Neuroscience

Single dopaminergic neuron DAN-c1 in *Drosophila* larval brain mediates aversive olfactory learning through D2-like receptors

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Abstract

The intricate relationship between the dopaminergic system and olfactory associative learning in *Drosophila* has been an intense scientific inquiry. Leveraging the formidable genetic tools, we conducted a screening of 57 dopaminergic drivers, leading to the discovery of DAN-c1 driver, uniquely targeting the single dopaminergic neuron (DAN) in each brain hemisphere. While the involvement of excitatory D1-like receptors is well-established, the role of D2-like receptors (D2Rs) remains underexplored. Our investigation reveals the expression of D2Rs in both DANs and the mushroom body (MB) of third instar larval brains. Silencing D2Rs in DAN-c1 via microRNA disrupts aversive learning, further supported by optogenetic activation of DAN-c1 during training, affirming the inhibitory role of D2R autoreceptor. Intriguingly, D2R knockdown in the MB impairs both appetitive and aversive learning. These findings elucidate the distinct contributions of D2Rs in diverse brain structures, providing novel insights into the molecular mechanisms governing associative learning in *Drosophila* larvae.

eLife assessment

This study presents a **valuable** finding on the role of dopamine receptor D2R in dopaminergic neurons DAN-c1 and mushroom body neurons (Y201-GAL4 pattern) on aversive and appetitive conditioning. The evidence supporting the claims of the authors is **solid** and promotes the investigation using fly larvae, which have interesting advantages in the time required for obtaining experimental animals and the use of optogenetics. The work will be of interest to researchers studying neuronal control of behaviour and learning and memory in general.

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Introduction

Learning defines a behavioral change that results from acquiring information about the environment, and memory refers to the process by which the information is encoded, stored, and later retrieved. Learning and memory forms the basis for higher brain functions, including cognition and decision making, which shapes our individuality[1]. On the cellular and physiological level, learning and memory is achieved by neuroplastic changes in circuits, including neuronal excitability and synaptic plasticity. Usually distinct types of neurotransmitters, such as dopamine, modulate these changes.

Dopamine (DA) plays an important role in many mammalian brain functions, including motor functions, motivation, reinforcement, addiction, and learning and memory[2–4]. Dopaminergic neurons (DAN) are mainly located in the mesencephalon: DANs in the substantia nigra (SN) are responsible for motor functions, while those in the ventral tegmental area (VTA) are important in reward, addiction, learning and memory[3,5]. Dopamine achieves its functions via two families of G protein-coupled receptors (GPCR): excitatory D1-like and inhibitory D2-like receptors[4]. All D1-like receptors are located post-synaptically; in contrast, D2-like receptors both function post-synaptically and play an important presynaptic role, regulating dopamine release through negative feedback[4]. All these receptors are important in mammalian associative learning[6,7]. D1-like receptors elevate intracellular cAMP by activating adenylyl cyclase (AC) via $G\alpha_s$, while D2-like receptors repress cAMP by inhibiting AC via $G\alpha_{i/o}$. cAMP activates protein kinase A (PKA), leading to the phosphorylation of DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein, 32kDa), ion channels, and CREB (cAMP response element-binding protein). In addition, dopamine receptors also activate the PLC-PKC, MAPK, and CaMKII pathways[2–4,8–10].

Although mammalian studies reveal mechanisms more relevant to human beings, the complexity of the nervous system impedes our understanding about the basic or more universal principles of learning and memory applicable generally to all nervous systems[11]. With a simple central nervous system (CNS) and powerful genetic tools, the fruit fly *Drosophila melanogaster* has become a popular model organism in learning and memory research[12,13]. With conserved genes in dopamine metabolism and signaling[14], as well as fundamental similarities in the olfactory circuitry compared to mammals[15,16], *Drosophila* can perform olfactory associative learning in both larvae[17–19] and adults[20–24]. Olfactory associative learning is a type of classical conditioning in which flies are trained under positive or negative reinforcement paired with an odorant. Different from the naïve reaction to the odorant, flies approach the odorant after being trained with rewards (e.g., sucrose; appetitive)[21], but avoid the odorant when trained with punishments (e.g., electric shock, bitter taste chemicals; aversive)[20]. Several genes related to the cAMP-PKA signaling pathway, including *dunce* (*dnc*)[25] and *rutabaga* (*rut*)[21], are expressed in the mushroom body (the center for *Drosophila* learning and memory)[26,27]. Mutations of these genes lead to learning deficiencies, indicating their roles in olfactory learning[21,25].

Drosophila larvae offer several advantages for studying olfactory learning compared to adults. Notably, compared to neural circuits underlying olfactory learning, their simpler neural circuitry[28], characterized by fewer olfactory receptor neurons (ORNs)[29,30], projection neurons (PNs) [31], mushroom body neurons (MBNs)[32], and dopaminergic neurons (DANs)[33,34], facilitates the elucidation of underlying mechanisms. Additionally, larvae exhibit simpler behavioral patterns, facilitating experimental manipulations and observations. Furthermore, their translucent cuticles enable convenient application of techniques such as optogenetics[35], further enhancing the experimental versatility of larval studies.

Like in mammalian brains, dopamine achieves its functions via four dopamine receptors in flies, two D1-like receptors dDA1[36] (or Dop1R1) and DAMB[37] (or Dop1R2), one D2-like receptor D2R[38] (or Dop2R), and one non-canonical receptor DopEcR[14,39]. dDA1 is mainly found in the mushroom body[40,41] and is necessary for appetitive and aversive olfactory learning in larvae and adults[41,42]. D2R in the mushroom body is necessary for anesthesia-resistant memory[43]. In addition, D2R in GABAergic anterior paired lateral (APL) neurons is known to secure aversive conditioning in adult flies[44]. Although D2R expression has been reported in the ventral nerve cord[45], neither its expression in larval brains, nor its functions in larval olfactory learning have been investigated.

By using a GFP-tagged D2R strain, we detected the expression pattern of D2R in the third- instar larval brain, specifically in dopaminergic and mushroom body neurons. Knockdown of D2Rs in DAN-c1 impaired aversive learning, while knockdown of D2R in mushroom body neurons led to deficits in both aversive and appetitive learning. These results revealed that D2Rs in distinct brain structures mediate different learning tasks. The newly discovered roles of D2R in the larval brain provides new insights into the mechanisms underlying larval associative learning.

Results

Distinct dopaminergic neurons innervate different compartments of the mushroom body

The connectome of larval learning has been investigated in both first- and third-instar larvae[28,46]. The mushroom body works as the learning center in *Drosophila*, which is composed by αβ, α'β', and γ neurons in adult brains[47]. In larvae, axons from γ neurons bifurcate and form the vertical and medial lobes, as αβ and α'β' neurons are not mature[33,48,49]. These lobes are divided into 11 compartments (refer to **Figure 1**) based on the coverage of neurites from both mushroom body extrinsic neurons (MBEN) and mushroom body output neurons (MBON)[28]. Around 21 dopaminergic neurons are found in each brain hemisphere, and categorized into 4 clusters: DM1 (dorsomedial), pPAM (primary protocerebral anterior medial, or DM2)[50], DL1 (dorsolateral), and DL2[34]. DL1 neurons project to the vertical lobe[41], while pPAM neurons innervate the medial lobe[50] (refer to **Figure 1a-c**).

In this study, we wanted to functionally identify individual DANs that mediate larval olfactory learning. The first step was to search for DAN-specific driver strains that mark single dopaminergic neurons, which subsequently can be used to target genetic manipulations of corresponding neurons. A total of 56 driver strains identifying dopaminergic neurons were screened in this study (**Table 1**). These strains were chosen based on previous studies, either identifying a single dopaminergic neuron in larvae, or identifying only several in adult brains and indicating the potential of identifying single DAN in larvae. TH-GAL4, a traditional dopaminergic neuronal driver[51], identifies all dopaminergic neurons except those in pPAM (**Figure 1d**, **Figure S1a**). Split-GFP reconstitution across synaptic partners (GRASP) technique was used to investigate the "direct" synaptic connections from DANs to the mushroom body (MB), in which portions of GFP were specifically expressed in corresponding neurons (**Figure S2d**). GRASP results showed neurons under TH-GAL4 formed synapses in the vertical lobe and lower peduncle (white dash lines in **Figure 1d**'), consistent with previous electron microscopy data[46]. We found three DAN driver strains that identify single dopaminergic neurons in the third-instar larval brain hemisphere. DAN driver R76F02-AD;R55C10-DBD identifies a dopaminergic neuron innervating the lower peduncle (LP), which would be DAN-c1 based on previous published nomenclature[28] (**Figure 1f**). MB296B driver identifies the dopaminergic neuron (DAN-d1) projecting to the lateral appendix (LA) (**Figure 1g**), as well as many non-dopaminergic neurons. SS1716 driver identifies one dopaminergic neuron forming synapses in the lower vertical lobe (LVL), indicating it is DAN- g1 (**Figure 1h**).

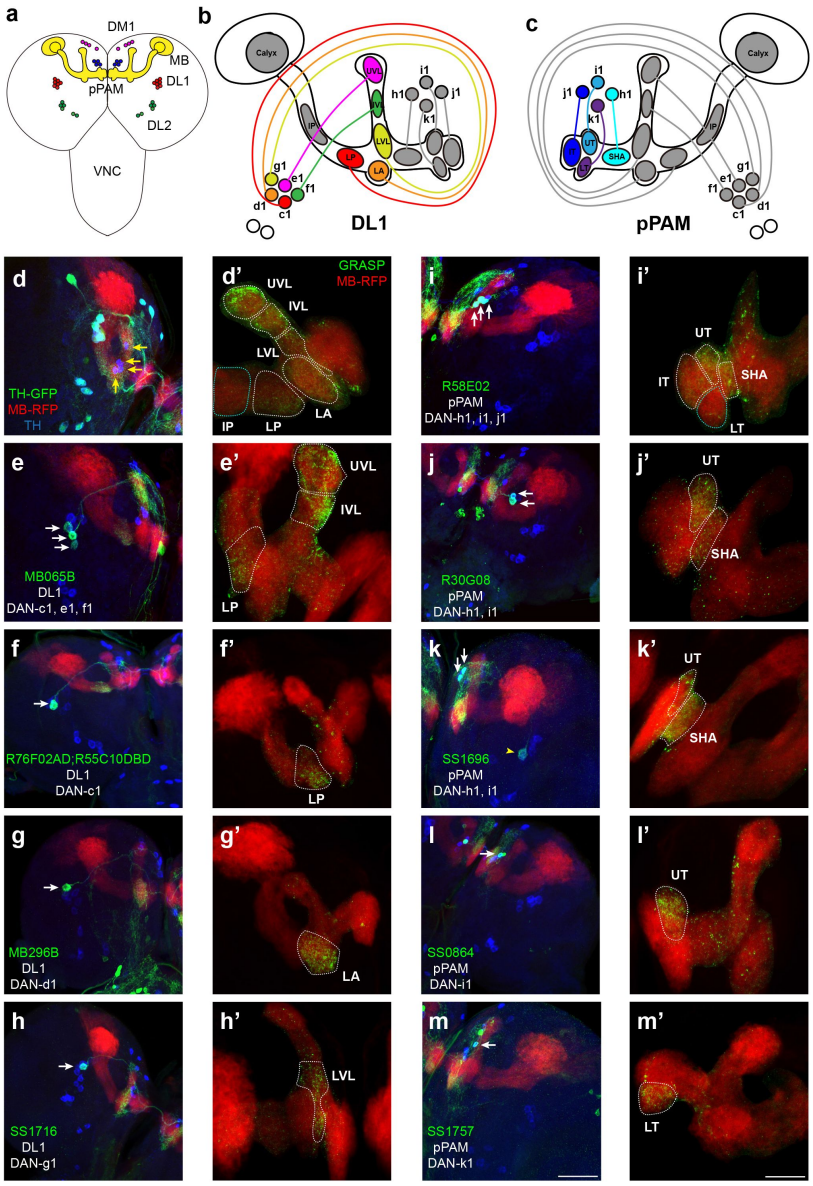


Figure 1.

