

Review

Roles of feedback and feed-forward networks of dopamine subsystems: insights from *Drosophila* studies

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Across animal species, dopamine-operated memory systems comprise anatomically segregated, functionally diverse subsystems. Although individual subsystems could operate independently to support distinct types of memory, the logical interplay between subsystems is expected to enable more complex memory processing by allowing existing memory to influence future learning. Recent comprehensive ultrastructural analysis of the *Drosophila* mushroom body revealed intricate networks interconnecting the dopamine subsystems—the mushroom body compartments. Here, we review the functions of some of these connections that are beginning to be understood. Memory consolidation is mediated by two different forms of network: A recurrent feedback loop within a compartment maintains sustained dopamine activity required for consolidation, whereas feed-forward connections across compartments allow short-term memory formation in one compartment to open the gate for long-term memory formation in another compartment. Extinction and reversal of aversive memory rely on a similar feed-forward circuit motif that signals omission of punishment as a reward, which triggers plasticity that counteracts the original aversive memory trace. Finally, indirect feed-forward connections from a long-term memory compartment to short-term memory compartments mediate higher-order conditioning. Collectively, these emerging studies indicate that feedback control and hierarchical connectivity allow the dopamine subsystems to work cooperatively to support diverse and complex forms of learning.

Dopamine systems are ubiquitously important for learning in both vertebrates and invertebrates. Although mammalian dopaminergic neurons (DANs) were once regarded as a relatively uniform population that broadcasts reward signals, it has become clear that they consist of functionally diverse groups, with some of them mediating aversive stimuli (Matsumoto and Hikosaka 2009; Lerner et al. 2015; Menegas et al. 2018; de Jong et al. 2019; Yuan et al. 2019). The heterogeneity of DANs is not limited to the signs of valence they convey. In the primate caudate nucleus, two subregions receiving projections from distinct populations of DANs (Kim et al. 2014) contribute to flexible short-lasting memory and stable long-lasting memory in reward conditioning tasks (Kim and Hikosaka 2013). Thus, anatomically distinct groups of DANs mediate distinct types of memory. Furthermore, the benefits of having multiple dopamine subsystems bearing diverse properties in reward predictions and learning rates have been theoretically predicted and are supported by physiological and behavioral experiments (Iigaya et al. 2019; Dabney et al. 2020). However, major outstanding questions include what circuit mechanisms underlie the distinct properties of dopamine subsystems and how those subsystems interact with each other to enable diverse forms of learning and memory.

The *Drosophila* mushroom body (MB) has been an important model to study the mechanisms of dopamine-dependent learning. The orderly arrangement of the dopaminergic input and genetic tractability of individual circuit elements allowed detailed functional characterization of each dopamine subsystem. Importantly, like mammalian systems, DANs in the *Drosophila* MB exhibit considerable heterogeneity in their contribution to olfactory learn-

ing. Moreover, advanced anatomical studies in this system revealed that DANs receive numerous direct and indirect inputs from the output neurons of the dopamine subsystems. In this review, we spotlight these feedback and feed-forward networks within and across dopamine subsystems.

Basic circuit principles of the *Drosophila* MB

The adult *Drosophila* MB consists of ~2600 neurons, of which ~2000 are Kenyon cells (KCs) that predominantly receive inputs from the second-order olfactory neurons in the antennal lobes (Li et al. 2020). Each KC is narrowly tuned to odors, and as a population, KCs accurately represent odor identity in a sparse format (Turner et al. 2008; Honegger et al. 2011). KCs can be divided into three major anatomical classes (α/β , α'/β' , and γ), each forming separate axon bundles that collectively make up five MB lobes (α , β , α' , β' , and γ) (Crittenden et al. 1998; Lee et al. 1999). Those MB lobes can be further divided into 15 anatomical compartments ($\alpha 1-3$, $\beta 1-3$, $\alpha' 1-2$, $\beta' 1-2$, and $\gamma 1-5$) defined by matching innervation patterns of the dendrites of the MB output neurons (MBONs), the postsynaptic neurons of the KCs, and the axons of DANs (Fig. 1A; Tanaka et al. 2008; Aso et al. 2014a). Thus, the overall structure of the MB can be described as repeated anatomical units formed by pairs of MBONs and DANs arranged along the axis of KC axon bundles.

