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RESEARCH REPORT

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Dopamine activity in projection neurons regulates short-lasting olfactory approach memory in *Drosophila*

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Abstract

Survival in many animals requires the ability to associate certain cues with danger and others with safety. In a *Drosophila melanogaster* aversive olfactory conditioning paradigm, flies are exposed to two odours, one presented coincidentally with electrical shocks, and a second presented 45 s after shock cessation. When flies are later given a choice between these two odours, they avoid the shock-paired odour and prefer the unpaired odour. While many studies have examined how flies learn to avoid the shock-paired odour through formation of odour-fear associations, here we demonstrate that conditioning also causes flies to actively approach the second odour. In contrast to fear memories, which are longer lasting and requires activity of D1-like dopamine receptors only in the mushroom bodies, approach memory is short-lasting and requires activity of D1-like dopamine receptors in projection neurons originating from the antennal lobes, primary olfactory centers. Further, while recall of fear memories requires activity of the mushroom bodies, recall of approach memories does not. Our data suggest that olfactory approach memory is formed using different mechanisms in different brain locations compared to aversive and appetitive olfactory memories.

KEYWORDS
D. melanogaster, D1-like dopamine receptor, olfactory approach memory, olfactory aversive learning, projection neurons

1 | INTRODUCTION

The ability to recognize that some sensory cues are associated with danger, while others are associated with safety, is important for survival. In *Drosophila melanogaster*, this ability has been studied using an aversive olfactory conditioning system (Tully & Quinn, 1985). In this system, flies are exposed sequentially to two odours, the first of which is paired with aversive electrical

Abbreviations: AL, antennal lobe; APL, anterior paired lateral; BEN, benzaldehyde; CRI, conditional response index; CS, conditioned stimulus; D1R, D1-like DA receptor; DA, dopamine; DANs, dopaminergic neurons; HLs, horizontal lobes; ISI, inter-stimulus interval; MBs, mushroom bodies; MCH, 4-methylcyclohexanol; OCT, 3-octanol; PAM, protocerebral anterior medial; PFA, paraformaldehyde; PPL1, protocerebral posterior lateral 1; shi^{ts1}, $shibire^{ts1}$; US, unconditioned stimulus.

shocks. The first odour is known as the paired conditioned stimulus or CS+, the second odour is known as the unpaired stimulus or CS− and the electrical shocks are referred to as the aversive unconditioned stimulus or US. Immediately after flies are conditioned using this system, they show an intense avoidance to the CS+ odour, a behaviour that arises from fear memory (Tully & Quinn, 1985).

Neuronal mechanisms underlying olfactory aversive memory are well identified. The mushroom bodies (MBs) are critical brain structures required for olfactory associations and receive input from various neurons, including projection neurons (PNs) from the antennal lobes (ALs), and dopaminergic neurons (DANs). The ALs are analogues of the vertebrate olfactory bulbs and receive inputs from olfactory receptor neurons and inhibitory interneurons. Each AL sends odour information directly to the MB via PNs. PNs form synaptic connections with MB neurons, which are called Kenyon cells, in a region of the MB known as the calyx. DANs in the protocerebral posterior lateral 1 (PPL1) cluster (Mao & Davis, 2009) project axons projecting to the vertical lobes of the MBs and are essential for olfactory aversive memory formation, since suppressing dopamine (DA) release from these neurons attenuates olfactory learning (Aso et al., 2010). Furthermore, activity of D1-like DA receptors (D1Rs) in the MBs is required for olfactory aversive learning (Kim et al., 2007; Qin et al., 2012).

In *Drosophila*, extensive studies of associative learning, both through paired and through unpaired presentations of reinforcers, have been conducted (Barth et al., 2014; Gerber et al., 2014; Jacob & Waddell, 2020; Niewalda et al., 2011; Paisios et al., 2017; Saumweber et al., 2011; Schleyer et al., 2015, 2018; Tanimoto et al., 2004; Yarali & Gerber, 2010). However, mechanisms of unpaired learning are to a large extent still unclear. If flies are subjected to electrical shocks, which are then terminated before exposing them to an odour, flies show conditioned preference to this odour, a behaviour that arises from association of the odour with relief from shock (relief memory), or from association of the odour with safety (safety memory) (Jacob & Waddell, 2020; Tanimoto et al., 2004; Yarali & Gerber, 2010).

Although relief and safety memory cause similar approach behaviours, they have some different properties. Formation of relief memory requires the time between cessation of the US and the exposure of the CS to be relatively short (within 45 s), suggesting that relief memory is an association of an odour with US cessation (Gerber et al., 2014). In contrast, safety memory requires a different training paradigm and is less dependent on a tight temporal linkage between US cessation and CS− presentation, suggesting that safety memory is an association between an odour and the absence, rather than the cessation, of the US (Gerber et al., 2014). Relief memory emerges immediately after olfactory conditioning and persists for less than 24 h, while safety memory occurs slowly and persists more than 24 h (Jacob & Waddell, 2020). In addition, recent studies have shown that different DA signalling is involved in relief memory and safety memory in flies (Aso & Rubin, 2016; Handler et al., 2019; Jacob & Waddell, 2020; Konig et al., 2018). DANs in the protocerebral anterior medial (PAM) cluster that project to the horizontal lobes of the MBs, such as PAM-γ3, PAM-β′2mp and PAM-β′1 DANs, are necessary for the formation of safety memory (Jacob & Waddell, 2020), while DANs that project to the vertical lobes of the MBs, such as PPL1-γ1pedc and PPL1-α3 DANs, have been suggested to participate in formation of relief memory (Aso & Rubin, 2016; Handler et al., 2019; Konig et al., 2018). However, it has still been unclear whether any type of approach memory is formed after training flies in the standard single-cycle olfactory avoidance conditioning paradigm.

In the present study, we demonstrate that single cycle olfactory aversive conditioning forms two different odour associations of opposite valence, olfactory avoidance memory and short-lasting olfactory approach memory. While avoidance memory is known to require D1R activity in the MBs, we find that formation of approach memory requires D1R activity in both the MBs and the PNs from the ALs. In addition, recall of olfactory approach memory does not require neuronal outputs from the MBs. Our results demonstrate that D1Rs outside the MBs are required for olfactory approach memory and contribute to altered behaviours after olfactory aversive conditioning. We discuss how olfactory approach memory may be related to relief and safety memories.

2 | MATERIAL AND METHODS

2.1 | Fly stocks

All fly strains (*D. melanogaster*) were raised on standard cornmeal at 25 ± 2°C in 60 ± 10% humidity under a 12-h/12-h light/dark cycle. The wild-type strain was wCS (10) (Tamura et al., 2003). *GAL4* drivers were *OK107-GAL4*, *TH-GAL4*, *GH146-GAL4* (Heimbeck et al., 2001), *NP225-GAL4* (Tanaka et al., 2004) and *APL-GAL4* (Wu et al., 2013). The $dumb^2$ mutant used has been previously described (Kim et al., 2007). UAS lines were *UAS-nSyb-GFP* (Bloomington stock center, Indiana, USA), *UAS-mCD8::GFP* (Bloomington stock center, Indiana, USA) and *UAS-shibire*^{ts1} (Kitamoto, 2001). *UAS-*

D1R RNAi lines were obtained from the Vienna *Drosophila* Resource Center (VDRC, www.vdrc.at).