Identification of driver strains for single dopaminergic neurons in the *Drosophila* larval brain.

(a) A schematic diagram shows the dopaminergic neuron (DAN) clusters and the mushroom body (MB) in the third-instar larval brain. **(b)** & **(c)** Schematic diagrams show the innervation patterns from distinct DANs to different compartments in the MB. All 11 MB compartments are shown, note that there is no synapse formed from dopaminergic neurons to the calyx and intermediate peduncle (IP). DANs in DL1 (b) and in pPAM (c). **(d-m)** Drivers covering DANs in the DL1 cluster (d-h). Drivers covering DANs in the pPAM cluster (i-m). The first column shows drivers covering distinct dopaminergic neurons. Neurons under the drivers are labeled by GFP, the mushroom body (MB) is labeled by RFP, and dopaminergic neurons (DANs) are marked by tyrosine hydroxylase (TH) antibody. The second column (d'-m') shows the GRASP signals from DANs under the driver to the corresponding compartments in the MB. The green channel represents the GRASP signals, and RFP marks the morphology of the MB. In the first column, white arrows mark the DANs under the driver strains. Yellow arrows in (d) show the pPAM neurons not labelled by TH-GAL4 driver strain. The yellow arrowhead in (k) showed the DL1 neuron not innervating the MB. Scale bars: 50 μm for the first column, and 20 μm for the second column. ***Abbreviations:*** DL, dorsolateral; DM, dorsomedial; IP, intermediate peduncle; IT intermediate toe; IVL, intermediate vertical lobe; LA, lateral appendix; LP, lower peduncle; LT, lower toe; LVL, lower vertical lobe; pPAM, primary protocerebral anterior medial; SHA, shaft; UT, upper toe; UVL, upper vertical lobe. *(Note) GFP expression patterns in the entire larval CNS by GAL4 driver strains used in this study can be found in* **Figure S1**.

	Strains	Name in this work	Neurons in third-instar larval brains		Analogs	Stock #	Source/Gift
			DANs	Non-DANs			
1	TH-Gal4	-	All DANs except pPAM	Some weak	All DANs except PAM (Adult)	-	Dr. J. Hirsh's Lab
2	TH-C'	-	1 DL1, 1 DM1, 3DL2a	Rare	PPL1 + PPM3 (Adult)	-	Dr. M. Wu's lab (Liu et al., 2012)
3	TH-C1	-	-	-	PPL1 + PPM3 (Adult)	-	
4	TH-D'	-	3DL1, 2DL2b	Rare	PPL1 + PPM3 (Adult)	-	
5	TH-D1	-	-	-	PPL1 + PPM3 (Adult)	-	
6	TH-D4	-	-	-	PPL1 + PPM3 (Adult)	-	
7	TH-F1	-	3DL1	Rare	PPL1 + PPM3 (Adult)	-	
8	Th-F2	-	3 DM1, 2-4 DL1, 2DL2a	-	PPL1 + PPM3 (Adult)	-	
9	Th-F3	-	3 DM1b, 1 DL1	-	PPL1 + PPM3 (Adult)	-	
10	TH-G1	-	2-3 DM1, 2-4 DL1, 2 DL2b	-	PPL1 + PPM3 (Adult)	-	
11	R30G08	-	h1, i1, weak k1	-	2 pPAM (L3)	48101	BDSC (Rohwedder et al., 2016)
12	R58E02	-	h1, i1, j1	1	3 pPAM (L3)	41347	
13	R64H06	-	h1, i1, j1, k1	Lots	4 pPAM (L3)	49608	
14	MB315C	-	1-3 pPAM (h1)	Lots	PAM-γ5 (Adult)	-	Janelia Farm (Aso et al., 2014)
15	MB109B	-	-	-	PAM-β'2a (Adult)	-	

Table 1.

Driver strains screened for dopaminergic neurons in the third-instar larval brain.

Strains are listed with their published names and names used in this work. The numbers of dopaminergic and non-dopaminergic neurons in the third-instar larval brain are described. The identities of dopaminergic neurons from distinct clusters are also listed, especially for those in DL1 and pPAM. The analogs column lists the labelled neurons in previous publications. Source/Gift column shows the original papers in which these strains were described, as well as the laboratories these strains were obtained from. Several, 2-5 neurons; some, 6-10 neurons; lots, >10 neurons.

16	MB301B	-	-	-	PAM-β2β'2a (Adult)	-	
17	MB056B	-	-	-	PAM-β'2p (Adult)	-	
18	MB032B	-	-	-	PAM-β'2m (Adult)	-	
19	MB312B	-	-	-	PAM-γ4/<γ1γ2 (Adult)	-	
20	MB194B	-	-	-	PAM-β1ped (Adult)	-	
21	MB063B	-	-	-	PAM-β1 (Adult)	-	
22	MB043B	-	h1, i1, j1	Weak, lots of MBN	PAM-α1 (Adult)	-	
23	MB441B	-	-	-	PAM-γ3 (Adult)	-	
24	MB025B	-	-	-	PAM-β'1ap/m (Adult)	-	
25	MB438B	-	-	-	PPL1-γ1pedc (Adult)	-	
26	MB296B	DAN-d1	d1	Some in VNC	PPL1-γ2α'1 (Adult)	-	
27	MB304	-	-	-	PPL1-α'3 (Adult)	-	
28	MB058B	-	-	-	PPL1-α'2α'2 (Adult)	-	
29	MB065B	-	c1, f1, e1	-	PPL1-α3 (Adult)	-	
30	GMR_SS01716	DAN-g1	g1	-	g1 (LVL) (L3)	-	Dr. M. Zlatic's Lab (Saumerber et al., 2018)
31	GMR_SS01696	DAN-h1	h1 + i1	-	h1 (SHA) (L3)	-	
32	GMR_SS00864	DAN-i1	i1	1	i1 (UT) (L3)	-	
33	GMR_SS1757	DAN-k1	k1	1	k1 (LT) (L3)	-	
34	R78E04	-	1 weak DL1	Several	d1 (LA) (L3)	39997	
35	R72B05	-	1 DL1	Lots	MBIN-e2 (L3)	39611	
36	R12C11	-	2 DM1b	Several	MBON-c2 (L3)	76324	
37	R30F04	-	weak d1	Rare	d1 (LA) (L3)	48614	

Table 1. (continued)

Table 1. (continued)

38	R76C04	-	c1, d1	Rare	c1 (LP) (L3)	48621	
39	R37D06	-	-	-	f1 (IVL) (L3)	47921	
40	R14E06	-	2 DL1	Several	MBIN-e2 (L3)	48643	
41	TH-C-AD;R76F05-DBD	-	1 weak DL1, 2 strong DM1	Lots	PPL1 (SMP-PED) (Adult)	-	Dr. M. Wu's Lab (Xie et al., 2018)
42	TH-F-AD;R61H03-DBD		2DL2, 3 DM1	Rare	PPL1 (SMP) (Adult)	-	
43	R76F02-AD;TH-F-DBD	-	c1, e1	Lots weak	PPL1 (MB-MP1) (Adult)	-	
44	TH-F-AD;R76F05-DBD	-	1 weak DL1, 1-2 strong DM1	Several	PPL1 (SMP-γ) (Adult)	-	
45	R76F02-AD;R60F07-DBD	-	c1, f1, e1	Rare	PPL1 (PED, MB-MP1) (Adult)	-	
46	R60F07-AD;R76F05-DBD	-	g1, e1	Strong SOG	PPL1 (SMP-γ) (Adult)	-	
47	DAT-B-AD;R76F05-DBD	-	2-3pPAM, 1 DL1	Rare	PPL1 (hSMP-γ) (Adult)	-	
48	R76F02-AD;R55C10-DBD	DAN-c1	c1	-	PPL1 (MB-MP1) (Adult)	-	
49	R76F02-AD;R76F01-DBD	-	1 weak DL1	Lots	PPL1 (dFB) (Adult)	-	
50	R76F02-AD;R76F05-DBD	-	-	Lots	PPL1 (dFB) (Adult)	-	
51	R76F05-AD;R61H03-DBD	-	1 DL1, 2 pPAM, 3 DM1	1	PPL1 (MB-MV1) (Adult)	-	
52	WED-1	-	-	-	WED (Adult)	-	Dr. M. Wu's Lab (Liu et al., 2017)
53	WED-2	-	-	-	WED (Adult)	-	
54	TH-FLP/UAS-FRT>>FRT-CD8::GFP;R39G05	-	-	-	PPL1 (bSMP-γ) (Adult)	50063	
55	TH-FLP/UAS-FRT>>FRT-CD8::GFP;R17C11	-	-	-	PPL1 (V1 & MP1) (Adult)	48763	
56	TH-FLP/UAS-FRT>>FRT-CD8::GFP;R39C07	-	2 DL1	-	PPL1 (MV1 & MP1) (Adult)	50039	
57	TH-FLP/UAS-FRT>>FRT-CD8::GFP;R67E08	-	-	-	PPL1 (V1) (Adult)	39445	

In pPAM, R58E02 driver identifies DAN-h1, i1, and j1, innervating the shaft (SHA), intermediate toe (IT) and upper toe (UT) (**Figure 1i**), and R30G08 driver identifies DAN-h1 and i1 (**Figure 1j**). As described in a previous report[28], SS864 driver identifies DAN-i1, innervating the upper toe (**Figure 1l**), and SS1757 driver identifies DAN-k1 which innervates the lower toe (LT) (**Figure 1m**). In contrast, SS1696 driver identifies not only DAN-h1, but also i1 and one DL1 neuron not innervating the mushroom body (**Figure 1K**).

In summary, our results show that five DL1 and four pPAM DANs innervate nine distinct mushroom body compartments in a one-to-one pattern (**Figure 1b and c**). DL1 neurons innervate the vertical lobe and peduncle, while pPAM neurons project to the medial lobe. The single neuronal driver strains screened can be used to investigate the roles of individually identified DANs in larval olfactory learning.

Dopamine release from DAN-c1 is both necessary and sufficient for larval aversive learning

Dopamine plays an important role during olfactory associative learning in both adults and larvae[52,53]. In adults, dopaminergic neurons in PPL1 regulate aversive learning[54–57], while those in PAM mediate reward signals in appetitive learning[58–60]. In larvae, DL1 neurons innervating the vertical lobe and the peduncle are required for aversive learning[18,41], while those in pPAM projecting to the medial lobe are involved in appetitive learning[50].

