and the simpler olfactory conditioning memory. First, STM (2–3 h) and LTM (9 d) for courtship suppression can be induced using principles similar to those used for olfactory conditioning (single cycle vs. spaced training) (Kamyshev et al. 1999; McBride et al. 1999; Keleman et al. 2007, 2012). Second, many mutants impaired in olfactory conditioning are also impaired in courtship suppression (cataloged and summarized in Griffith and Ejima 2009; Raun et al. 2021). Finally, the neural circuitry supporting courtship memory appears to be more similar to the circuits supporting appetitive, rather than aversive, olfactory memories. The PAM reward subset of DANs, particularly PAM-γ5, is necessary for courtship conditioning but not PPL1 aversive DANs (Krüttner et al. 2012, 2015; Montague and Baker 2016). The reason why reward DANs are necessary for the memory of courtship rejection is not well understood. Ablating MBNs or silencing MBNs or MBONs broadly using *shibire*^{ts} impaired courtship memory (McBride et al. 1999; Montague and Baker 2016).

Social learning

In social learning, observers gain information about a stimulus or another individual from demonstrator individuals without having direct experiential knowledge (Kavaliers et al. 2017; Nieberding et al. 2021; Paletta et al. 2022). Social learning is conceptualized as a teacher–student relationship and is demonstrated by humans and many different animal species, including *Drosophila*. The neurobiology supporting social learning is not well understood.

Mate choice copying

In the mate-copying paradigm for *Drosophila*, male flies are dusted with either green or pink powder (Mery et al. 2009). A female fly then observes a green male successfully mating with a demonstrator female and then a pink male being rejected by a recently mated female (colors are counterbalanced; demonstrator females can also be placed simultaneously with the green and pink males) (Mery et al. 2009; Dagaeff et al. 2016). The observer females then display a significant preference for the green male during the test (Fig. 2B, panel i). Observer females will also alter their innate preference for a healthy male raised on normal food for a poor-health male raised on nutrient-poor food if the poor-health male was observed copulating with another female (Fig. 2B, panel ii; Mery et al. 2009). Similar mate-copying preferences can also be seen for males carrying detrimental genes (Nöbel et al. 2018b). Mate-copying memory is long-lasting (24 h) and can be abolished by protein synthesis inhibitor cycloheximide, similar to other forms of LTM (Danchin et al. 2018). This arbitrary preference for colored male flies can also be transferred to larger groups of flies, altering population genetics (Danchin et al. 2018). It remains unclear whether mate-copying involves an aversive memory to the rejected male phenotype, an appetitive memory to the successful male phenotype, or a