2.1.1 | Behavioural tests

The procedure for measuring olfactory memory is described elsewhere (Tully & Quinn, 1985). Briefly, ~100 flies were exposed sequentially to two aversive odours, 3-octanol (OCT, Tokyo Chemical Industry Co., Ltd., Japan) and 4-methylcyclohexanol (MCH, Sigma-Aldrich Co. LLC, UK), for 1 min with an interval of 45 s between each odour exposure. When the flies were exposed by the first CS+ odour (either OCT or MCH), they were simultaneously exposed to 1.5-s pulses of 60-V DC electric shocks every 5 s (US). To test olfactory memory, flies were placed at the choice point of a T-maze where both CS+ and CS− odours were delivered in each arm and allowed to choose between the odours. After 1.5 min, flies choosing each odour were counted, and avoidance behaviour was calculated as a performance index, such that a 50:50 distribution (no memory) yielded a performance index of zero and a 0:100 distribution away from the CS+ yielded a performance index of 100.

To measure olfactory avoidance memory and approach memory separately, ~100 flies were stored in vials containing a 2 × 6-cm Whatman 3MM filter paper soaked with 5% sucrose overnight. Flies were then subjected to either normal olfactory aversive conditioning or mock conditioning (Figure 1a). For mock conditioning, the flies were treated the same as in normal conditioning except that the US was omitted during CS+ exposure. Both conditioned and mock conditioned flies were separated into two groups. One group was tested for responses to the CS+ odour, while the second group was tested for responses to the CS− odour. To test responses to the CS+ odour, flies were placed at the choice point of a T-maze and allowed to choose between the CS+ odour

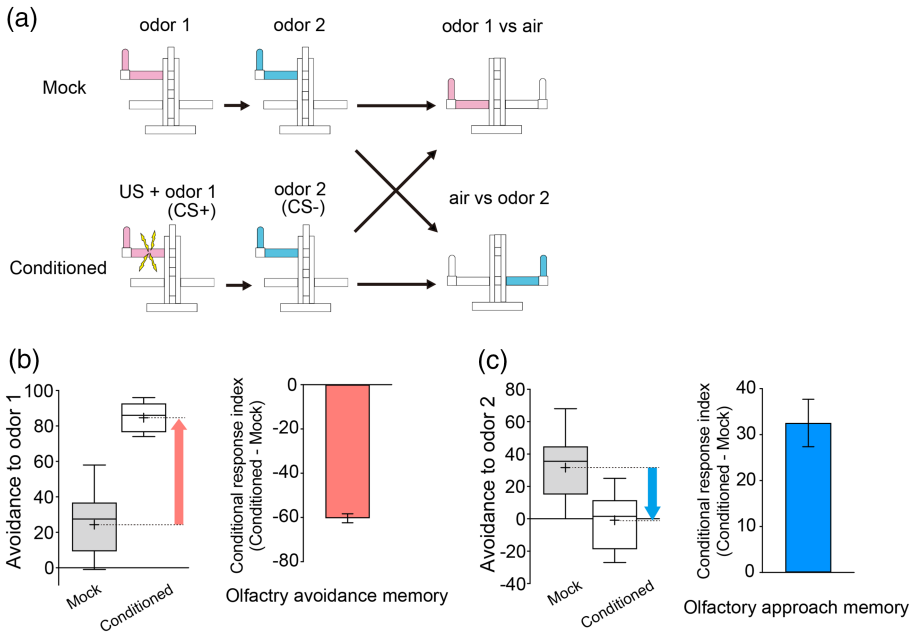

FIGURE 1 Traditional olfactory conditioning changes fly behaviours towards both the CS+ and the CS− odours. (a) A schematic showing the procedures for mock conditioning (Mock) and olfactory aversive conditioning (Conditioned). In mock conditioning, about a hundred of flies are exposed sequentially to two odours in the absence of electrical shocks. For aversive conditioning, odour 1 (the CS+ odour) is paired with electrical shocks (the unconditional stimulus or US), while odour 2 (the CS− odour) is not. Odour avoidance scores for conditioned and mock conditioned flies are measured for odour 1 versus air and odour 2 versus air. (b) Box plots represent odour avoidance scores to odour 1 after mock conditioning (grey) and after aversive conditioning (white). '+' in box plots represents mean value of odour avoidance score. We defined formation of CS+ avoidance memory as a significant difference in behaviour to the CS+ when comparing conditioned versus mock conditioned flies (Figure 1b, red arrow). Conditional response index (CRI) is calculated by subtracting mock conditioned scores from conditioned scores (Figure 1b, red column, see Methods). (c) Box plots represent odour avoidance scores to odour 2 after mock conditioning (grey) and after aversive conditioning (white). Formation of CS− avoidance memory is defined as a significant change in behaviour towards the CS− (Figure 1c, blue arrow). Again, a CRI is calculated by subtracting mock conditioned scores from conditioned scores (Figure 1c, blue column, also see Methods). 4-Methylcyclohexythanol (MCH) and 3-Octanol (OCT) were used as odours. $N = 16$ for all data. In boxplots, upper whisker and lower whisker represent the minimum and the maximum value of the data set, respectively.

and air. To test responses to the CS− odour, flies were allowed to choose between the CS− odour and air. Odour response scores were measured manually as described above. To test odour response behaviours towards a novel odour, conditioned flies were allowed to choose between a novel odour, including benzaldehyde (BEN, Tokyo Chemical Industry Co., Ltd., Japan), and air. An undiluted solution of each odour was used for behavioural experiments.

2.2 | Calculation for the conditional response index

All data in bar graphs are expressed as means ± *SEM*s. To calculate conditional response indices (CRIs), we subtracted average odour response scores after mock conditioning from average odour response score after normal conditioning.

$$\text{Odour response scores after normal conditioning}: x_1 \ldots\ldots x_{n1}$$
$$\text{Odour response scores after mock conditioning}: y_1 \ldots\ldots y_{n1}$$
$$\text{CRI}: m = \bar{x} - \bar{y}$$

where $\bar{x}$ and $\bar{y}$ are averaged odour response scores.

Standard deviation:

$$\text{S.D.} = \sqrt{\frac{\sum (\text{x}_\text{i} - \bar{\text{x}})^2}{\text{n}_1 - 1} + \frac{\sum (\text{y}_\text{i} - \bar{\text{y}})^2}{\text{n}_1 - 1}}$$

Standard error:

$$\text{S.E.} = \frac{\text{S.D.}}{\sqrt{\text{n}_1}}$$

2.2.1 | Statistics

Unpaired two-tailed *t* tests were used to determine whether there were any statistically significant differences between normal conditioned datasets and mock conditioned datasets.