Just like anatomically segregated dopamine subsystems in mammals mediate distinct valences and types of memory (as introduced above), *Drosophila* MB compartments represent functionally diverse memory units. Distinct populations of DANs are involved

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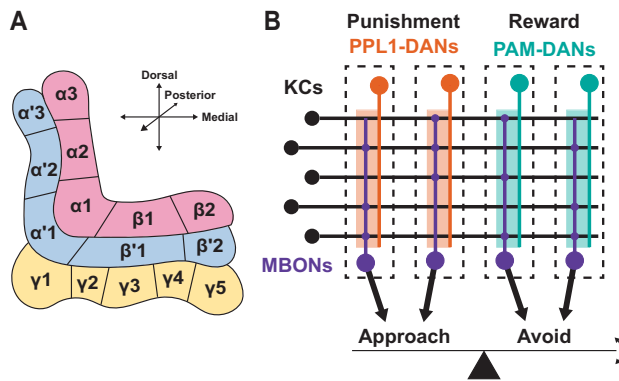


Figure 1. Basic anatomy and function of the *Drosophila* MB. (A) Arrangement of the MB lobes and 15 MB compartments. (Red) α/β Lobes, (blue) α'/β' lobes, (yellow) γ lobe. (B) Schematic illustrating the anatomy underlying the valence balance model, which proposes that odor-directed behavior is executed based on the balance of valence-related output from the MBON population. In general, punishment-encoding PPL1-DANs (orange) innervate compartments that activate approach-promoting MBONs, and reward-encoding PAM-DANs (teal) innervate compartments that activate avoidance-promoting MBONs. The depression of KC–MBON synapses by dopamine therefore promotes the appropriate behavior by biasing behavior toward avoidance (in the case of modulation by DANs activated by punishment) or approach (in the case of modulation by DANs activated by reward).

in appetitive and aversive learning. The compartments innervated by DANs belonging to the protocerebral anterior medial (PAM) cluster are generally appetitive memory compartments, while those innervated by the protocerebral posterior lateral 1 (PPL1) cluster DANs are aversive memory compartments (Fig. 1B; Schwaerzel et al. 2003; Burke et al. 2012; Liu et al. 2012; Aso and Rubin 2016). Moreover, memory dynamics are also distinct across compartments. Memories in some compartments develop slowly but are resistant to extinction, while those in other compartments are quickly formed but easily overwritten by subsequent conditioning (Yamagata et al. 2015; Aso and Rubin 2016). The key step of memory formation is cAMP-dependent long-term depression (LTD) at KC–MBON synapses (Yamada and Hige 2023) induced by coactivation of KCs and DANs (Cohn et al. 2015; Hige et al. 2015; Berry et al. 2018), which is thought to occur during simultaneous presentation of odor and punishment or reward. Since optogenetic activation of each MBON can promote either approach or avoidance behavior, they are considered to signal either positive or negative valence (Aso et al. 2014b; Oswald et al. 2015; Perisse et al. 2016). Thus, compartment-specific induction of LTD should tip the balance of the overall valence signaled by the MB and bias the behavioral choice (Fig. 1B). In support of this valence balance model (Oswald and Waddell 2015; Hige 2018), a recent study identified a cluster of neurons that integrate synaptic inputs from multiple MBONs, whose odor-evoked activity is unmasked upon LTD induction in one of the presynaptic MBONs (Aso et al. 2023).

As expected from the nonoverlapping tiling pattern of the MB compartments, recent studies using dopamine sensors revealed compartment-specific spatial patterns of dopamine signals (Sun et al. 2020; Noyes and Davis 2023; Yamada et al. 2023), which explains compartment-specific modulation of MBON activity (Cohn et al. 2015; Hige et al. 2015) and transmitter release from KCs (Davidson et al. 2023). However, MB compartments are not entirely functionally independent from each other or from their own output. Anatomical analysis of the total population of MBONs identified five neuropils where their axon terminals converge (Aso et al. 2014a). Four of those neuropils collectively contain 90% of the dendritic arbors of DANs, suggesting the presence of