In **Figure 1** and **Table 1**, three driver strains identifying single dopaminergic neurons in DL1 were discovered, which could be candidates to investigate their roles in larval aversive learning. The R76F02-AD;R55C10-DBD strain identifies MB-MP1 in the adult brain[61], which is a dopaminergic neuron involved in adult aversive learning[62]. This driver strain identifies one neuron per hemisphere in the third-instar larval brain (**Figure 2a-c**, **Figure S1c**). Using a UAS- DenMark;UAS-sytGFP strain, its dendrites were labeled with RFP and axonal terminals were marked by GFP. Its dendrites were localized in the dorsomedial protocerebrum (dml), and its axonal terminals located in the lower peduncle of the mushroom body (**Figure 2a and d**), with GRASP results supporting the existence of synapses in this compartment (**Figure 2E**). All these characteristics are consistent with the previously published nomenclature[28], indicating that this neuron is DAN-c1 (**Figure 2f**), thus, this strain will now be referred to simply as DAN-c1.

To reveal the role of DAN-c1 in larval olfactory learning, a single odor learning paradigm and thermogenetic tools were applied[17,18,63]. Compared to those trained with distilled water (DW), control larvae exhibited repulsion to the odorant pentyl acetate (PA) after being trained with quinine (QUI) paired with PA, reflecting aversive learning. In contrast, larvae were attracted to PA after being trained with sucrose (SUC) paired with PA, reflecting appetitive learning. The extent of repulsion or attraction was represented with a response index (R.I.) that is compared to the DW group (**Figure 2g**).

To examine the role of DAN-c1 in aversive learning, we used a *Shibire^{ts1}* strain, which encodes a thermosensitive mutant of dynamin blocking neurotransmitter release when the ambient temperature is higher than 30°C by repressing endocytosis and vesicle recycling functions[17]. When trained at 34°C, the complete inactivation of dopamine release from DAN-c1 with *Shibire^{ts1}* impaired aversive learning (**Figure 2h**), while *Shibire^{ts1}* did not affect learning when trained at room temperature (22°C). These data indicated that dopamine release from DAN-c1 is necessary for larval aversive learning to occur.

In the next experiments, a fly carrying temperature-sensitive cation channel, dTRPA1, was used to excite the DAN-c1 neuron because it can be activated at temperatures higher than 30°C[58]. Activation of DAN-c1 with dTRPA1 at 34°C during training induced repulsion to PA in the distilled

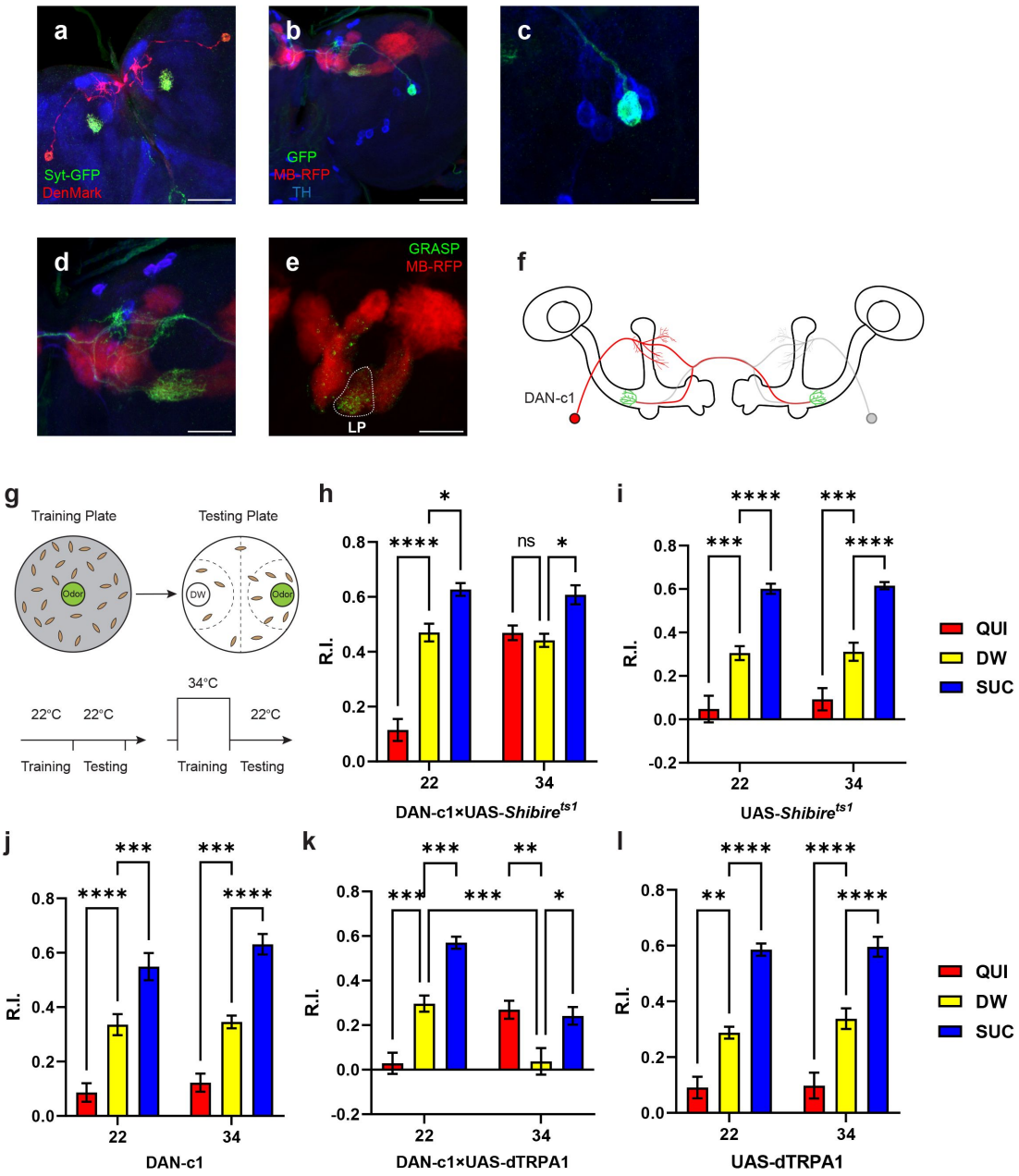


Figure 2.

DAN-c1 is both necessary and sufficient for larval aversive learning.

(a-f) R76F02-AD;R55C10-DBD driver is used as it covers one dopaminergic neuron, DAN-c1, in each brain hemisphere. **(a)** Dendrites and axons of DAN-c1 are labeled by DenMark and sytGFP, correspondingly. **(b-e)** The innervation patterns of DAN-c1. DAN-c1 is labeled with GFP, the MB with RFP, and DANs with TH antibody (blue color). Only one DAN soma is identified (c), axons from DAN-c1 innervate the lower peduncle (LP) of the MB (d), and synapses from DAN-c1 to the LP of the MB are shown by GRASP (e). A schematic diagram (f) shows the innervation patterns of DAN-c1. Modified from Eichler *et al.*[46], and Saumweber *et. al.*[28]. **(g)** A schematic paradigm for larval olfactory learning (top) and two different training paradigms for thermogenetics (bottom). **(h-j)** Blocking dopamine release from DAN-c1 during learning using *shibire*^{ts1} strain under 34°C impairs larval aversive learning. **(k-l)** Activation of DAN-c1 with dTRTPA1 under 34°C induces aversive learning. **QUI,** quinine. **DW,** distill water. **SUC,** sucrose. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S2**. Scale bars: 50 μm (a and b), 20 μm (c, d and e).

water group (**Figure 2k**). These data suggested that DAN-c1 excitation and presumably increased dopamine release is sufficient for larval aversive learning in the absence of gustatory pairing.

Combining the blockade results with *Shibire*^{ts1}, these data revealed that dopamine released from DAN-c1 activation is both necessary and sufficient in larval aversive learning. However, when paired with a gustatory stimulus (QUI or SUC), activation of DAN-c1 during training impairs both aversive and appetitive learning (**Figure 2k**). We suggest that these data indicate a critical role for the amount of DAN-c1 dopamine release in larval associative learning, as dTRPA1 stimulation may result in excessive dopamine release.

The expression pattern of D2R in the third-instar larval brain

Although dopamine D1-like receptors have been proven important for learning[42], the role of D2-like receptors has not been fully investigated. In addition, the expression pattern of D2R in fly brains was not reported. A fly strain expressing GFP-tagged D2R (BDSC#60276) was used to reveal the expression pattern of D2R in the third-instar larval brain (**Figure 3a**). D2Rs were found in dopaminergic neurons and the mushroom body. In dopaminergic neurons (**Figure 3b-g**), D2Rs were found in DM1, pPAM, DL2b, and some DL1 neurons. In the mushroom body, D2Rs were expressed in the soma and lobes, but not in the calyx (**Figure 3h and i**). Even though D2Rs were widely found in vertical lobes, medial lobes, and peduncles, they were not expressed in every mushroom body neuron. A transection of the peduncle region showed the absence of D2Rs in the core area (**Figure S3i**), which is composed of densely packed newly created fibers and lacks Fasciclin II (FAS II)[49].

To inspect whether the pattern of GFP signals indeed reflected the expression of D2R, three D2R enhancer driver strains (R72C04, R72C08, and R72D03-GAL4) were crossed with the GFP-tagged D2R strain. R72C08-GAL4 covered three DM1 dopaminergic neurons (**Figure S3c**), and R72C04-GAL4 labeled one DM1 and two DL2b dopaminergic neurons (**Figure S3d** and e). R72D03-GAL4 identified parts of mushroom body neurons, whose axons spread on the surface of the mushroom body lobes (**Figure S3f**); R72C08-GAL4 also identified a subset of neurons from four MB neuroblasts, with soma in four clusters and a converged bundle of axons (**Figure S3g** and h). These results revealed the expression of D2R in the mushroom body and dopaminergic neurons in the third-instar larval brain.

D2R in DAN-c1 influences larval aversive learning

Our previous work reported that D2R knockdown (UAS-RNAi) in dopaminergic neurons driven by TH-GAL4 impaired larval aversive learning[63]. Using a microRNA strain (UAS-D2R-miR)[61], a similar deficit was observed (**Figure S5c**). To further understand the roles of D2R in aversive learning, its expression in distinct DANs, as well as corresponding learning assays were both investigated. Crossing the GFP-tagged D2R strain with a DAN-c1-mCherry strain demonstrated the expression of D2R in DAN-c1 (**Figure 4a**).

To reduce the expression of D2R in dopaminergic neurons, a microRNA strain UAS-D2R-miR was used when crossing with distinct driver strains. The efficiency of D2R knockdown was confirmed by crossing the GFP-tagged D2R strain with TH-GAL4;UAS-D2R-miR. In these larval brains, GFP signals in DM1 were not detected, while those in pPAM were still intact (**Figure 4b and c**). Quantification showed a significant decrease of GFP signals in the knockdown group compared to the control (**Figure 4d**), indicating reduced transcripts of D2R linked GFP by D2R-microRNA (**Figure S4f**).