To determine whether CRIs between two groups had statistically significant differences, we calculated *z* scores.

$$z = \frac{m_1 - m_2}{\sqrt{se_1^2 + se_2^2}}$$

where m_1 and m_2 are CRIs under two different conditions, and se_1 and se_2 are their respective standard errors. We compared *z* scores to values from a standard normal distribution table to obtain *P* values. *P* values < .05 were considered to be statistically significant. To determine whether CRIs between three groups had statistically significant differences, we employed Tukey's multiple comparison test to obtain *P* values. In this case, statistical analyses were performed using Prism 9 (GraphPad Software, US).

2.3 | Whole mount immunohistochemistry

Terminals of DANs were visualized by expressing *nSyb-GFP* from a *TH GAL4* driver. Brains were dissected, fixed in PBS containing 4% PFA and blocked in .3% PBSTx containing 4% BLOCK ACE (KAC co., Ltd., Japan). Brains were then incubated with primary antibodies (a 1:250 dilution of chick anti-GFP polyclonal antibody, Abcam, UK, or a 1:250 dilution of anti-D1R antibody gifted from Dr. Wolf; Kong et al., 2010), overnight at 4°C. Alexa Fluor488-conjugated donkey anti-chick IgG (1:400; Jackson ImmunoResearch Inc., USA) and Alexa Fluor555-conjugated donkey anti-rabbit IgG (1:400; Thermo Fisher Scientific Inc., USA) were used as secondary antibodies. Images were captured using an A1R confocal microscope (NIKON instruments Inc., Japan) with a ×60 water immersion objective lens (×60 NIKON CFI APO NIR 1.0 NA).

3 | RESULTS

3.1 | Single cycle olfactory conditioning forms two different odour associations of opposite valence

We examined whether flies simultaneously form associations between the CS+ odour and electrical shocks and between the CS− odour and lack/cessation of shocks after single cycle aversive olfactory conditioning. Normally, conditioned flies are tested by having them choose between CS+ and CS− odours in a T maze. We modified this testing procedure by having one set of trained flies choose between the CS+ odour and air, and having another set choose between the CS− odour and air (Figure 1a). Control flies that had been mock trained by exposing them to the odours in the absence electric shocks showed a slight avoidance to these odours (Figure 1b mock and Figure 1c mock), since we use odour concentrations that are slightly aversive in our experiments to verify that flies are able to detect these odours. Conditioned flies, on the other hand, showed significant changes in behaviour towards both the CS+ and

CS− odours (Figure 1b,c, conditioned). They showed an increased avoidance to the CS+ odour and a loss of avoidance towards the CS− odour (Figure 1c, conditioned). This indicates that the CS− odour does not behave as a passive control odour during olfactory conditioning. Instead, flies show an active behavioural change towards this odour, suggesting that it becomes associated with either the cessation or lack of an aversive stimulus. To characterize avoidance memory and approach memory further, we defined avoidance memory as a significant change in behaviour to the CS+ odour when comparing conditioned to mock-conditioned flies (Figure 1b, red arrow). Likewise, we defined approach memory as a significant change in behaviour towards the CS− odour (Figure 1c, blue arrow). To quantify avoidance memory, we subtracted CS+/air scores of mock trained flies from those of conditioned flies (Figure 1b, red column), and to quantify approach memory, we