MBON–DAN connections. The EM connectome indeed confirmed that there are extensive monosynaptic and disynaptic connections from MBONs to DANs, constituting feedback and feed-forward networks (Li et al. 2020). Those connections imply that MB compartments can dynamically tune their function based on ongoing activity of themselves, history of learning, and the hierarchical relationships between compartments. This interplay enables the influence of existing memories on future learning. This dense MBON–DAN network was originally reported in the connectome of the *Drosophila* larval MB (Eschbach et al. 2020). The connectome-based computational modeling predicted that both direct and indirect MBON–DAN connections are important for multiple types of learning, including classical conditioning, higher-order conditioning, context-dependent conditioning, and extinction (Eschbach et al. 2020). Simulated neuronal activity showed altered firing rates of modulatory neurons after conditioning, which may underlie at least some forms of learning. However, some of the conditioning paradigms have been experimentally demonstrated so far to be effective only in adult flies. Below, we introduce recent advances in understanding the functions of MBON–DAN networks in the adult brain by classifying them into three categories: memory consolidation, memory update, and higher-order conditioning.

Memory consolidation—feedback mechanism

Perhaps the simplest form of the circuit mediated by MBON–DAN connections is a feedback loop, where MBONs of a given MB compartment synapse onto the DANs projecting back to the same compartment. Among all MB compartments, $\alpha1$ is the only compartment whose resident MBONs and DANs do not directly communicate with DANs or MBONs in other compartments (Li et al. 2020). However, they do form a feedback loop from α/β KCs to glutamatergic MBON- $\alpha1$ to a subpopulation of PAM- $\alpha1$ (DANs in $\alpha1$) (Fig. 2A), and the activity of this recurrent circuit is implicated in the consolidation of appetitive memory. While there are several appetitive memory compartments (Burke et al. 2012; Liu et al. 2012; Huetteroth et al. 2015), $\alpha1$ is particularly essential for long-term memory (LTM) formed by nutritious sugar rather than for short-term memory (STM) formed by nonnutritious sweetness (Yamagata et al. 2015; Aso and Rubin 2016). Transient blockade of any one of the three components of the feedback loop (i.e., α/β KCs, MBON- $\alpha1$, and PAM- $\alpha1$) during or shortly after sugar conditioning impairs memory measured after 24 h, representing LTM (Ichinose et al. 2015). However, synaptic outputs from those neurons are no longer needed between 22 h posttraining and the test period. These results led to the model that self-sustained activity of the feedback loop after learning contributes to the consolidation of memory. However, the postulated reverberant circuit activity during the consolidation period is yet to be demonstrated. The memory consolidation function of this circuit depends on both Dop1R1 dopamine receptors on α/β KCs and N-methyl-D-aspartate (NMDA)-type glutamate receptors on PAM- $\alpha1$ (Ichinose et al. 2015). While dopamine can depolarize the postsynaptic neurons (Cohn et al. 2015; Takemura et al. 2017), glutamatergic transmission in the central brain of *Drosophila* has been generally considered inhibitory (Liu and Wilson 2013). In particular, transmission from glutamatergic MBON- $\alpha1$ to another group of postsynaptic neurons is also inhibitory (Aso et al. 2023). Cell type-specific transcriptome analysis suggests that PAM- $\alpha1$ abundantly expresses glutamate-gated chloride channels in addition to NMDA receptors (Aso et al. 2019). Thus, it is entirely possible that an additional indirect pathway from MBON- $\alpha1$ to PAM- $\alpha1$ is responsible for the function of this feedback loop. Interestingly, a systematic study on the behavioral contribution of each



transient activity of dopamine in $\gamma 1$ pedc, which is known as one of the aversive memory compartments (Claridge-Chang et al. 2009; Aso et al. 2010), helps consolidate the appetitive memory and how DAN activity outside the narrow time window disrupts the consolidation process.