To investigate the roles of D2R in distinct dopaminergic neurons during larval associative learning, UAS-D2R-miR strain was crossed with distinct single dopaminergic neuronal drivers. Among them, the knockdown of D2R in DAN-c1 impaired aversive learning with the odorant

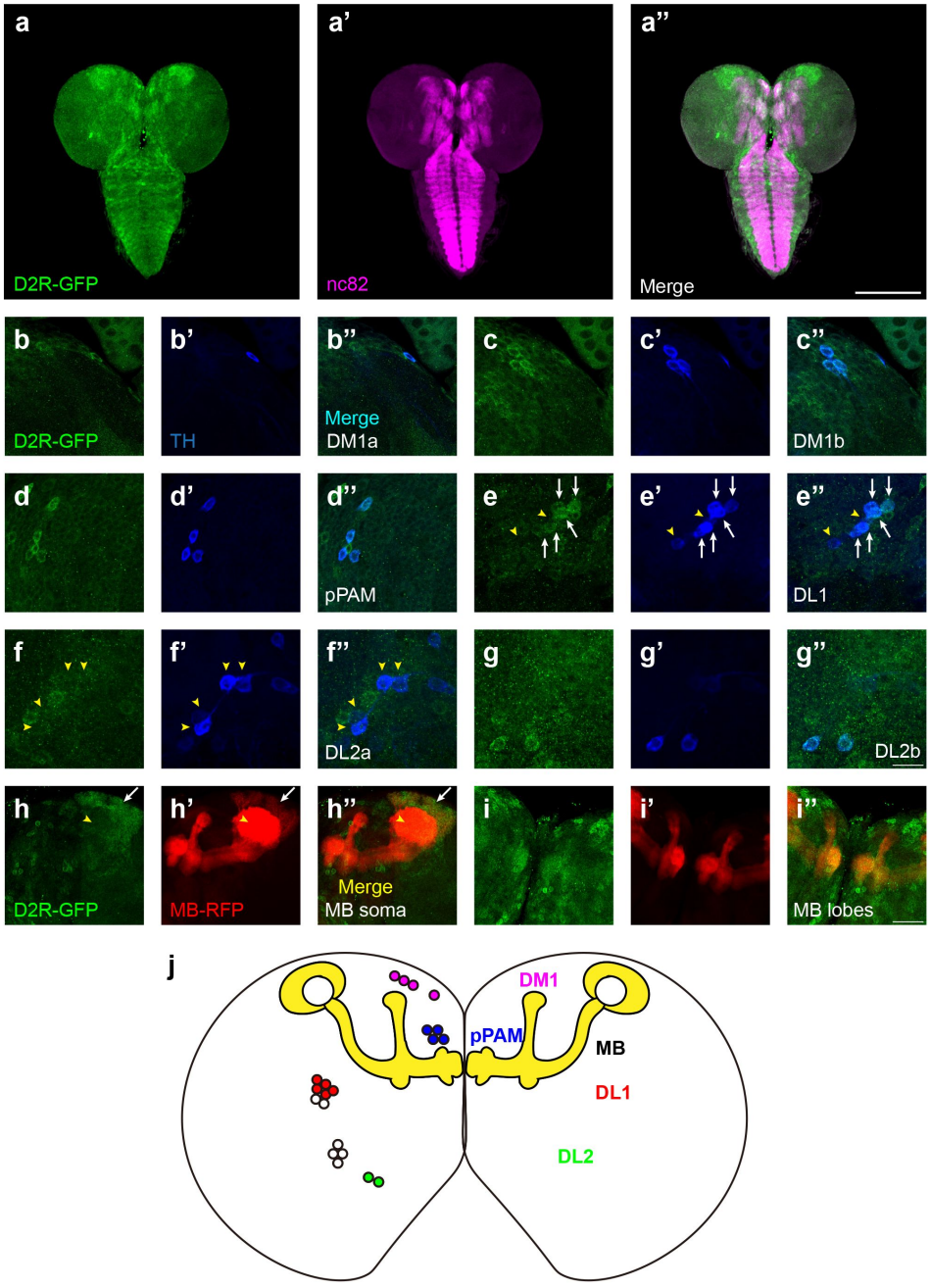


Figure 3.

D2Rs are expressed in dopaminergic neurons and mushroom body in *Drosophila* larval brains.

(a) The expression pattern of D2R in a general view. D2R is shown with tagged GFP (D2R-GFP). Magenta represents neuropils marked by nc82 antibody (a'). **(b-g)** D2Rs (presynaptic) are found in most DANs: DM1a (**b**) and DM1b (**c**), pPAM (**d**), DL1 **(e)**, DL2a **(f)**, and DL2b **(g)** clusters. D2Rs are expressed in parts of DL1 neurons (white arrows in **e**) but not in DL2a neurons (yellow arrow heads in **f**). **(h-i)** D2Rs (postsynaptic) are found in soma of mushroom body (MB) neurons (white arrows in **h**), and MB lobes and peduncles (**i**), but not in calyx (yellow arrow heads in **h**). **(j)** A schematic diagram shows the expression pattern of pre- and postsynaptic D2R in DANs and MB (yellow) in the *Drosophila* larval brain, respectively. Scale bars: 200 μm **(a)**; 50 μm (**b-g**); 20 μm **(h, i)**. ***Abbreviations:*** DL, dorsolateral; DM, dorsomedial; pPAM, primary protocerebral anterior medial.

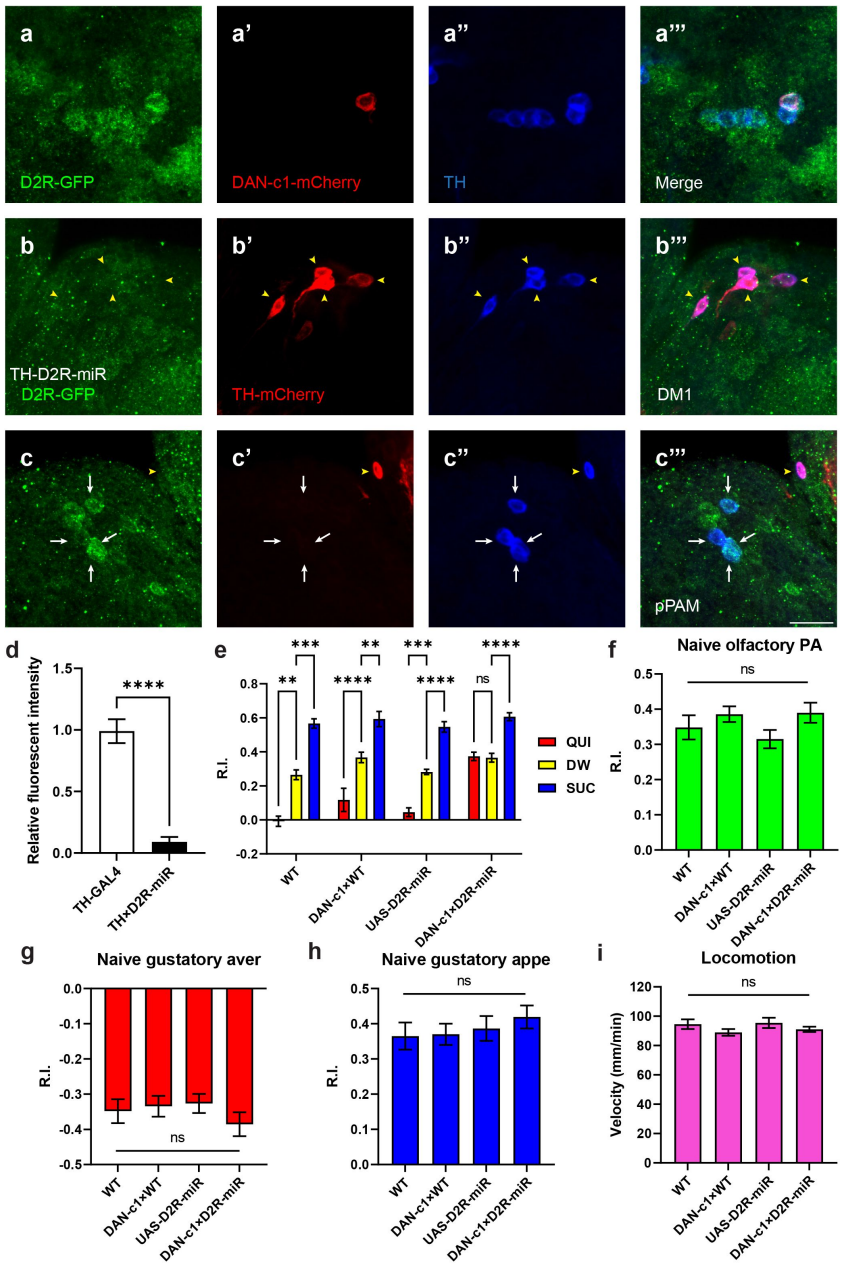


Figure 4.

Presynaptic D2R in DAN-c1 is necessary for larval aversive learning.

(a) D2R is expressed in DAN-c1. The expression pattern of D2R is shown with tagged GFP, DAN-c1 is marked by mCherry, and all DANs are marked with TH antibody (blue). **(b-c)** Knockdown of D2R by D2R-miR reduces fluorescent intensities of D2R-tagged GFP (D2R-GFP). The TH-GAL4 driver is used to express mCherry. The intensity of D2R-tagged GFP is reduced in the DM1 cluster (yellow arrowheads in b), which is still intact in pPAM neurons (white arrows in c). **(d)** D2R-GFP fluorescent intensity is quantified by standardizing the values in DM1 with those in the pPAM. Data are shown as mean ± SEM. For the TH-GAL4 group, N=32 from 8 brains; for the TH×D2R-miR group, N=24 from 6 brains. Unpaired t test, ****p < 0.0001. Scale bar: 20μm. **(e)** Knockdown of D2R in DAN-c1 by D2R-miR impairs larval aversive learning. **QUI,** quinine. **DW,** distill water. **SUC,** sucrose. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, **p < 0.01, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S3**. **(f-i)** D2R knockdown in DAN-c1 does not affect naïve sensory and motor functions. Data are shown as mean ± SEM. One-way ANOVA, Tukey multiple comparisons test. For N numbers, see **Table S3**. *(Note)* **Figures S5** *and* **S6** *show additional information on naïve sensory and motor functions in larvae related to D2R-miR experiments*.

pentyl acetate, while appetitive learning was unaffected (**Figure 4e**). In contrast, although D2R was also found in DAN-d1 and DAN-g1, neither D2R knockdown in DAN-d1 nor in DAN-g1 affected larval olfactory learning (**Figure S4c-e**). As the naïve sensory and motor functions were not affected, this deficiency was caused by impairment in learning abilities (**Figure 4f-i**). Similar learning deficits were observed in the same strain trained with another odorant, propionic acid (**Figure S5a**), as well as in larvae with D2R knockdown using UAS-RNAi (**Figure S5b**). These results demonstrated that D2Rs are expressed in DAN-c1, and they are necessary for larval aversive learning. Presumably the knockdown of presynaptic inhibitory D2R autoreceptors on DAN-c1 will result in increased and excessive dopamine release, which leads to aversive learning deficiency. These results are consistent with the activation studies with dTRPA1 above, in which increased dopamine release during training results in impaired aversive learning (**Figure 2k**).