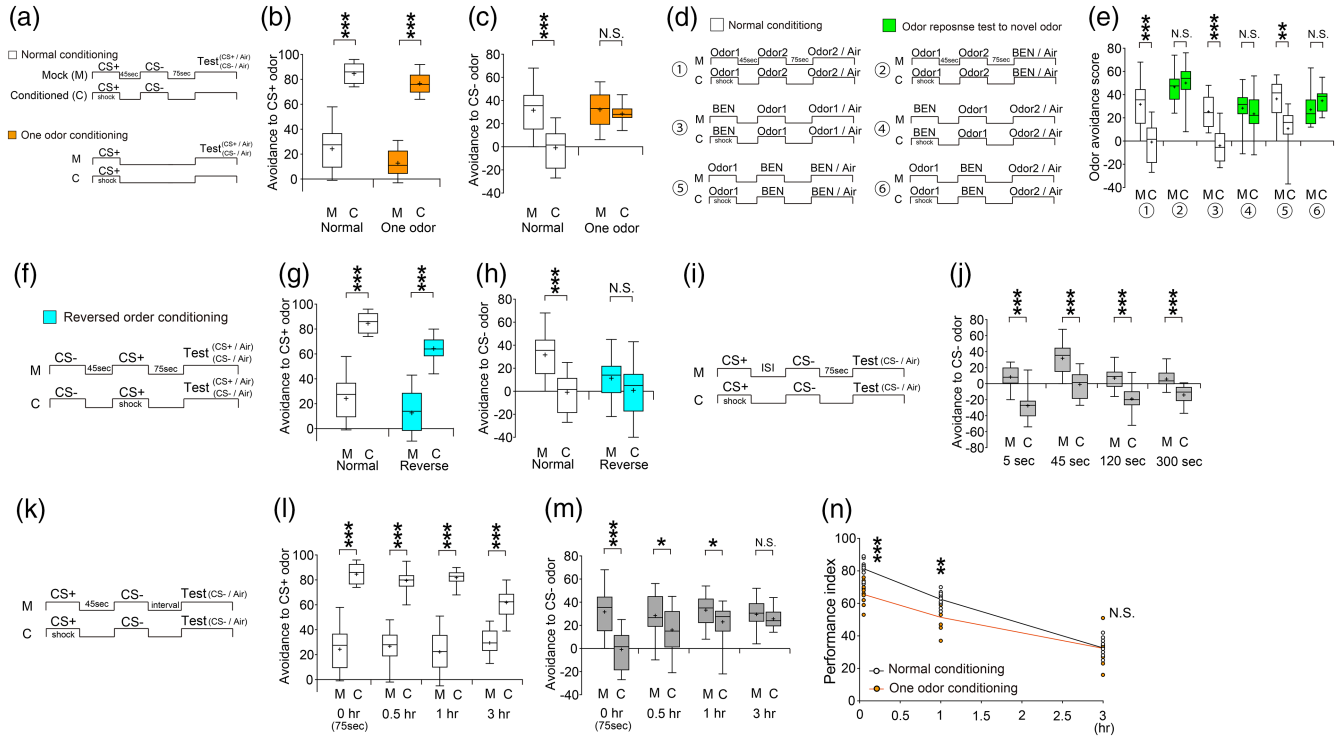

FIGURE 2 Formation and retention of olfactory avoidance memory and approach memory. (a) Schematics show conditioning procedures. Also, see Methods. (b) Odour avoidance scores for the CS+ odour after normal conditioning (white box plots) and after one-odour conditioning (orange box plots). $N = 16$ for normal conditioning, and $N = 14$ for one-odour conditioning. (c) Odour avoidance scores for the CS− odour in wild-type flies. $N = 16$ and $N = 15$ for normal conditioning and for one-odour conditioning, respectively. (d) Schematics show conditioning procedures for odour discrimination test. (e) Odour avoidance scores after normal conditioning (white box plots) and after odour discrimination test (green box plots). $N = 16$ for normal conditioning, and $N = 14$ for single odour conditioning. (f) Schematics show conditioning procedures for reversed order conditioning. (g) Odour avoidance scores for the CS+ odour after normal conditioning (white box plots) and after reversed order conditioning (blue box plots). $N = 16$ for normal conditioning and for reversed order conditioning. (h) Odour avoidance scores for the CS− odour after normal conditioning and after reversed order conditioning. $N = 16$ and $N = 17$ for normal conditioning and for reversed order conditioning, respectively. (i) Schematics show conditioning procedures for varying inter-stimulus interval (ISI) between CS+ and CS−. (j) Odour avoidance scores for the CS− odour after conditioning (grey box plots). $N = 17$ for 5-s interval, $N = 16$ for 45-s interval, $N = 16$ for 120-s interval and $N = 16$ for 300-s interval. (k) Schematics show conditioning procedures for memory retention. (l) Odour avoidance scores for the CS+ odour after conditioning. $N = 16$ for all time points after normal two-odour conditioning. (m) Odour avoidance scores for the CS− odour after conditioning. $N = 16$ for 75-s memory, $N = 22$ for 30-min memory, $N = 15$ for 1-h memory and $N = 16$ for 3-h memory after normal two-odour conditioning. (n) Memory retention curves obtained by allowing flies to choose between CS+ and CS− odours at different time points after normal conditioning (white circles), and after one odour conditioning (orange circles). $N = 16$ for 3-mi memory, $N = 10$ for 1-h memory and $N = 10$ for 3-h memory after normal two-odour conditioning. $N = 15$ for 3-min memory, $N = 7$ for 1-h memory and $N = 10$ for 3-h memory after single-odour conditioning. The P value between normal conditioning and single-odour conditioning was <.001 for 3-min memory, .005 for 1-h memory and .954 for 3-h memory. *** $P < .001$, ** $P < .01$, * $P < .05$, N.S., not significant. In boxplots, upper whisker and lower whisker represent the minimum and the maximum value of the data set, respectively. '+' represents mean value of odour avoidance score.

subtracted CS−/air scores of mock trained flies from those of conditioned flies (Figure 1c, blue column). In both cases, if the behavioural response to the odour is unchanged by olfactory conditioning, the score is close to zero. We refer to these subtracted scores as conditioned response indices (CRIs).

3.2 | Formation and retention of approach memory after single cycle olfactory conditioning

The change in behaviour towards the CS− after training could be specific to the CS− odour, or it could be a general increase in preference to any odour not associated with electrical shocks. To test which of these possibilities is correct, we trained flies using normal CS+ and CS− conditioning, or by one-odour conditioning where exposure to the CS− was omitted (Figure 2a). Both types of conditioning resulted in the formation of CS+ avoidance memory (Figures 2a and S1a), but only normal conditioning produced CS− approach memory (Figures 2c and S1a). Furthermore, if flies were conditioned normally and then tested using a third novel odour, they showed no approach memory (Figures 2d,e and S1b). In normal olfactory aversive conditioning, flies are first exposed to the CS+ odour paired with US foot-shocks, followed later by exposure to the CS− odour. To verify that this order is important, we examined whether approach memory is formed when flies are exposed to the CS− odour before the CS+ odour and US (reversed order conditioning) (Figure 2f). Although reversed order conditioning formed avoidance memory (Figures 2g and S1c), it produced no significant CS− approach memory (Figures 2h and S1c). Our results indicate that approach memory is formed specifically towards an odour that flies are exposed to after cessation of the aversive US.

Does the formation of approach memory depend on the time interval between cessation of the aversive US and exposure to the CS−? We altered the time interval between US cessation and CS− exposure (interstimulus interval, ISI) from 5 to 300 s (Figure 2i) and found that significant CS− approach memory was formed at all time points (Figures 2j and S1d). This suggests that CS− approach memory may be associated with the absence, rather than the cessation, of the US.

To determine how long CS− approach memory lasts, we tested both avoidance memory and approach memory at various time points after training (Figure 2k). While avoidance memory was retained for over 3 h (Figures 2l and S1e, white circles), approach memory disappeared within 3 h (Figures 2m and S1e, grey circles). Consistent with this result, we found that normal CS+ and CS− conditioning produced memory scores significantly higher than single odour CS+ conditioning at 3-min and 1-h time points, but not at 3-h time points (Figure 2n). These results suggest that both avoidance and approach memories contribute to odour behaviours within the first 3 h after conditioning, while only avoidance memories contribute to behaviours at longer times.

3.3 | D1-like dopamine receptors in the MBs are necessary and sufficient for olfactory avoidance memory

Activity of D1Rs in the MBs is required for olfactory aversive learning (Kim et al., 2007; Qin et al., 2012). Further, Handler and colleagues have shown that the timing between odour and DAN activation can alter the valence of an olfactory memory and that D1Rs in the MBs contribute to this temporal sensitivity (Handler et al., 2019). Based on these results, we hypothesized that D1R activities in the MB may contribute to CS− approach memory. Thus, we examined CS+ avoidance and CS− approach memory in *dumb*^{2} mutants (Kim et al., 2007), which have a mutation in a D1R, and found that *dumb*^{2} mutants were unable to form either type of memory (Figures 3a,b and S2a). We next examined whether D1R expression in the MBs could rescue these memory defects. *dumb*^{2} mutants have a piggyBac transposon in the first intron of the D1R gene (Kim et al., 2007; Thibault et al., 2004), which contains UAS sequences. Thus, crossing *dumb*^{2} mutants to MB GAL4 driver lines will induce D1R expression in the MBs and can be used for D1R rescue experiments. When we crossed *dumb*^{2} with *OK107*, a pan-MB GAL4 driver (Lee et al., 1999), we found that avoidance memory, but not approach memory, was restored in an otherwise *dumb*^{2} background (Figures 3c,d and S2b). Furthermore, knocking down D1R expression in the MBs by crossing D1R RNAi with *OK107* resulted in reduced avoidance memory (Figures 3e and S2c), but not approach memory (Figures 3f and S2c). These results indicate that D1R activities in the MBs are necessary and sufficient for olfactory avoidance memory, but not olfactory approach memory.