One potential problem shared by the consolidation models involving sustained recurrent activity is the maintenance of the stimulus specificity of memory. That is, during the consolidation period, there is no longer an olfactory input that evokes odor-specific activity in KCs; meanwhile, DANs keep releasing dopamine to all KCs in the compartment. This may result in nonspecific modulation of KC outputs and therefore a decrease in stimulus specificity. Such a decline in stimulus specificity of LTM as compared with STM has been shown in appetitive memory (Ichinose et al. 2015). In the case of courtship learning, this trade-off may be less significant because the role of accurate sensory coding in KCs is not well defined in this behavioral paradigm. Another related issue that remains to be solved is how sustained input from DANs modulates KC outputs. At this point, bidirectional modulation of KC–MBON synapses is only demonstrated as dependent on the relative timing of the activity of KCs and DANs (Handler et al. 2019), the size of odor-evoked calcium transient in the KC presynaptic terminal (Davidson et al. 2023), or the second-messenger pathway implicated in a cotransmitter of DANs (Aso et al. 2019; Yamada and Hige 2023), none which can easily explain the action of dopamine during consolidation. Aversive LTM formation also depends on protein synthesis in a small subset of MBONs at specific time points during the consolidation period (Wu et al. 2017), suggesting the involvement of plasticity on the postsynaptic neurons. The presence of transcriptional memory trace has been also suggested in at least one of the MBONs in the context of appetitive LTM (Widmer et al. 2018).

Memory consolidation—feed-forward mechanism

Multiple lines of evidence indicate that different classes of KCs preferentially contribute to different phases of memory. In general, γ KCs serve as an important “gateway” of memory or primary association site for olfactory and punishment/reward signals, whereas output from α/β KCs is critical for LTM (Isabel et al. 2004; Yu et al. 2006; Krashes et al. 2007; Krashes and Waddell 2008; Blum et al. 2009; Trannoy et al. 2011; Qin et al. 2012; Huang et al. 2013). These findings are in line with MB compartment-specific contributions to STM and LTM. For appetitive learning, a series of studies using artificial activation and blockade of DANs or MBONs identified $\alpha 1$ as one of the LTM compartments and $\gamma 5\beta 2a$ as an STM compartment (Huetteroth et al. 2015; Yamagata et al. 2015; Aso and Rubin 2016). Similarly, for aversive learning, $\gamma 1$ pedc was identified as one of the STM compartments, and $\alpha 3$ as an LTM compartment (Pai et al. 2013; Aso and Rubin 2016; Jacob and Waddell 2020), although $\alpha 3$ is also implicated in appetitive LTM (Pla  ais et al. 2013). The observations that memories with different retention times depend on distinct neuronal substrates of the MB invited active debates over the origin of LTM in relation to STM. In an extreme scenario, STM and LTM could form and develop in parallel in completely independent, segregated circuits. The LTM induction mechanisms that we reviewed so far do not contradict this view because they reside within a single MB compartment, even though they may involve additional neurons outside the MB. However, the mechanisms that we introduce next, which are mediated by MBON–DAN feed-forward circuits, are in support of another view: Formation of STM sequentially induces LTM, involving multiple KC classes and MB compartments.

Aversive olfactory LTM is typically induced by so-called spaced training, in which odor–shock conditioning is repeated multiple times with certain intervals (Tully et al. 1994; Beck et al. 2000), and the optimum interval is at least in part determined by the level of protein phosphatase activity (Pagani et al. 2009). During later cycles of the spaced training, GABAergic MBON– $\gamma 1$ pedc and a subset of the presynaptic γ KCs decrease their odor-evoked activity (measured by the level of phosphorylation of extracellular signal-related kinase [pERK]) in a Dop1R1-dependent manner (Awata et al. 2019). In contrast, PPL1– $\alpha'2\alpha 2$, one of the DANs postsynaptic to MBON– $\gamma 1$ pedc, shows a concurrent increase in the pERK reported activity, suggesting disinhibition from MBON– $\gamma 1$ pedc (Fig. 2D). This suggests that in the later cycles of spaced training, both KCs and DANs are strongly activated in the $\alpha'2\alpha 2$ compartment. Simultaneous thermogenetic activation of PPL1– $\alpha'2\alpha 2$ and α/β s KCs, which are the subset of α/β KCs identified as particularly essential for LTM retrieval (Huang et al. 2013), induces *Arc2* mRNA expression in α/β s KCs, which is implicated in LTM formation. Thus, these observations suggest that LTM induction involves the coordinated action of multiple MB compartments.