Over-excitation of DAN-c1 during learning impairs larval aversive learning

To exclude possible chronic effects of D2R knockdown during development, optogenetics was applied at distinct stages of the learning protocol. Channelrhodopsin2 (ChR2) is a blue light activated cation channel from algae, which can be used to activate target neurons[64,65]. Over-excitation of DANs under a TH-GAL4 driver with ChR2 during training impaired aversive learning but left appetitive learning intact (**Figure S7**), which is consistent with D2R knockdown results. To investigate the mechanisms with a better temporospatial resolution, ChR2 was expressed in DAN-c1, and blue light was applied at distinct stages of the learning protocol (**Figure 5a**). Optogenetic activation of DAN-c1 during training impaired aversive learning, not appetitive learning (**Figure 5b-d**). This result is consistent with the effect of D2R knockdown in DAN-c1, indicating that increased, excessive dopamine release during training leads to impaired aversive learning.

D2R in mushroom body mediates larval learning through inhibition

We have shown that D2R in DAN-c1 plays a critical role in larval aversive learning. Since D2Rs are also expressed in soma and axons in most mushroom body neurons (**Figure 3h and i**), we examined the role of D2R in MB neurons, the center for learning in *Drosophila*. Knockdown of these D2Rs by D2R-miR impaired both appetitive and aversive learning (**Figure 6a**). Similarly, optogenetic activation of mushroom body neurons during training led to deficiencies in both appetitive and aversive learning (**Figure 6b and d**). These deficiencies were not observed in larvae with activation during the resting stage. As D2Rs are inhibitory receptors, and optogenetic activation leads to greater neuronal excitation like what may occur with knockdown of D2Rs, these data show that the inhibitory effect of D2Rs in mushroom body neurons is necessary for larval olfactory associative learning to occur.

Discussion

The dopaminergic system plays an important role in *Drosophila* olfactory associative learning, but the roles of D2R in this process have not been fully explored. In this study, we systematically investigated the expression pattern of D2R in the third-instar larval brain as well as its role in larval aversive and appetitive learning. One driver strain singly identifying the DAN-c1 neuron was discovered and learning assays with thermogenetic tools ($Shibire^{ts1}$, dTRPA1) determined that the blockade of dopamine release from DAN-c1 impeded aversive learning, while its activation during training led to repulsion toward the odor in the absence of unconditioned stimulus (i.e., QUI). These results revealed that DAN-c1 activation (i.e., presumably leading to the release of

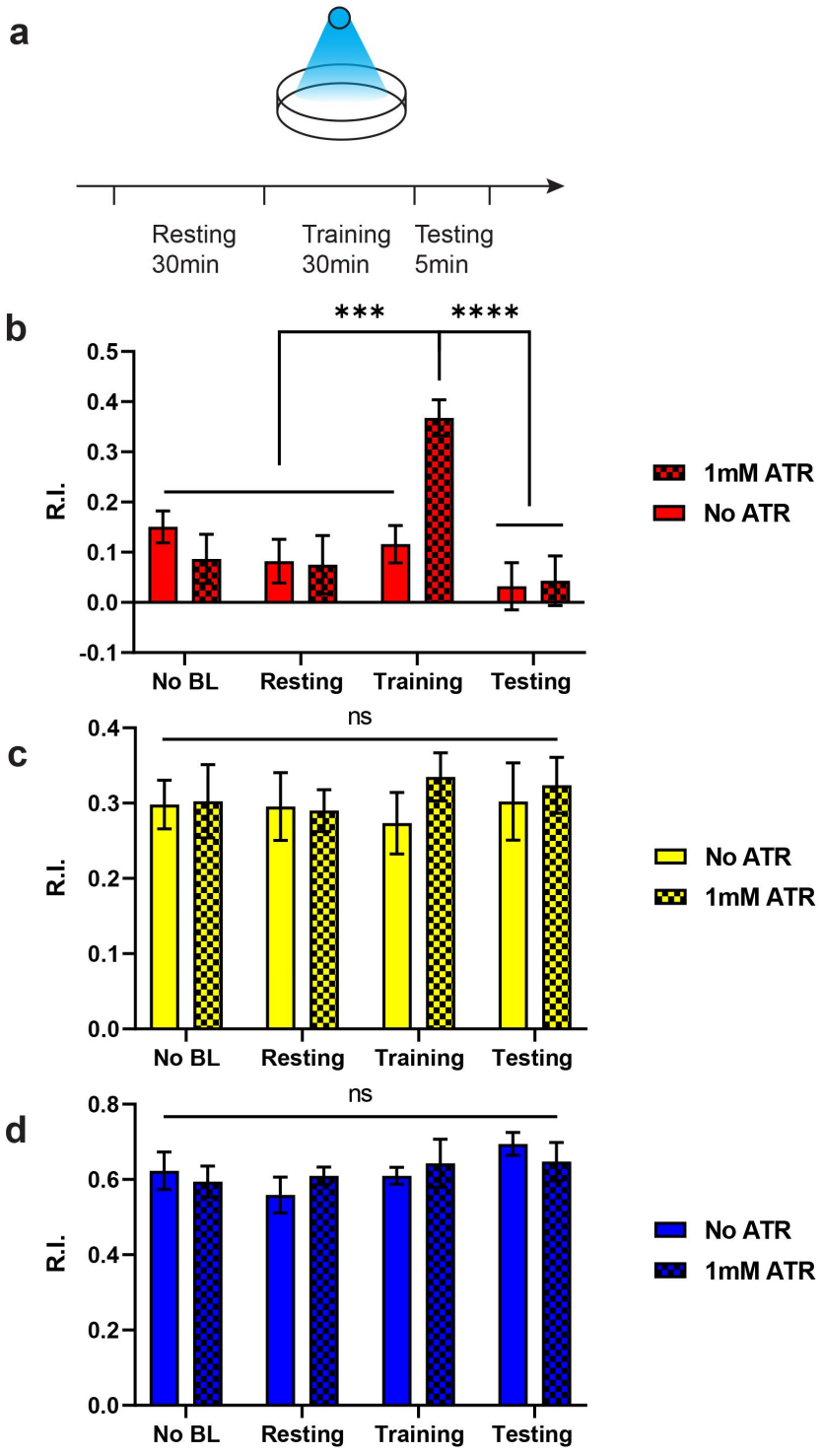


Figure 5.

Over-excitation of DAN-c1 impairs larval aversive learning.

(a) A schematic diagram of optogenetic manipulations of neuronal excitability in DAN-c1 during distinct stages in learning. **(b-d)** Activation of DAN-c1 during training impairs larval aversive learning (**b**), while keeps appetitive learning intact (**d**). Unconditioned stimuli used were quinine (b) and sucrose (d). No learning behaviors are observed in the control distilled water (DW) groups (**c**). Third-instar larvae with ChR2 expression in DAN-c1 are used. *ATR,* all-trans-retinal. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S4**.

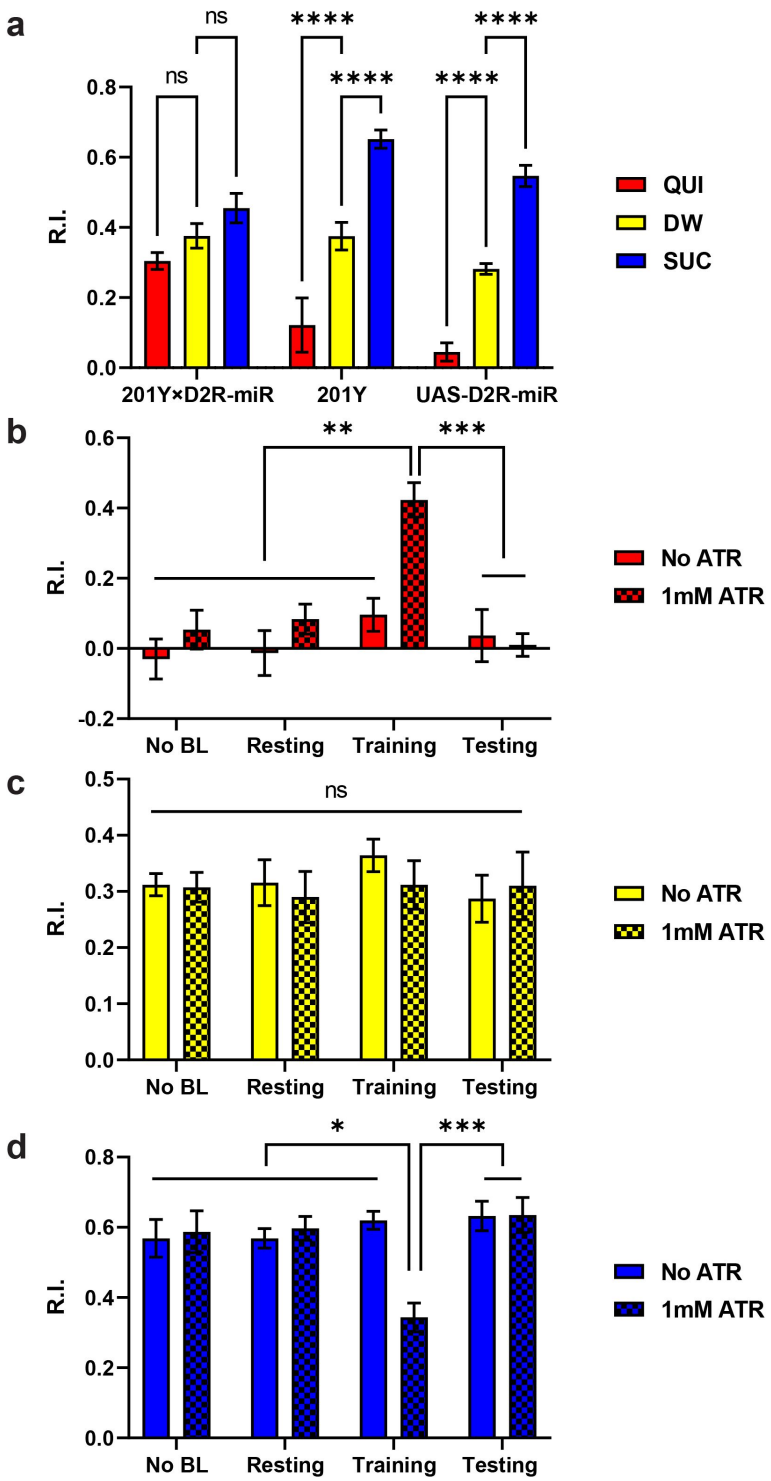


Figure 6.

D2R in mushroom body is necessary for both aversive and appetitive learning.