3.4 | D1-like dopamine receptor activity in the mushroom bodies and projection neurons contribute to formation of olfactory approach memory

The above results indicate that D1R expression in the MBs is insufficient for olfactory approach memories and

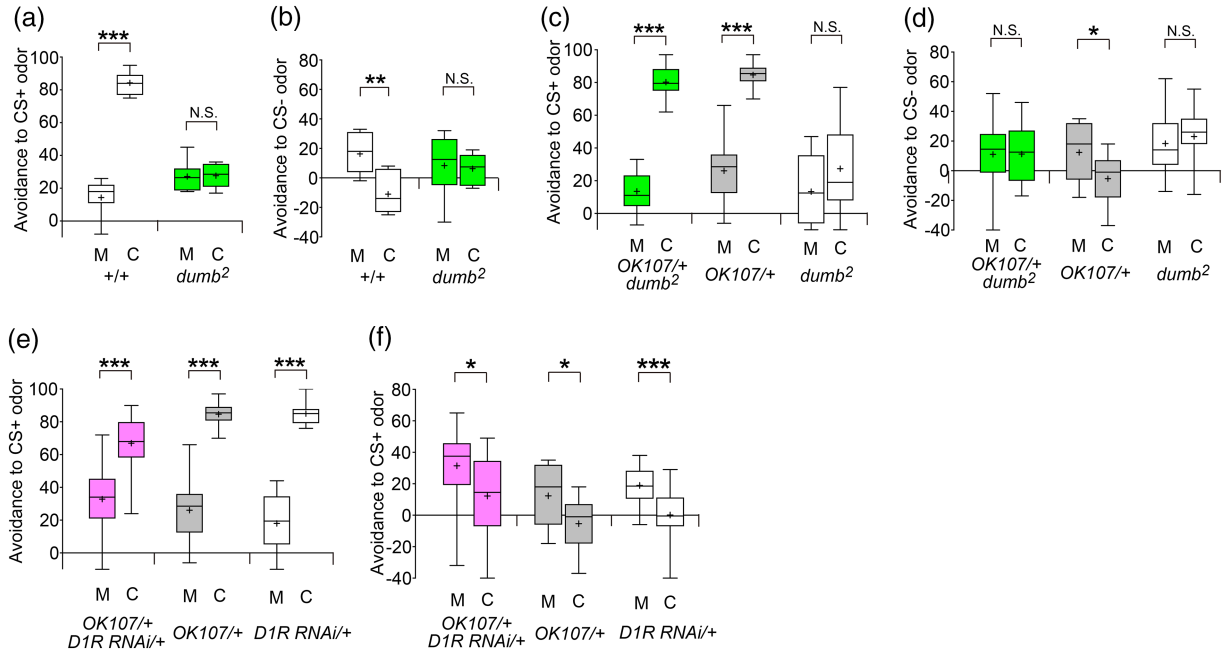

FIGURE 3 D1 dopamine receptor activity in the mushroom bodies (MBs) is not sufficient for olfactory approach memory. (a) Odour avoidance scores for the CS+ odour. $N = 7$ for wild-type flies (white), and $N = 8$ for *dumb*^{2} (green). (b) Odour avoidance scores for the CS− odour. $N = 7$ for wild-type flies, and $N = 8$ for *dumb*^{2}. (c) Odour avoidance scores for the CS+ odour. $N = 18$ for *OK107/+, dumb*^{2}, $N = 18$ for *OK107/+* and $N = 16$ for *dumb*^{2}. (d) Odour avoidance scores for the CS− odour. $N = 20$ for *OK107/+, dumb*^{2}, $N = 13$ for *OK107/+* and $N = 15$ for *dumb*^{2}. (e) Odour avoidance scores for the CS+ odour. $N = 22$ for *OK107/+, D1R RNAi/+*, $N = 18$ for *OK107/+* and $N = 20$ for *D1R RNAi/+*. (f) Odour avoidance scores for the CS− odour. *** $P < .001$, ** $P < .01$, * $P < .05$, N.S., not significant. In boxplots, upper whisker and lower whisker represent the minimum and the maximum value of the data set, respectively. '+' represents mean value of odour avoidance score.

expression in other brain regions is necessary. Previous studies report that the PNs in the ALs retain short-term memory (Liu & Davis, 2006; Tamura et al., 2010; Thum et al., 2007). Therefore, we next examined whether D1Rs may be required in PNs to regulate approach memory. We first conducted immunohistochemistry experiments using anti-D1R antibody (Kong et al., 2010) and found that presynaptic dopaminergic terminals (visualized as GFP-positive signals in *TH/+, UAS-nSyb-GFP/+* flies) and D1R signals co-localized in the ALs (Figure 4a). Next, we knocked down D1R expression in PNs using *GH146-GAL4* (Stocker et al., 1997) and found that knockdowns using this driver abolished approach memory (Figures 4c and S3a) without affecting avoidance memory (Figures 4b and S3a). *GH146-GAL4* drives *GAL4* expression in anterior paired lateral (APL) neurons as well as PNs (Liu & Davis, 2009; Pitman et al., 2011; Wu et al., 2013), while a second driver, *NP225-GAL4* (Tanaka et al., 2004), expresses strongly in the PNs, but not in APL neurons (Zhou et al., 2019). Knocking down D1R expression in PNs using *NP225-GAL4* also extinguished CS− approach memory (Figures 4e and S3b) without affecting avoidance memory (Figures 4d and S3b). Furthermore, we knocked down D1R expression in APL neurons using *APL-GAL4* (Wu et al., 2013) and found that approach memory was intact (Figures 4f,g and S3c). These results suggest that D1R activity in PNs is necessary for the formation of olfactory approach memory.

To determine whether PN expression of D1R could restore approach memory in *dumb*^{2} mutants, we measured approach memory in a *GH146/+; dumb*^{2} background but did not observe restoration (Figures 4i and S3d). Interestingly, expressing D1Rs both in the MBs and in the PNs restored approach memory in a *dumb*^{2} background (Figures 4j and S3e), indicating that D1R activities both in the MBs and in the PNs are sufficient for approach memory.

3.5 | Neuronal outputs from the mushroom bodies are required for recall of avoidance but not approach memory

During aversive olfactory conditioning, neuronal activity in the MBs is necessary for formation of associations and also for recall of associations when flies are re-exposed to training-associated odours. Since conditioning forms both olfactory avoidance memories and approach memories,