Although care must be taken when interpreting these results because of the use of indirect measurement of neuronal activity and plasticity (Awata et al. 2019), a similar circuit mechanism was independently proposed by another study using elegant voltage imaging tools (Schnitzer et al. 2022). This work also points to the importance of MBON– $\gamma 1$ pedc plasticity as a trigger to open the gate for plasticity in other MB compartments in the α lobe. Repetitive odor–shock pairing induces an odor-specific decline in spike responses in MBON– $\gamma 1$ pedc in later cycles, as expected from the above-mentioned pERK reported activity (Awata et al. 2019) as well as other studies (Hige et al. 2015; Perisse et al. 2016; Felsenberg et al. 2018; Cervantes-Sandoval et al. 2020; McCurdy et al. 2021; Hancock et al. 2022; Davidson et al. 2023; Zeng et al. 2023). This activity decline is accompanied by the odor-specific activity increase in PPL1– $\alpha 3$, which is also postsynaptic to MBON– $\gamma 1$ pedc (Scheffer et al. 2020), as well as in PPL1– $\alpha'2\alpha 2$ (Fig. 2D; Schnitzer et al. 2022). Importantly, MBON– $\alpha 3$ shows odor-specific depression for up to 48 h after conditioning (Schnitzer et al. 2022), whereas MBON– $\gamma 1$ pedc recovers from depression after 1 h (Cervantes-Sandoval et al. 2020; Schnitzer et al. 2022; but see Perisse et al. 2016; Felsenberg et al. 2018). Thus, MBON–DAN feed-forward circuits assign hierarchical relationships between MB compartments, enabling STM formation in one compartment to gate the plasticity in another compartment for LTM induction. Since bypassing this circuit mechanism by direct optogenetic activation of DANs in $\gamma 1$ pedc and $\alpha 3$ results in the formation of STM and LTM, respectively, with different learning rates (Aso and Rubin 2016), intrinsic properties of the MB compartments, such as compartment-specific expression of a DAN cotransmitter (Aso et al. 2019), are also an important determinant of the induction of LTM.

Memory update

While forming, stabilizing, and maintaining a memory are certainly important aspects of learning, memories also require sufficient flexibility to be updated; for example, in response to more recent experience that does not align with the previously learned association. We next introduce the circuit mechanisms of various types of memory update involving MBON–DAN connections.

After forming an association between an odor (conditioned stimulus+ [CS+]) and shock, the aversive olfactory memory can be extinguished by subsequent presentation of the CS+ alone (Tempel et al. 1983; Schwaerzel et al. 2002), and this extinction involves an excitatory MBON–DAN connection (Felsenberg et al.

2018) that also plays a role in the aforementioned consolidation process of courtship memory (Zhao et al. 2018). As repeatedly mentioned, aversive conditioning induces CS⁺-specific depression in MBON- γ 1pedc. This GABAergic MBON sends axon collaterals to the MB lobes to make contacts with multiple MBONs, including MBON- γ 5 β 2a (Fig. 2E), which belongs to an appetitive memory compartment. After aversive training, the CS⁺ response of the axon of MBON- γ 5 β 2a is potentiated (as compared with control odors), presumably due to disinhibition from MBON- γ 1pedc (Owald et al. 2015; Perisse et al. 2016; Felsenberg et al. 2018). This relative potentiation of the CS⁺ response is expected to contribute to conditioned avoidance of CS⁺ because direct activation of a cluster of MBONs, including MBON- γ 5 β 2a, drives avoidance behavior (Aso et al. 2014b; Oswald et al. 2015). During re-exposure to CS⁺ or extinction training, this potentiated CS⁺ response drives excitation of a subset of the PAM- γ 5 DANs via direct synaptic contacts (Otto et al. 2020), which in turn induces depression in the γ 5 branch of the MBON- γ 5 β 2a dendrites (Felsenberg et al. 2018). Although MBON- γ 1pedc remains depressed after extinction training, the dendritic depression of MBON- γ 5 β 2a neutralizes the potentiation effect in its axon. Thus, this excitatory feedback connection, perhaps representing the “reward” of the absence of shock or prediction error, induces plasticity in the appetitive memory compartment, which counteracts that induced by the original aversive training. A similar antagonism between coexisting opposing memories is considered to underlie the extinction of appetitive memory (Felsenberg et al. 2017). In this case, activation of punishment-encoding PPL1-DANs via corresponding MBONs during extinction training is crucial, although a less detailed process compared with aversive memory extinction is known.