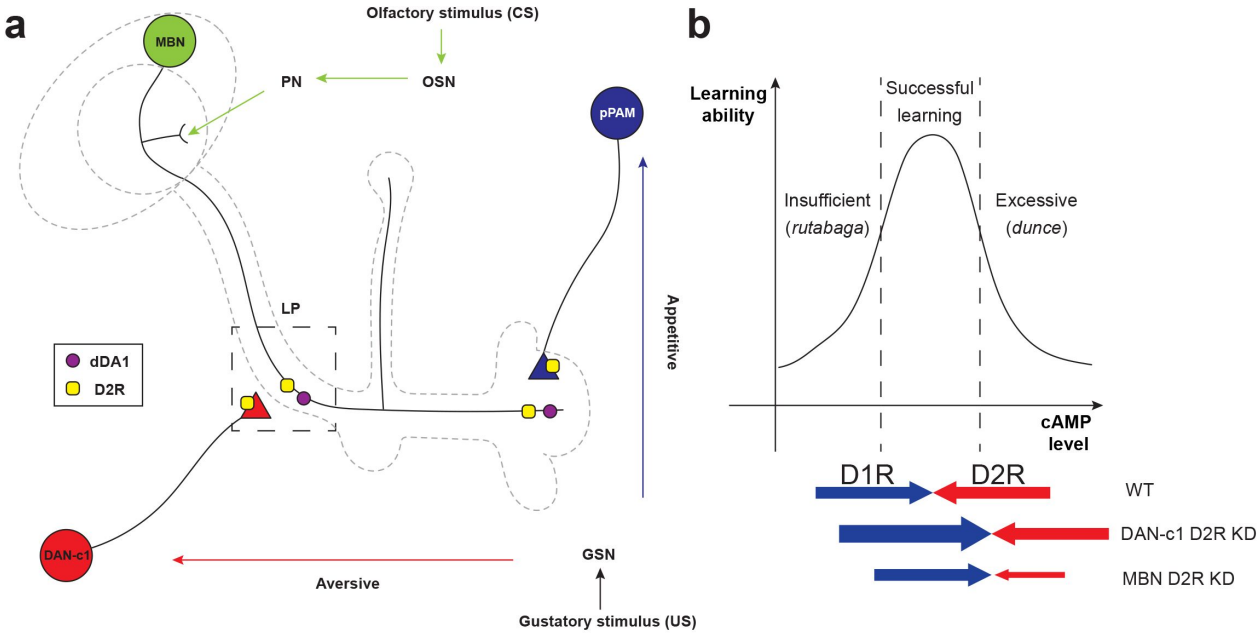
(a) Knockdown D2R in mushroom body neurons (MBNs) impairs larval aversive and appetitive learning. **(b-d)** Activation of MBNs during training impairs both larval aversive and appetitive learning. Unconditioned stimuli used were quinine (b) and sucrose (d). No learning behaviors are observed in the control distilled water (DW) groups (**c**). Third-instar larvae with ChR2 expression in MBNs (201Y-GAL4) are used. *ATR,* all-trans-retinal. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S3** and **S4**.

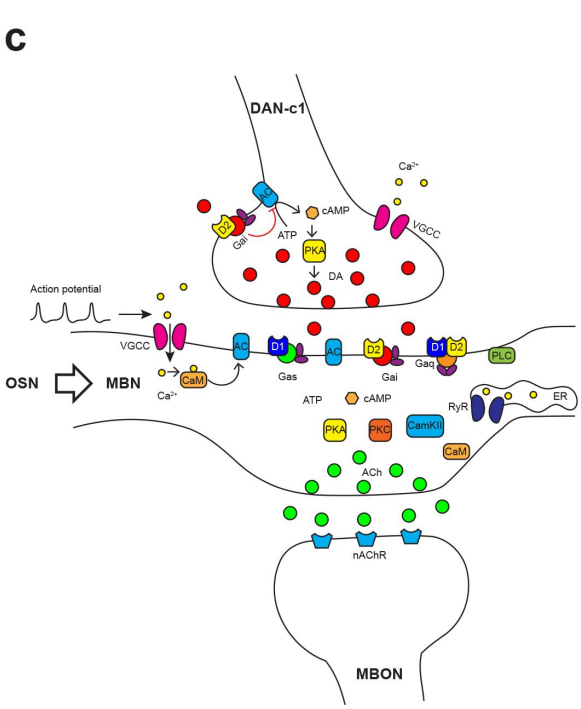
synaptic dopamine) is both necessary and sufficient for larval aversive learning to occur. Subsequently, the expression pattern of D2R was explored by using a GFP-tagged D2R strain, including distinct dopaminergic and mushroom body neurons.

D2Rs were expressed in DAN-c1, and the knockdown of these receptors resulted in aversive learning deficiency. These data suggest that presynaptic D2Rs in a single dopaminergic neuron, DAN-c1, regulate dopamine release during excitation whereas knockdown of these same receptors leads to excessive dopamine release, causing deficits in aversive learning to occur. Furthermore, the activation of DAN-c1 with optogenetic tools during training, resulting in excessive dopamine release, impaired aversive learning, as well. Finally, it was demonstrated that either the knockdown of postsynaptic D2R or activation of mushroom body neurons led to learning deficits. These data demonstrate that D2Rs in distinct brain locations are critically involved in associative learning.

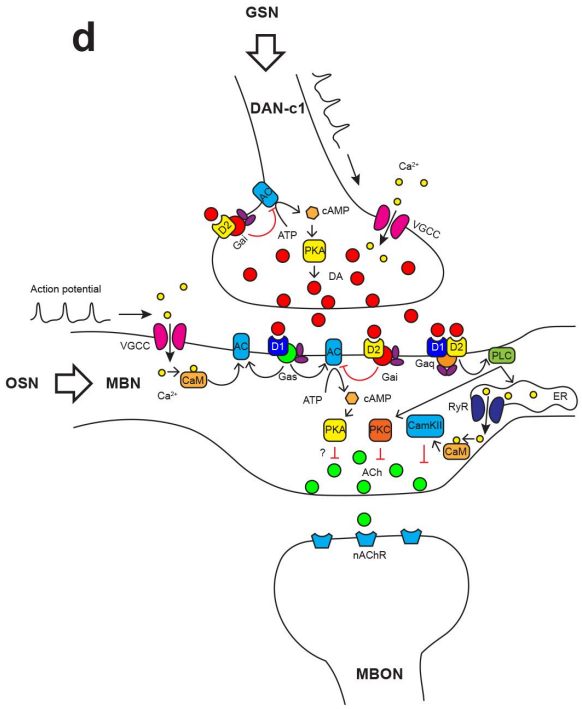
Insights into the neuronal circuits underlying larval olfactory associative learning

Mushroom body and dopaminergic neurons play important roles in *Drosophila* associative learning[66–69]. In larvae, olfactory information (odors, Conditioned Stimulus, CS) is received by olfactory sensory neurons (OSN), then transmitted to the mushroom body via projection neurons (PN)[70]. The mushroom body (MB) is the learning center of *Drosophila* and composed of Kenyon cells (KC, or mushroom body neurons, MBN)[71–73]. Their dendrites form the calyx, receiving olfactory information from projection neurons. The axons converge into peduncles, then branch into the vertical and medial lobes. Distinct gustatory cues (taste, Unconditioned Stimuli, US) are sensed by gustatory sensory neurons (GSN) and transferred to dopaminergic neurons in different clusters (**Figure 7A**). DAN-c1 in the DL1 cluster mediates aversive cues, projects to the lower peduncle (LP) in the mushroom body. The plasticity of synapses from MBNs to MB output neurons (MBON) is negatively modulated by dopamine[67,74,75]. The MBN-MBON synapses in the vertical lobe and peduncle are responsible for attraction, while those in the medial lobe are for repulsion[67,75].





Naive



Aversive learning

Figure 7.

Roles of D2Rs in dopaminergic and mushroom body neurons during larval olfactory learning.

(a) A schematic diagram shows the roles of D2R in dopaminergic neurons (DAN) and mushroom body neurons (MBN) in larval olfactory associative learning. During learning, olfactory stimuli (conditioned stimulus, CS) are received by olfactory sensory neurons (OSN) and transmitted to MBNs (green) via projection neurons (PN). Distinct gustatory stimuli (unconditioned stimulus, US) are received by gustatory sensory neurons (GSN) and transferred to different dopaminergic neurons. Aversive stimuli are sent to DAN-c1 (red) in the DL1 cluster which connects the lower peduncle compartment (LP, square in dash line), while appetitive stimuli are received by pPAM neurons (blue) innervating the medial lobe (ML). D2Rs (yellow square) are expressed in DAN-c1 and pPAM as autoreceptors, regulating dopamine release. Both D2R and dDA1 (magenta circle) are expressed in the MBNs. **(b)** A hypothetical curve showing the relationship between learning ability and cAMP level in the mushroom body. Insufficient cAMP (*rutabaga mutant*) cannot induce learning, while excessive cAMP (*dunce mutant*) also impairs learning. Only the appropriate level of cAMP regulated by the opposing actions of D1R (dDA1) and D2R leads to successful learning in wild type larvae (WT). Knockdown of D2R in DAN-c1 causes excessive dopamine release, elevating cAMP and resulting in impaired learning. D2R knockdown in MBNs relieves the inhibition effect of D2R, resulting in excessive intracellular cAMP and learning failure. **(c-d)** Potential molecular mechanisms underlying *Drosophila* olfactory learning in the square region shown in (a). **(d)** During aversive learning, olfactory stimuli induce depolarization of MBNs, which activates voltage-gated calcium channels and induces calcium influx. Gustatory stimuli, such as quinine, activates dopaminergic neurons and elevates dopamine (DA) release. D1 receptor (dDA1) activates adenylyl cyclase (AC) and elevates cAMP via $G\alpha_s$, while D2 receptor (D2R) inhibits AC and suppresses cAMP via $G\alpha_{i/o}$. In *Drosophila*, the coincidence detector *rutabaga* (AC) is activated by the existence of both calcium and $G\alpha_s$, converging the olfactory and gustatory stimuli. cAMP activates the PKA signaling pathway, elevating the neuronal excitability. D1 and D2 receptors can also form heteromeric receptors and activate the PLC-PKC and CaMKII signaling pathways via $G\alpha_q$. These pathways inhibit acetylcholine (ACh) release from MBNs to MB output neurons (MBON), which leads to avoidance of the learned odor. ***Abbreviations:*** AC, adenylyl cyclase; ACh, acetylcholine; ATP, adenosine triphosphate; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cAMP, cyclic adenosine monophosphate; CS, conditioned stimulus; DA, dopamine; DAN, dopaminergic neurons; DL, dorsolateral; ER, endoplasmic reticulum; $G\alpha_{i/o}$, $G_{i/o}$ protein α subunit; $G\alpha_q$, G_q protein α subunit; $G\alpha_s$, G_s protein α subunit; GSN, gustatory sensory neurons; LP, lower peduncle; MBN, mushroom body neurons; MBON, mushroom body output neurons; ML, medial lobe; nAChR, nicotinic acetylcholine receptor; OSN, olfactory sensory neurons; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PN, projection neurons; pPAM, primary protocerebral anterior medial; RyR, ryanodine receptor; US, unconditioned stimulus; VGCC, voltage gated calcium channel.

When only the odorant appears, the subset of Kenyon cells representing this odor are depolarized, inducing calcium influx[73]. As a balance exists between compartments across the mushroom body lobes, the response to the odor depends on the naïve olfactory circuits from projection neurons to the lateral horn (LH). In aversive learning, in addition to olfaction induced calcium influx, gustatory stimuli also lead to dopamine release from DAN-c1 and subsequently, activation of $G\alpha_s$ in LP[54]. The co-existence of calcium and $G\alpha_s$ activates a Ca^{2+}-dependent adenylate cyclase (AC), rutabaga (rut)[76–79]. Rutabaga converges information from both olfaction and gustation, working as the coincidence detector of associative learning. Its downstream signaling inhibits attractive MBN-MBON synapses in the LP, breaking the balance between distinct compartments. As a result, after learning, the larvae will exhibit repulsion when exposed to the odor again. In contrast, dopaminergic neurons in the pPAM convey appetitive cues, which project to compartments in the medial lobe. The co-existence of olfactory and appetitive gustatory stimuli leads to inhibition of these repulsive MBN-MBON synapses, inducing attraction.