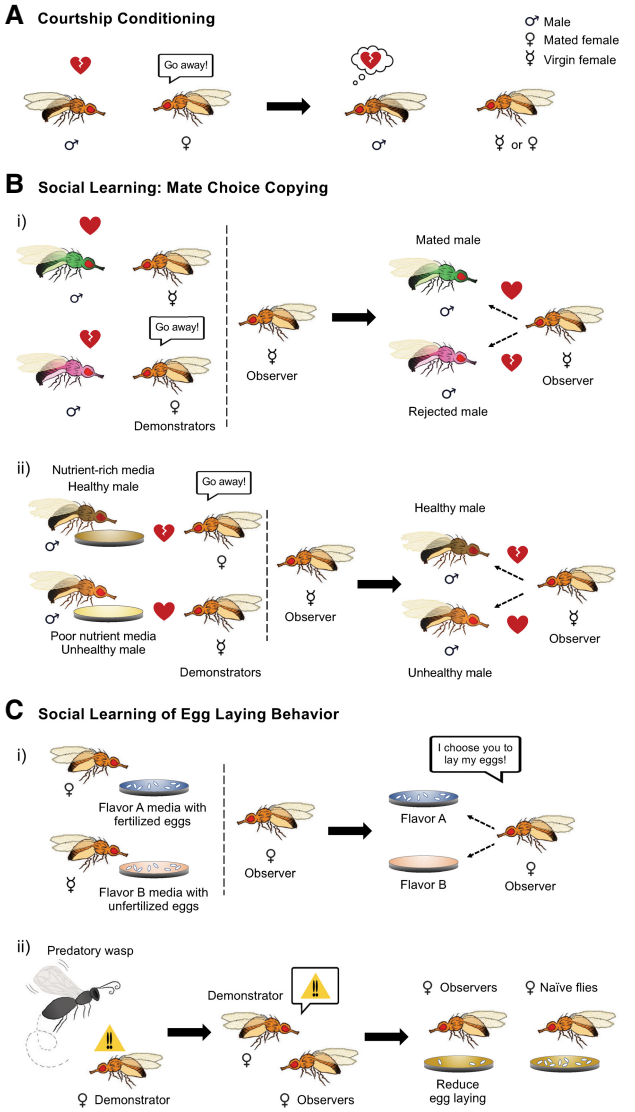

Figure 2. Courtship and social learning paradigms. (*A*) In courtship conditioning paradigms, male flies are conditioned to have their courtship advances rejected by a mated or immature female, reducing their subsequent courtship attempts with other females. (*B*) During mate-copying, an observer female will prefer to mate with a green-colored male (panel *i*) or with an unhealthy male if they previously observed a demonstrator female mating with them (panel *ii*). (*C*) Social learning of egg-laying preference information is transmitted from the demonstrator to the observer to lay eggs at sites preferred by other mated demonstrator females (panel *i*) or to reduce egg laying due to the presence of parasitoid wasps (panel *ii*).

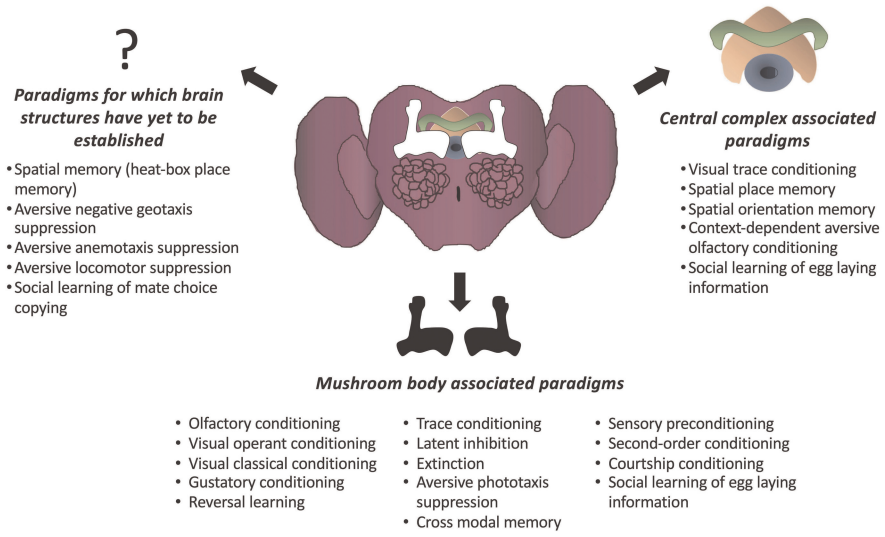

Figure 3. *Drosophila* learning and memory paradigms and their known neuroanatomical associations. *Drosophila* learning and memory paradigms investigated thus far are generally dependent on the MB structure or central complex structures. The brain structures necessary for several types of memory are not yet known.

combination of both. The neural circuits supporting mate-copying in female flies are not known, but pharmacological blockade of DA and serotonin synthesis suggest that both are important for mate-copying (Monier et al. 2019). Male flies can also mate copy in a reciprocal paradigm in which female flies are colored green and pink (Nöbel et al. 2018a).

Social learning of egg-laying information

During training, female observer flies are exposed to two differently flavored foods: flavors A and B (Sarin and Dukas 2009). The observer female encountered flavor A with other mated females and their eggs on the food, whereas flavor B was encountered alone or with virgin females and unfertilized eggs (Fig. 2C, panel i). Immediately after, observer females laid more eggs on flavor A versus flavor B food, demonstrating social learning for egg-laying sites.