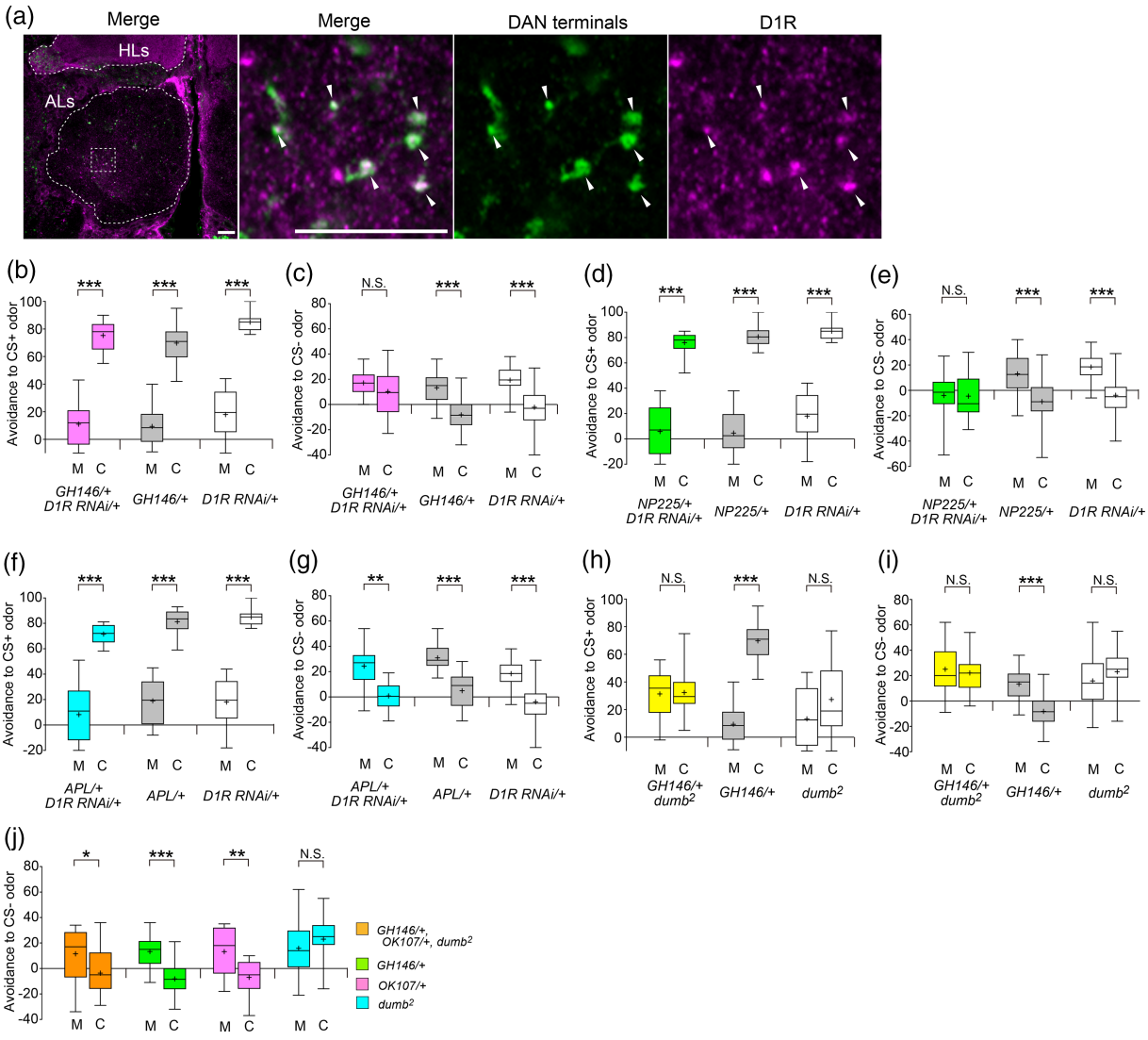

FIGURE 4 D1-like DA receptors (D1Rs) in projection neurons are required for olfactory approach memory. (a) Immunohistochemistry of the ALs to detect D1Rs and pre-synaptic dopaminergic neuron (DAN) terminals. D1Rs were detected using anti-D1R antibody (magenta), and DAN terminals were visualized using *TH/+, UAS-nSyb-GFP/+* lines and anti-GFP antibody (green). HLs: mushroom body (MB) horizontal lobes. ALs: antennal lobes. The second, third and fourth panels from the left are magnified views of the dotted square in the leftmost panel. Arrowheads indicate co-localization of D1R and DAN terminal signals. Scale bar represents 10 μm. (b) Odour avoidance scores for the CS+ odour. $N = 16$ for *GH146/+, D1R RNAi/+* (magenta), $N = 18$ for *GH146/+* (grey) and $N = 20$ for *D1R RNAi/+* (white). (c) Odour avoidance scores for the CS− odour. $N = 24$ for *GH146/+, D1R RNAi/+*, $N = 22$ for *GH146/+* and $N = 24$ for *D1R RNAi/+*. (d) Odour avoidance scores for the CS+ odour. $N = 16$ for *NP225/+, D1R RNAi/+* (green), $N = 16$ for *NP0225/+* (grey) and $N = 20$ for *D1R RNAi/+* (white). (e) Odour avoidance scores for the CS− odour. $N = 18$ for *NP0225/+, D1R RNAi/+*, $N = 18$ for *NP0225/+* and $N = 28$ for *D1R RNAi/+*. (f) Odour avoidance scores for the CS+ odour. $N = 13$ for *APL-GAL4/+, D1R RNAi/+* (blue), $N = 14$ for *APL-GAL4/+* (grey) and $N = 20$ for *D1R RNAi/+* (white). (g) Odour avoidance scores for the CS− odour. $N = 12$ for *APL-GAL4/+, D1R RNAi/+*, $N = 12$ for *APL-GAL4/+* and $N = 28$ for *D1R RNAi/+*. (h) Odour avoidance scores for the CS+ odour. $N = 14$ for *GH146/+, dumb*^{2} (yellow), $N = 18$ for *GH146/+* (grey) and $N = 16$ for *dumb*^{2} (white). (i) Odour avoidance scores for the CS− odour. $N = 21$ for *GH146/+, dumb*^{2}, $N = 22$ for *GH146/+* and $N = 16$ for *dumb*^{2}. (j) Odour avoidance scores for the CS− odour. $N = 17$ for *GH146/+, OK107/+, dumb*^{2} (orange), $N = 22$ for *GH146/+* (green), $N = 15$ for *OK107/+* (magenta) and $N = 16$ for *dumb*^{2} (blue). ***$P < .001$, **$P < .01$, *$P < .05$. N.S., not significant. In boxplots, upper whisker and lower whisker represent the minimum and the maximum value of the data set, respectively. '+' represents mean value of odour avoidance score.

we next examined whether synaptic output from the MBs is required for formation and recall of both types of memory by expressing *shibire*^{ts1} (*shi*^{ts1}), a dominant-negative, temperature-sensitive *dynamin* allele that represses synaptic vesicle recycling at restrictive temperatures (Kitamoto, 2001), in the MBs (*UAS-shi*^{ts1}*/+, OK107/+*).

When we conditioned these flies, at restrictive temperature (30°C) and tested at permissive temperature (20°C) (Figure 5a), both CS+ avoidance and CS− approach memories were fully disrupted (Figures 5b,c and S4a), indicating that synaptic output from the MBs is necessary for formation of both types of memory. However, when we conditioned flies at permissive temperature and tested at restrictive temperature (Figure 5d), avoidance memory was disrupted (Figures 5e and S4b), while approach memory was unaffected (Figures 5f and S4b), indicating that recall of avoidance memories requires MB output, but recall of approach memories does not. These results