Reversal learning is somewhat similar to extinction but requires more flexible updates of memory, as it completely flips the stimulus contingency between trainings rather than simply omitting the reinforcement. In this paradigm, after normal associative learning, the cue that was presented without reinforcement during original training (conditioned stimulus⁻ [CS⁻]) is paired with reinforcement, while the original CS⁺ is presented alone without reinforcement (Shuai et al. 2010; Cervantes-Sandoval et al. 2016; Berry et al. 2018; McCurdy et al. 2021). Unsurprisingly, the reversal of aversive learning induces neuronal activity changes that overlap with those observed after the extinction of aversive memory (McCurdy et al. 2021). As mentioned above, aversive conditioning potentiates the CS⁺ response of MBON- γ 5 β 2a presumably via disinhibition from MBON- γ 1pedc. Just like in extinction, the central mechanism of attenuation of CS⁺ avoidance after reversal learning is also neutralization of the plasticity (or depotentiation) of MBON- γ 5 β 2a. However, in reversal learning, the depression that counteracts the ongoing potentiation is mediated by PAM- β 2a DANs, not PAM- γ 5 as seen in the case of extinction (Felsenberg et al. 2018; Otto et al. 2020). The CS⁺ response of PAM- β 2a, previously depressed by aversive conditioning, regrows during reversal training to depotentiate MBON- γ 5 β 2a (McCurdy et al. 2021). This activity pattern of PAM- β 2a mirrors that of cholinergic MBON- γ 2 α 1 (Berry et al. 2018; McCurdy et al. 2021), which is presynaptic to PAM- β 2a (Fig. 2F). Thus, although slightly different in the routes leading to plasticity, both reversal learning and extinction induce parallel opposing memory traces via MBON–DAN pathways to attenuate the conditioned behavior. Interestingly, a closely overlapping mechanism is used for learning of relative aversive value (Villar et al. 2022). In this learning, flies experience strong electric shocks with odor A and then milder shocks with odor B, after which they develop a relative preference of B over A (Yin et al. 2009; Perisse et al. 2013). During pairing with odor B, a subset of reward-encoding PAM-DANs (namely, PAM- β 2a and PAM- γ 5) shows an increased activity, likely driven by elevated activity of MBON- γ 2 α 1 (Fig. 2F; Villar et al. 2022). Thus, the circuitry

around PAM- β 2a and PAM- γ 5 may have a specialized role of representing “relative reward” based on the animal’s past experience.

Memory update does not always mean that memory is extinguished or reversed; some memory just needs to be refreshed and re-established, especially since memory appears to become temporarily labile after retrieval (Nader et al. 2000; Schafe and LeDoux 2000), often triggering reconsolidation to maintain the originally learned association. In flies, moderate reactivation of aversive memory by re-exposure to CS⁺ alone triggers reconsolidation, whereas intense reactivation results in extinction (Lagasse et al. 2009). Reconsolidation of appetitive memory can be initiated by postlearning re-exposure to CS⁻ alone, and this process relies on the excitation of multiple types of DANs via both feedback and feed-forward MBON–DAN networks (Felsenberg et al. 2017). After appetitive learning, MBON- γ 2 α 1 shows an increased response to CS⁻, which is counterintuitive because MBON- γ 2 α 1 promotes approach behavior (Aso et al. 2014b); the elevated response to CS⁻ could counteract the conditioned approach to CS⁺. However, this cholinergic MBON feeds a critical excitatory drive to its partner DAN PPL1- γ 2 α 1 (Fig. 2G) via a feedback loop and also to PAM-DANs via feed-forward connections, both of whose activity is required for reconsolidation with different timing; PPL1- γ 2 α 1 is required during CS⁻ re-exposure, and PAM-DANs are required right after it (Felsenberg et al. 2017). Although the detailed process is still elusive, sequential action of those DANs driven by MBON–DAN connections is expected to induce destabilization and restabilization of memory.