More recently, it was reported that female demonstrator flies exposed to parasitoid wasps that target *Drosophila* larvae (but not adults) reduce oviposition for at least 72 h after exposure (Kacsoh et al. 2015a,b). When these demonstrators are housed with naive observer females in the absence of the wasps, the observers also reduce oviposition (Fig. 2C, panel ii). If the demonstrators are exposed to wasps in the dark or the observers are paired with demonstrators in the dark, the reduction of oviposition does not occur. Thus, this social learning is communicated via visual cues. Demonstrator flies with only one wing cannot teach observer females to reduce oviposition, even though the demonstrators themselves had reduced oviposition. However, it remains unclear what wing-based information is used to communicate the presence of a parasitic wasp.

Kacsoh et al. (2015b) tested several mutant flies known to be impaired in classical olfactory conditioning (*rutabaga*, *dunce*, *Adf1*, *amnesiac*, *FMR1*, and *Orb2*$^{\Delta Q}$) to discover that although they show an acute reduction in oviposition when exposed to wasps, their oviposition rates are normal 24 h after exposure. These memory mutants cannot serve as demonstrators to either wild-type or mutant observers, and mutant observers also cannot learn from wild-type demonstrators. Therefore, normal memory function is necessary for (1) the long-term reduction in egg laying upon exposure to parasitoid wasps and (2) social learning of reduced egg laying. Demonstrator flies with MBN output inhibited using tetanus toxin do not have prolonged reduction of egg laying after wasp removal and cannot serve as demonstrators to observer flies (Kacsoh et al. 2015b). Inhibiting MBN communication in observer flies also prevented learning from demonstrators. However, disruption of the MBNs does not alter the acute reduction of egg laying after exposure to the wasp, suggesting that different circuits underlie this short-term response.

Social learning can occur between demonstrator and observer females of different *Drosophila* species, with those species more closely related having more efficient communication (Kacsoh et al. 2019). Interestingly, cohabitation between two fly species, *D. melanogaster* (observer) and *Drosophila ananassae* (demonstrator), before social learning training increases the communication between demonstrator and observer, suggesting that flies can learn species-specific "dialects." This facilitation of social learning by species cohabitation was impaired when the antennal lobe, optic lobe, MBNs, LH, fan-shaped body neurons, and ellipsoid body neurons were inhibited using *shibire*^{ts} during cohabitation. Within these regions, it was found that olfactory receptor Or69a mutants or Or69a RNAi impaired dialect learning in observers. Or69a is expressed in olfactory sensory neurons that innervate the D glomerulus of the antennal lobe. In addition, inactivation of the motion-detecting neurons in the optic lobe (L2 and L4) and layer 5 of the fan-shaped body using *shibire*^{ts} impaired dialect learning.

Discussion

In this review, we attempted to synthesize the different types of learning and memory paradigms developed in *Drosophila* and assess the similarities and differences in the assays themselves and the genes and neural circuits involved (Fig. 3). Several overall themes are emerging in the field of *Drosophila* learning and memory. (1) The MBNs are important not only for olfactory memory, but for memories of a variety of sensory modalities and multimodal memories, and the genes and neural wiring revealed to support olfactory memory are helping to decode how other associative memories are established. (2) The brain structures in the central complex are important for spatial-, orientation-, and/or navigation-based memories. How these are integrated with information processed in the MBNs is of great interest moving forward. (3) Different memory paradigms exist for *Drosophila* that assess a variety of memories. For many of these, we have very little understanding of the mechanisms and neurons supporting these types of memories. (4) The reconstruction of the *Drosophila* hemibrain connectome (Li et al. 2020; Scheffer et al. 2020) is instrumental for determining the neurobiology of memory and offers a monumental advantage to neuroscience studies in *Drosophila*. The wiring diagram of the MB has led to a "high-resolution" understanding of olfactory memory and is now aiding in the study of more complex memories such as multimodal memories and SOC memories (Okray et al. 2023; Yamada et al. 2023). Undoubtedly, this information will be used in many future studies to decipher how more complex memories are encoded, stored, and retrieved.

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