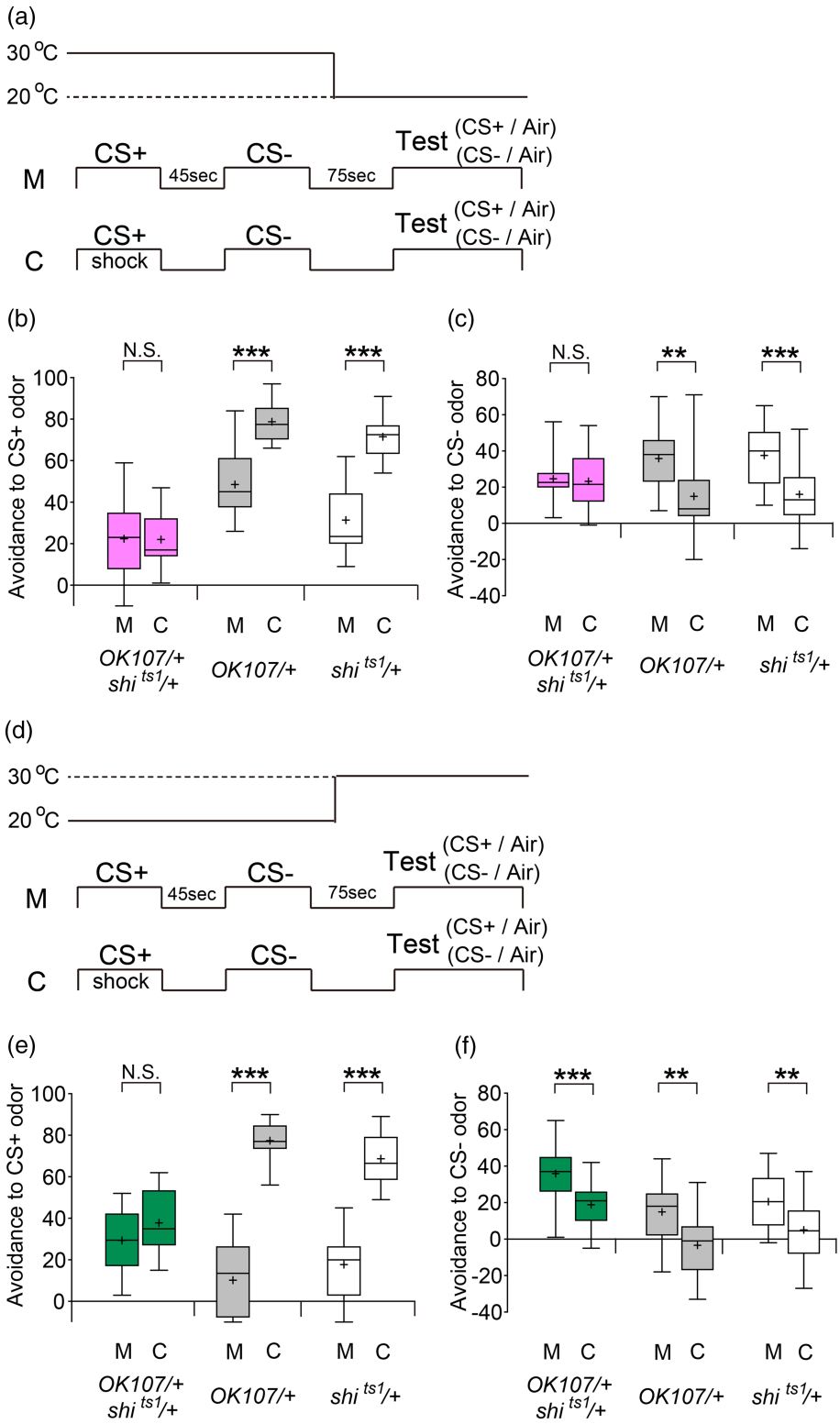

FIGURE 5 Recall of olfactory approach memory does not require neuronal output from the mushroom bodies (MBs). (a) A schematic diagram indicating temperature shifts. MB output is blocked during conditioning in an *OK107/+, UAS-shi^{ts1}/+* line. (b) Odour avoidance scores for the CS+ odour. $N = 16$ for *OK107/+, shi^{ts1}/+* (magenta), $N = 16$ for *OK107/+* (grey) and $N = 16$ for *shi^{ts1}/+* (white). (c) Odour avoidance scores for the CS− odour. $N = 18$ for *OK107/+, shi^{ts1}/+*, $N = 19$ for *OK107/+* and $N = 17$ for *shi^{ts1}/+*. (d) A schematic indicating temperature shifts. MB output is blocked during testing. (e) Odour avoidance scores for the CS+ odour. $N = 14$ for *OK107/+, shi^{ts1}/+* (green), $N = 16$ for *OK107/+* (grey) and $N = 16$ for *shi^{ts1}/+* (white). (f) Odour avoidance scores for the CS− odour. $N = 19$ for *OK107/+, shi^{ts1}/+*, $N = 19$ for *OK107/+* and $N = 24$ for *shi^{ts1}/+*. *** $P < .001$, ** $P < .01$, N.S., not significant. In boxplots, upper whisker and lower whisker represent the minimum and the maximum value of the data set, respectively.

further support the idea that cellular mechanisms involved in olfactory approach memory are at least partially distinct from those involved in currently characterized olfactory avoidance memory processes.

4 | DISCUSSION

Our current study demonstrates that traditional single cycle aversive olfactory conditioning generates two different odour associations that can be studied separately. CS+ odour-associated avoidance memories are relatively stable, lasting for up to 24 h, while CS− odour-associated approach memories are more labile and disappear within 3 h. Traditional behaviour scores, which compare odour preferences between CS+ and CS− odours, seem to be the sum of behavioural changes due to olfactory avoidance and approach. Avoidance memory scores and traditional CS+/CS− scores converge 3 h after conditioning when short-lasting olfactory approach memory is no longer present.

In our studies, we evaluated behavioural changes by subtracting response scores to the CS+ or CS− odours after mock conditioning from scores after normal conditioning. This allowed us to measure behavioural changes to each odour individually normalized to mock conditions. During mock conditioning, we exposed flies to the same odours as normal conditioning in the absence of electrical shocks. However, it has been reported that electrical shocks can alter naïve responses to odours in certain situations (Preat, 1998), raising the possibility that mock conditioning may not be an appropriate normalization control. We do not believe this is the case. In our one-odour conditioning experiments, responses to a non-shocked odour were indistinguishable in either conditioned or mock conditioned situations (Figure 2). Thus, under our conditions, shocks during training do not have any measurable non-associative effects on odour-responses during testing.

A previous study (Preat, 1998) demonstrated that previous electrical shock exposure can reduce subsequent odour avoidance behaviours. However, we do not believe this effect is related to CS− approach memories. Under Preat's conditions, electrical shocks reduced subsequent odour avoidance to several different odours including odours that the flies had likely not been previously exposed. Thus, the Preat effect is a non-associative, general effect. In contrast, CS− approach memories are formed towards a specific odour that becomes associated with the absence of electrical shocks; CS− approach memories are not formed to novel odours. Furthermore, Preat used higher voltages than we did (120 volts), and his effects were greatest when shocks were not paired to any odour. In our experiments, we used lower voltages for shocks (60 volts), and paired our shocks with a CS+ odour, conditions in which we were unable to observe Preat's effects.