Higher-order conditioning

The examples so far show that the hierarchical interconnection between dopamine subsystems enables existing memory to gate LTM or update it based on new experiences. In general, this circuit architecture allows previous learning to influence future learning. This feature also enables a complex form of learning, in which animals use a previously learned, rather than an innate, valence of stimuli to learn a new association. Second-order conditioning is one such type of indirect learning that has been observed in many different species, including *Drosophila* (Rizley and Rescorla 1972; Rashotte et al. 1977; Bitterman et al. 1983; Hawkins et al. 1998; Brembs and Heisenberg 2001; Mizunami et al. 2009; Tabone and de Belle 2011; Yamada et al. 2023). In appetitive unimodal olfactory second-order conditioning, starved flies first undergo odor (S1)–sugar conditioning. This S1 serves as a reward in the following round of training, in which flies are presented with another odor (S2) followed by S1 but not by sugar. After this training, flies develop a robust preference toward S2 even though it was never presented with the primary reward (Yamada et al. 2023). Optogenetic imprinting of S1 memory in targeted reward memory compartments followed by S2–S1 conditioning identified α 1, the LTM compartment, as the most efficient “teacher” compartment capable of instructing second-order memory. Paradoxically, as mentioned above, α 1 is the most “isolated” compartment in terms of direct connectivity with other compartments, lacking monosynaptic connection between MBON- α 1 and non- α 1 DANs (Li et al. 2020). It turned out, however, that there is a robust network indirectly connecting MBON- α 1 to multiple PAM-DANs innervating reward memory compartments. A cluster of cholinergic neurons (SMP353/354) receiving inhibitory inputs from MBON- α 1 acquires an excitatory S1 response upon induction of depression in α 1, mimicking S1 memory formation (Aso et al. 2023). SMP353/354 converge on a single cholinergic neuron named SMP108, which also acquires an excitatory S1 response (Yamada et al. 2023). SMP108 in turn synapses on PAM-DANs and, upon activation, can induce dopamine release in multiple “student”

compartments, including the STM compartments $\gamma 5$ and $\beta' 2a$ (Fig. 2H). S2–S1 conditioning after S1 memory formation in $\alpha 1$ indeed induces S2-specific depression in MBON- $\gamma 5\beta' 2a$. Successful second-order conditioning generally involves repetitive S2–S1 pairing. This requires S1 memory to be resistant to extinction because no reward is presented during S2–S1 pairing. This requirement may explain why $\alpha 1$, which can hold stable and long-lasting memory, is most effective as a teacher compartment. On the other hand, the retention time of S2 memory is known to be short across species (Gewirtz and Davis 2000). This feature could be explained by the memory properties of the student compartments. Thus, the identified circuit mechanisms of second-order conditioning may account for distinct properties of first- and second-order memory. A somewhat similar mechanism may underlie aversive second-order learning because suppression of MBON- $\gamma 1$ pedc, which mimics the plasticity following first-order conditioning, paired with an odor presentation induces aversive memory (Ueoka et al. 2017; König et al. 2019), although the exact mechanisms are yet to be studied.

Current limitations and future prospects

Apart from a notable exception of the second-order learning study (Yamada et al. 2023), the majority, if not all, of the studies mentioned in this review focused on, or attributed the identified functions to, direct MBON–DAN connections. However, experimental evidence to support the specific role of the direct connections is typically circumstantial and does not formally exclude the potential involvement of indirect connections. For example, even the presence of an EM-confirmed monosynaptic connection from an MBON to DANs and corresponding behavioral evidence of the requirement of DAN expression of receptors for the transmitter released by the MBON do not rule out the possibility that other neurons that use the same transmitter and synapse on the DANs play an important role. Additionally, although the relationship between the ultrastructural arrangement of synapses and physiologically measured synaptic strength has been studied in certain cell types (Liu et al. 2022), it is unclear whether we can extrapolate the results to other cells. In general, we need more information about the subcellular localization of transmitter receptors to validate the connectome-identified synapses. We also need tools to manipulate specific synapses or axon branches rather than switching the entire output of a given neuron on and off to understand the function of the specific connections of interest. For example, a recent study in T4/T5 neurons of the fly visual system used epitope-tagged endogenous transmitter receptors together with expansion microscopy to demonstrate subtype-specific localization of receptors to dendritic domains within a neuron (Sanfilippo et al. 2023). Subtype compositions of the receptors are also distinct among synapses within the same dendritic domains. Such high-resolution information may enable input-specific manipulation of synapses by, for example, cell type-specific knockout of a specific receptor subtype. To explore the presence of indirect connections, the combination of EM connectome data and the neurotransmitter prediction from the EM images (Eckstein et al. 2023) would be a powerful tool. However, to test the functions of the identified connections, genetic drivers for the interneurons connecting MBONs and DANs are required. Although such an approach has proven to be successful in identifying the circuit mediated by two interneurons (Yamada et al. 2023), the creation of specific genetic drivers for interneurons can be a rate-limiting step.