The conserved role of DAN-c1 in aversive learning throughout *Drosophila* development

Adult *Drosophila* share similar neuronal circuits of learning with larvae[54,66–69]. In adult brains, dopaminergic neurons are classified into 13 clusters, named as PAM (protocerebral anterior medial), PAL (protocerebral anterior lateral), PPM (protocerebral posterior medial), PPL (protocerebral lateral), and PPD (protocerebral posterior dorsal) clusters[32]. DANs in the PAM cluster innervate the medial lobe, while those in PPL1 project to the vertical lobe[69].

R76F02-AD;R55C10-DBD identifies two dopaminergic neurons in adult brains, MB-MP1 in PPL1 and ALT-PLPC in PPL2ab [61]. MB-MP1 is also named PPL1-γ1pedc, innervating both γ1 and the peduncle of the β lobe[69]. Activation of this neuron induced aversive learning[62], and activation of its corresponding MBON-γ1pedc>α/β led to approach[80]. During metamorphosis, dopaminergic neurons in DL1 develop into the PPL1 cluster, DL2a neurons develop into PPL2ab, and those in pPAM will develop into the PAM cluster[81]. This driver strain only identifies DAN-c1 from DL1 in larvae, which innervates the lower peduncle of the mushroom body. Interestingly, previous reports revealed that memory can be transferred from larvae to adults[82], indicating the maintenance of neuronal circuitry architecture during metamorphosis. This evidence supports that DAN-c1 is the corresponding neuron of PPL1-γ1pedc in larvae, which performs similar functions in aversive learning.

Pre- and post-synaptic D2Rs regulate cAMP in the mushroom body during aversive learning

The molecular mechanisms underlying *Drosophila* learning have not been fully determined. In a traditional view, gustatory cues elevate dopamine release, which binds to D1 receptors and then activates $G\alpha_s$. The co-existence of $G\alpha_s$ and calcium elicited by olfactory cues activates rutabaga in axons of MBNs. Rutabaga transforms ATP into cAMP, activating PKA signaling pathway. Mutant flies with either insufficient (*rutabaga*) or excessive cAMP (*dunce*) showed aversive learning deficiency, indicating that the level of cAMP should be kept in an optimal range to achieve aversive learning[21,25] (**Figure 7B**).

Our results have shown that D2Rs in dopaminergic neurons and the mushroom body are important for larval aversive learning, suggesting a "*dual brake*" role in regulating cAMP levels in the MBNs through both pre- and postsynaptic components. On the presynaptic side, D2R in DAN-c1 decreases the release of dopamine under gustatory stimuli, reducing the probability of postsynaptic D1R activation in MBNs. On the postsynaptic side, D2R in MBNs inhibits the coincidence detector rutabaga (AC) both via activation of $G\alpha_{i/o}$ and inhibition of voltage-gated calcium channels[2], indicating postsynaptic D2R functioning as a "*brake of the coincidence detector*". Combining these, "*dual brake*" D2R ultimately regulates the mushroom body cAMP level within a physiologically optimal range during aversive learning. In addition, D2R fine tunes the functional concentration spectrum of dopamine with higher resolutions, as its dopamine affinity is 10- to 100-fold greater than D1 receptors. Overall, D2Rs work in a "dual brake" system both expanding the representation of a dynamic range of gustatory signal intensity with high signal to noise ratio and preventing postsynaptic overexcitation, which increases the reliability of DAN-c1 and MBN circuits for the larval aversive learning (**Figure 7C** & D).

Recent studies showed that the approach/repulsion in learning is achieved via inhibition of the repulsive/attractive representing compartments[67,74,75], which indicates dopamine inhibits acetylcholine release from MBN to MBON[83]. However, the PKA signaling pathway usually elevates neuronal excitability and increases neurotransmitter release[2,3], which is contradictory to the recent findings. In addition to the "*dual brake*" role of D2R, our results suggest

a third role of D2R in aversive learning. D1 and D2 receptors can form heteromeric receptors, whose downstream $G\alpha_q$ activates PKC and CaMKII signaling pathways. The activation of these signaling pathways may reduce acetylcholine release[84] (**Figure 7C** & D).

Explaining the results of thermogenetic and optogenetic experiments

Activation of DAN-c1 with dTRPA1 induced aversive learning, while the repulsion disappeared when DAN-c1 was activated in the quinine group (**Figure 2K**). Our explanation is that quinine stimulation and temperature activation led to over-excitation of DAN-c1, which impaired aversive learning. This is consistent with the learning deficiency in larvae with D2R knockdown in DAN-c1 (**Figure 4E**). Results from optogenetic activation of DAN-c1 during aversive learning also support this (**Figure 5B**). However, in contrast to results with thermo-activation, larvae with optogenetic activation of DAN-c1 showed neither repulsion after being trained with distilled water (**Figure 5C**), nor did they show reduced attraction in the sucrose group (**Figure 5D**). One possible explanation is that the thermo-activation is relatively mild compared to the optogenetic activation. Based on this, thermo-activation of DAN-c1 is still in the physiological range of cAMP under the upper cutoff (**Figure 7B**), resulting in repulsion in the distilled water (DW) group, neutralized attraction in SUC group, and impaired repulsion in QUI group. In contrast, optogenetic activation of DAN-c1 overwhelmed the physiological conditions, leading to failure of repulsion in DW group (**Figure 5C**). This repulsive failure did not affect appetitive learning (**Figure 5D**), and a stronger over-excitation in QUI group induced similar failure (**Figure 5B**).

Distinct dopaminergic neurons may have different roles in larval aversive learning

Previous work reported that aversive olfactory learning was induced through the optogenetic activation of DAN-d1, f1, or g1, but not DAN-c1[85]. This discrepancy can be explained as the optogenetic overexcitation of DAN-c1, similar to our optogenetic or D2R knockdown results. Our learning assay results from larvae with D2R knockdown in DAN-d1 or g1 also supported this: aversive learning was not affected by D2R knockdown (**Figure S4E**). These data indicate D2Rs in DAN-d1 or g1 are not involved in larval aversive learning. Additionally, the DAN-c1 strain used in the previous work (SS02160-split-GAL4) not only labels DAN-c1, but also marks other non-dopaminergic neurons, which may affect the results. Besides, live calcium imaging showed that DAN-d1, f1, and g1 responded to the activation of mechanosensory and nociceptive neurons[85], indicating a functional differentiation from gustatory activated DAN-c1[62,86].

In future studies, the molecular signaling downstream of D2R needs to be explored, as well as the comprehensive neuronal circuit architectures of larval learning. The neuronal circuits underlying learning and memory are complex networks, sharing similarities with the regulatory networks of gene expression. Studies of the mechanisms of learning and memory help us understand the essential principles of the non-linear dynamic characteristics in these complex systems. On one hand, the progress in larval learning provides useful information for helping our understanding about more complex systems, from brains in adult flies to those in mammals. On the other hand, the architecture of larval learning circuits could improve either hardware design or algorithm structures in artificial intelligence, which may bring more powerful tools, such as navigation systems regulating multiple auto-drive vehicles in complex 3-dimentional environments.

In conclusion, we explored the expression pattern of D2R in the third-instar larval brain and investigated their roles during larval olfactory learning. D2Rs were found in DAN-c1, and their knockdown induced deficiency in aversive learning. D2Rs were also expressed in MBNs, knockdown of which impaired both aversive and appetitive learning. This research revealed the important roles of D2Rs in *Drosophila* larval olfactory learning and enriched our understanding regarding the mechanisms underlying the learning process.

Materials and Methods

Fly stocks

All fly strains used in this study are listed in **Table 1** and **Table S1**. Flies were maintained on standard medium, which consists of cornmeal, yeast, dextrose, sucrose and agar in water. Flies were kept in a 12/12-hour light/dark cycle at 25°C. Canton-S genotype (WT) and yw^{1118} were used as wild-type. Strains carrying more than one transgene were constructed by standard genetic crosses with the w^{1118}; CyO/Sco; TM2/TM6 multiple balancer chromosome strain. Strain UAS-Syb::spGFP1-10, LexAop-CD4::GFP11, LexAop-rCD2::RFP /CyO; MB247-LexA::Up16/TM6B was made by chromosome swapping between UAS-Syb::spGFP1-10, LexAop-CD4::GFP11/CyO (II, second chromosome) and LexAop-rCD2::RFP (II).

The D2R gene is located on the X chromosome, with 6 different alternative splicing products. The GFP-tagged D2R strain is inserted with a GFP gene in the second intron, generating D2R molecules tagged with GFP[87,88]. The D2R-miR strain produces microRNA recognizing the sequence across the third and fourth exons, which is not affected by the GFP insertion (**Figure S4F**).

GRASP

Split-GFP reconstitution across synaptic partners (GRASP) was used to investigate whether neurons formed synapses[89] (**Figure S2d**). Half of split GFP tethered to the presynaptic synaptobrevin (UAS-syb::spGFP1-10) was expressed in one type of neuron using UAS/Gal4 binary system, and the complementary split GFP linked to a membrane protein (LexAop-CD4::spGFP11) was expressed in another category of neuron with LexA/LexAop. If synapses between these neurons exist, the split GFPs would form a complete one and be recognized by a mouse antibody (**Figure S2a-c**).

Immunohistochemistry

All staining processes were performed in 1.5 ml Eppendorf tubes. Late third-instar (96-100h after egg laying) larval brains were dissected in dissection solution (300 mOsmol/L). Brains were fixed in 4% paraformaldehyde (PFA, Electron Microcopy Sciences, Cat. No. 15713) for 1 hour on ice. After three washes (0.1% bovine serum albumin in 10mM PBS, Sigma Life Sciences A9647), brains were incubated in the blocking and permeabilization solution (0.2% Triton X-100 in 10mM PBS with 5% normal goat serum; Triton-X 100, Sigma, T8532; NGS, Sigma-Aldrich, G9023) for 2 hours at room temperature. Incubation with primary antibodies was done overnight at 4°C. After three washes, brains were incubated in the secondary antibodies for two hours. Both the primary and secondary antibodies were diluted in the blocking and permeabilization solution. GFP antibody (Rabbit, Thermo Fisher Scientific, Cat. No. A6455, 1:1000), TH antibody (Mouse, Immunostar, Cat. No. 22941, 1:1000), goat anti-rabbit with green fluorescence (Invitrogen, Alexa Fluor 488 conjugate, Cat. No. A-11035, 1:1000), and goat anti-mouse IgG with far-red fluorescence (Alexa Fluor 633 conjugate, Cat. No. 21052, 1:500) secondary antibodies were used. After three washes, brains were transferred and mounted in the Fluoro-Gel with Tris Buffer (Electron Microcopy Sciences, Cat. No. 17985-10) on a piece of micro cover glasses (Electron Microcopy Sciences, Cat. No. 72200-41). Finally, the samples were covered with another piece of micro cover glasses.