While the role of olfactory approach memory in traditional olfactory conditioning has not previously been examined, other types of olfactory approach memories including relief and safety memories have been studied. Is CS− approach memory related to relief or safety memories? Relief memory is formed after multiple trainings where flies are exposed to a CS odour within 45 s of cessation of a US (Jacob & Waddell, 2020; Tanimoto et al., 2004; Yarali et al., 2008). This tight temporal connection between US cessation and CS exposure suggests that relief memory is associated with US cessation (Gerber et al., 2014). In contrast, safety memory is formed after multiple spaced trainings, in which a CS+ odour and electric shock are given simultaneously, followed by exposure to CS− odour in the absence of shocks. Safety memory is formed at many different ISIs between the US and the CS− (Jacob & Waddell, 2020), indicating that safety memory is associated with the absence, rather than the cessation, of the US (Gerber et al., 2014). Our CS− approach memory is formed after a single cycle of the same training trials used to form safety memory, suggesting that CS− memory may be a short-lasting form of safety memory. Consistent with this idea, both CS− memory and safety memory are less sensitive to the ISI between US cessation and CS− presentation compared to relief memory. A major difference between relief memory and both CS− and safety memories is that relief memories are formed in the absence of a CS+. Thus, only a single odour association is made for relief memory, and a short ISI between US cessation and CS presentation seems to help in forming this association. In contrast, during training where CS− and safety memories are generated, two different odour associations are formed. Because of the presence of the CS+, flies first form an odour association between the CS+ odour and electrical shocks. This first association may help when flies learn the second association between the CS− and lack of the US. Thus, formation of the second CS− approach memory may have a less rigid requirement for a short, tight ISI. If this idea is correct, flies may have a rudimentary higher order memory system where the formation of new associations can be aided by previous experiences or memories. Altogether, we believe that relief and safety memories have some similarities, but also distinct differences and that CS− approach memory may be a short-lasting form of safety memory.

Traditionally, olfactory associative memories in *Drosophila* have been thought to form and be stored in the MBs. Supporting this idea, D1Rs are required in the

MBs for aversive memory formation and maintenance (Kim et al., 2007). DA functions as a reinforcement signal that induces plastic changes (Ueno et al., 2017), indicating that plasticity in the MBs is required for olfactory avoidance memories. In contrast, D1Rs in both the MBs and the PNs are required for olfactory approach memory, suggesting that plasticity likely occurs in one or both of these regions for olfactory approach memory. It has been reported that suppression of DA synthesis by inhibiting the activity of tyrosine hydroxylase in PPL1-γ1pedc DAN did not affect the formation of relief-type memory (Konig et al., 2018). In addition, we have shown that RNAi-dependent knockdown of D1Rs in the MBs attenuates CS+ avoidance memory, but not CS− approach memory, suggesting that olfactory approach memories are less sensitive to perturbations in DA signalling in the MBs. Further, recall of olfactory approach memories does not require MB activity. Altogether, these results and ours suggest that these memories may not be stored in the MBs but instead may depend on plasticity in the PNs.

Supporting the idea of memory-related plasticity in the PNs, Yu et al. previously showed that olfactory classical conditioning induces odour-specific changes in PN activity (Yu et al., 2004). In addition, Thum et al. showed that activities of *rutabaga*-type adenylate cyclase in the PNs regulate olfactory appetitive memory (Thum et al., 2007), Tamura et al. showed that NMDA receptor activity in the PNs is required for normal memory acquisition (Tamura et al., 2010) and Ashraf et al. demonstrated that protein synthesis necessary for LTM occurs in specific PNs (Ashraf et al., 2006). Altogether, these results and ours suggest that certain types or aspects of memory are formed and stored in the PNs.

How can olfactory approach memory be encoded in the PNs? The ALs are composed of glomeruli, and each glomerulus consists of a cluster of synapses that receive input from sensory receptor neurons expressing a specific olfactory receptor (Couto et al., 2005; Laissue & Vosshall, 2008). Thus, different odours activate different subsets of glomeruli, and odours can be identified by the combination of glomeruli they activate (Silbering et al., 2008). Shock information is also conveyed to the ALs and strongly activates all glomeruli (Yu et al., 2004). From this, we propose that after strong activation of all glomeruli (after electric shocks), plasticity mechanisms become activated in the PNs from the ALs. Subsequently, if a subset of glomeruli is activated during odour exposure, plasticity is induced such that flies display approach behaviour when these glomeruli are reactivated.

Prior studies have demonstrated that stimulation of *TH-GAL4* positive DANs prior to odour presentation induced subsequent approach behaviour towards the odour (Aso & Rubin, 2016; Handler et al., 2019; Konig et al., 2018). We found that *TH-GAL4* positive DANs project axons to the ALs (Figure 4a) as well as the MBs. Previously, Hartenstein et al. found that neurons in the D1 cluster of DANs project axons to the ALs (Hartenstein et al., 2017), suggesting that these neurons may be required for short-lasting olfactory approach memory.

Our studies indicate that both CS+ avoidance and CS− approach memories require D1R activity in the MBs. Both avoidance and approach memories in D1R-deficient $dumb^2$ flies require expression of D1Rs in the MBs. However, avoidance memory strongly reduced, while approach memory is unaffected, by knocking down D1Rs in the MBs. This indicates that avoidance memory is more sensitive to perturbations in MB D1R expression, and relatively low amounts of D1Rs in the MBs are sufficient for approach memories. Furthermore, MB activity is required during formation, but not recall of olfactory approach memory. Thus, it is possible that the MBs function upstream of the PNs and are necessary to transfer odour, shock or some other type of information to the PNs. Supporting this idea, Hu et al. showed that activation of Keyon cells results in depolarization of AL neurons (Hu et al., 2010), suggesting the existence of feedback connections transmit information from the MBs back to the PNs from the ALs. In addition, a prior study also demonstrates that there are feedback recurrent activity loops between Kenyon cells and PNs within the MB calyx (Christiansen et al., 2011). Recently, it has become evident that functional feedback loops in the MBs are important in olfactory memory formation (Horiuchi, 2019; Ichinose et al., 2015; Lin et al., 2014; Wu et al., 2017). We propose that similar feedback connections between the MBs and the PNs may be important in certain aspects of memory formation including olfactory approach memory.

5 | CONCLUSIONS

Flies learn to associate certain sensory cues with pain, and others with safety from pain. These types of learning require dopaminergic activity and synaptic plasticity. We have shown that olfactory approach memory requires activity of D1-type dopamine receptors in the MBs and the PNs, while olfactory avoidance memory requires activity of D1-type dopamine receptors in the MBs but not in the PNs. Furthermore, neuronal output from the MBs is not required for recall of olfactory approach memory but is required for recall of olfactory avoidance memory. Our work indicates that olfactory avoidance memory and approach memory are formed and recalled using distinct cellular mechanisms in *Drosophila*.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHORS' CONTRIBUTIONS

S.N. designed and performed all experiments and wrote the manuscript with J.H. K.U. contributed to the basic idea of olfactory approach memory in olfactory aversive conditioning and to experimental design. M.S. supervised the work. All authors read and approved the final manuscript.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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