Another complicating factor is the heterogeneity of DANs projecting to the same MB compartment. While there is only a single cell per cell type of PPL1-DANs, which occupies the entire MB compartment, there are a few up to dozens of neurons per cell

type of PAM-DANs (Li et al. 2020). A given PAM-DAN cell type, as a population, occupies the entire MB compartment, but individual cells project to a subregion of the compartment. In the $\gamma 5$ compartment, which is innervated by ~20 PAM-DANs, such anatomical heterogeneity has been successfully linked to their functional segregation (Otto et al. 2020). However, the ability to interrogate the different roles across subclasses of PAM-DANs is again limited by the availability of specific genetic drivers.

Finally, to monitor the dynamic sequential changes of neuronal activity across multiple MB compartments, simultaneous recording of neurons and behavioral changes would be ideal. The recent development of optical probes represented by genetically encoded voltage sensors made it possible to report the spiking activity of neurons in head-fixed-behaving flies (Schnitzer et al. 2022). However, monitoring the activity of neurons while flies show learning-induced behavioral changes is still challenging, presumably due to the impact of surgery and head fixation on their internal state. One avenue to mitigate the problem might be to record from free-moving flies using less invasive imaging with bioluminescence probes (Mercier et al. 2018). Given the dense network of MBONs and DANs, however, the flip side of monitoring actual learning-induced changes is that it may make it difficult to discriminate the primary location of plasticity from the regions undergoing plasticity induced by the primary plasticity. To probe these relationships, artificial learning induced by site-directed memory imprinting using methods such as optogenetic activation of a specific DAN population should be useful.

Conclusions

In a simplified view, the *Drosophila* MB can be regarded as repeating memory modules controlled in parallel by dopamine and operating with different intrinsic parameters for plasticity induction and maintenance. However, the intricate network interconnecting the MB compartments revealed by the EM connectome suggests that these memory modules are far from independent from each other. Recent studies confirm that DANs in a given module are under the effect of its own or other modules' output, which enables previous learning to influence future learning. Computational modeling studies have also begun to account for such connectivity, illustrating the importance of these connections in processes like extinction learning and testing reward prediction capabilities of DANs (Bennett et al. 2021; Jiang and Litwin-Kumar 2021; Springer and Nawrot 2021; Zhao et al. 2021; Gkaniyas et al. 2022). It should be noted that, as evident from the circuit underlying second-order conditioning, even MBONs and DANs that are three synapses away can mediate a robust function. Thus, the potential impact of MBON–DAN connections should be even more significant than what one would expect from direct connectivity, which already appears dense. Indeed, there are more functions of the MB that are likely to involve this MBON–DAN network (Hattori et al. 2017; Jacob and Waddell 2020). Moreover, DAN activity is influenced not only by previous learning but also by a complex combination of the innate value of stimuli, the internal state of the animal, ongoing locomotion, and microcircuits inside the MB compartments (Krashes et al. 2009; Cohn et al. 2015; Cervantes-Sandoval et al. 2017; Tsao et al. 2018; Lyutova et al. 2019; Siju et al. 2020; Zolin et al. 2021; Schnitzer et al. 2022; Kato et al. 2023). Thus, the MB offers ample opportunities to explore the functions of higher-order connections between dopamine subsystems.

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