A seven-day staining protocol was used for staining of GFP-tagged D2R or GRASP, in which brains were fixed in 1% paraformaldehyde in Schneider's insect medium (Sigma Life Sciences, Cat.NO. S0146) overnight at 4°C. In the second day, the brains were rinsed and washed twice with PAT3 solution (0.5% Triton X-100 in 10mM PBS with 0.5% BSA), each for 1 hour. Then brains were incubated in the blocking and permeabilization solution (3% NGS in PAT3) for 2 hours at room temperature. Incubation of primary antibodies was done overnight at 4°C. On the third day, brains

were rinsed and washed twice with PBT solution, then incubated in the secondary antibodies for five days. Both the primary and secondary antibodies were diluted in the PBTN solution. In the staining of GFP-tagged D2R, GFP antibody (Rabbit, ThermoFisher Scientific, Cat. No. A6455, 1:1000), TH antibody (Mouse, Immunostar, Cat. No. 22941, 1:1000), and mCherry antibody (Rat, ThermoFisher Scientific, Cat. No. M11217, 1:1000) were used. For staining of GRASP, GFP antibody (Mouse, ThermoFisher Scientific, Cat. No. G6539, 1:100) was used. Goat anti-rabbit with green fluorescence (Invitrogen, Alexa Fluor 488 conjugate, Cat. No. A-11035, 1:1000), goat anti-mouse with green fluorescence (Alexa Fluor 488 conjugate, Cat. No. A11029, 1:1000), goat anti-mouse with far-red fluorescence (Alexa Fluor 633 conjugate, Cat. No. A21052, 1:500), and goat anti-rat with red fluorescence (Alexa Fluor 546 conjugate, Cat. No. A-11081, 1:1000) secondary antibodies were used. In the seventh day, brains were rinsed and washed twice with PBT solution, and then transferred and mounted in the Fluoro-Gel with Tris Buffer on a piece of micro cover glasses. Finally, the samples were covered with another piece of micro cover glasses.

Confocal imaging

All images were obtained with a Zeiss Laser Scanning Microscope 510 (LSM510, Carl Zeiss, Inc., USA). Under 40× and 100× objective magnifications, images were collapsed from confocal stacks of 1.0 μm optical slices. Under 25× objective magnifications, images were collapsed from confocal stacks of 2.0 μm optical slices. Under 10× objective magnifications, images were collapsed from confocal stacks of 12.5 μm optical slices. ImageJ software was used to remove other signals outside of the mushroom bodies, as the background noise in GRASP is strong.

Larval olfactory learning assays

A single odor learning paradigm was slightly modified from previous publication[17,18]. In brief, 25 to 50 third-instar larvae (92–96 h after egg laying) were trained on a 2.5% agar plate (100mm petri dish) covered with 2 mL of 1 M sucrose solution (SUC, Sigma, Cat. No. S1888) or 0.1% quinine hemisulfate solution (QUI, Sigma, Cat. No. 22640). Distilled water (DW) was used as a control. An odorant pentyl acetate (PA, 10μL, Sigma-Aldrich, CAT. No. 109584) was placed on a small piece of filter inside the lid. After 30 minutes, larvae were rinsed and transferred to the middle line of a new 2.5% agar plate. A small piece of filter paper with 2.5μL pentyl acetate was placed on one side of the plate, while distilled water on the other side. After 5 min, the numbers of larvae in the two semicircular areas were counted and the response index (R.I.) was calculated with the following equation (**Figure 2g**):

$$R.I. = \frac{\#\ of\ larvae\ on\ odorant\ side\ in\ dashline - \#\ of\ larvae\ on\ DW\ side\ in\ dashline}{Total\ \#\ of\ larvae\ on\ two\ sides\ in\ dashlines}$$

Naïve Olfactory Test

Larvae were transferred into the midline of test plates. 2.5μL of odorant were added on one side and distilled water on the other side. The number of larvae in two semicircular areas were counted and the R.I. was calculated after 5 min.

Naïve Gustatory Test

A petri dish with a median separator was used. Both sides were filled with 1% agar, with 2 mL of distilled water on the control side, and with 1 M sucrose (SUC) solution, or 0.1% quinine hemisulfate (QUI) solution on the test side. Twenty larvae were put on each side near the midline and allowed to move for 5 min. Gustatory R.I. was calculated using the larvae numbers on two sides[90].

Larval locomotion assay

Individual larvae were placed on the surface of a plate of 2.5% agar mixed with 1mL India ink. They were allowed to acclimate for 1 min, and then a video was recorded for 30 seconds using a Moticam3 digital camera (Motic) and Motic Images Plus 2.0 software. The video was analyzed by the MTrack2 plug-in (from http://valelab.ucsf.edu/~nico/IJplugins/MTrack2.html) in ImageJ. The path was recorded; scores were quantified as the length traveled per minute as previously described[91]. As the locomotion speed of DAN-c1 homozygous was slow, DAN-c1 × WT was used as the control group.

Learning assays with thermogenetics

In learning assays with thermogenetics, 25 to 50 third-instar larvae (92–96 h after egg laying) were trained on a 2.5% agar plate (100mm petri dish) covered with 2 mL of 1 M SUC or 0.1% QUI. DW was used as a control. An odorant PA was placed on a small piece of filter inside the lid. Training plates were put in a water bath either under 22°C or 34°C. After 30 minutes, larvae were rinsed and transferred to the testing plate. After 5 min, the response index (R.I.) was calculated.

Learning assays with optogenetics

In learning assays with optogenetics, egg laying plates with 1mM ATR (all-trans retinal, Sigma, Cat. No. R2500) were used. All-trans retinal (ATR) is a necessary light-isomerizable chromophore for ChR2, which is not synthesized by *Drosophila*.[92,93] Around 50 third-instar larvae were trained in a 35mm petri dish with 2 mL of either 1 M SUC or 0.1% QH solutions. DW was used as a control. During training, an odorant was placed on a small piece of filter inside the lid. To activate channelrhodopsin2, a LED (Luxeon Rebel Color LEDs, 07040 PB000-D, wavelength 470 nm) with a power supply (GW Instek, Laboratory DC power supply Model GPS-1830D) was used. The intensity of the blue light was 25 mW, measured by a laser power meter (Sanwa, LP1). After being trained for 30 minutes, larvae were rinsed and transferred to the middle line of a 2.5% agar plate in 100 mm test plate. A small piece of filter paper with pentyl acetate was placed on one side of the plate, while distilled water on the other side. Then the number of larvae in the two semicircular areas were counted and the R.I. was calculated after 5 min.

Quantification of D2R knockdown

Quantification of the fluorescent intensity of D2R knockdown was performed as follows. TH signals were used to define dopaminergic neurons, and the mean fluorescent intensity of GFP in each neuron was calculated with subtraction of the background. The mean intensity of DM1 was divided by that of pPAM, and the value in the knockdown group was subsequently normalized with the control group.

Statistical Analysis

Information for statistical analysis is provided in figure legends.

Author contributions

Qi. C.: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization, **Qian. C.:** Writing – Review & Editing, **Steijvers. E.:** Writing – Review & Editing **Colvin. R.:** Writing – Review & Editing **Lee. D.:** Conceptualization, Writing – Review & Editing, Supervision, Funding acquisition

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Declaration of Interest

The authors declare no competing interests.

Supplemental information

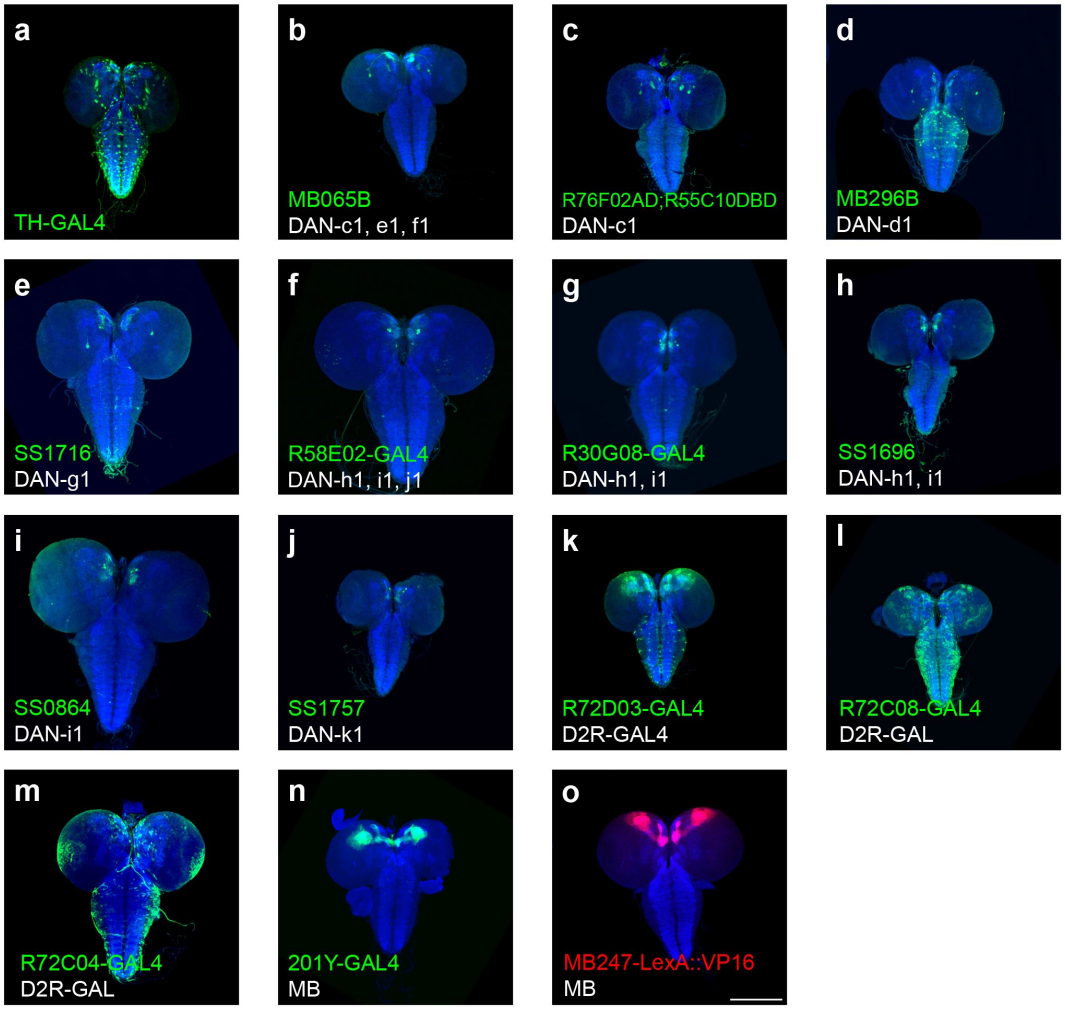

Figure S1.

GFP expression patterns in the larval CNS by GAL4 driver strains used in this study.

(a-n) Representative pictures show the patterns of distinct GAL4 driver strains crossed with a UAS-GFP strain. **(o)** A representative picture shows the pattern of MB247-LexA driver (MB247-LexA::VP16) crossed with a LexAop-RFP strain (LexAop-rCD2::RFP). Blue channel represents neuropils marked by nc82 antibody. Driver strains in **(a-j)** were used in **Figure 1d-m**. Driver strains in **(k-m)** are strains used in supplementary **Figure S3**. 201Y-Gal4 (N) is the driver strain used in **Figure 6**. Scale bar: 200 μm.

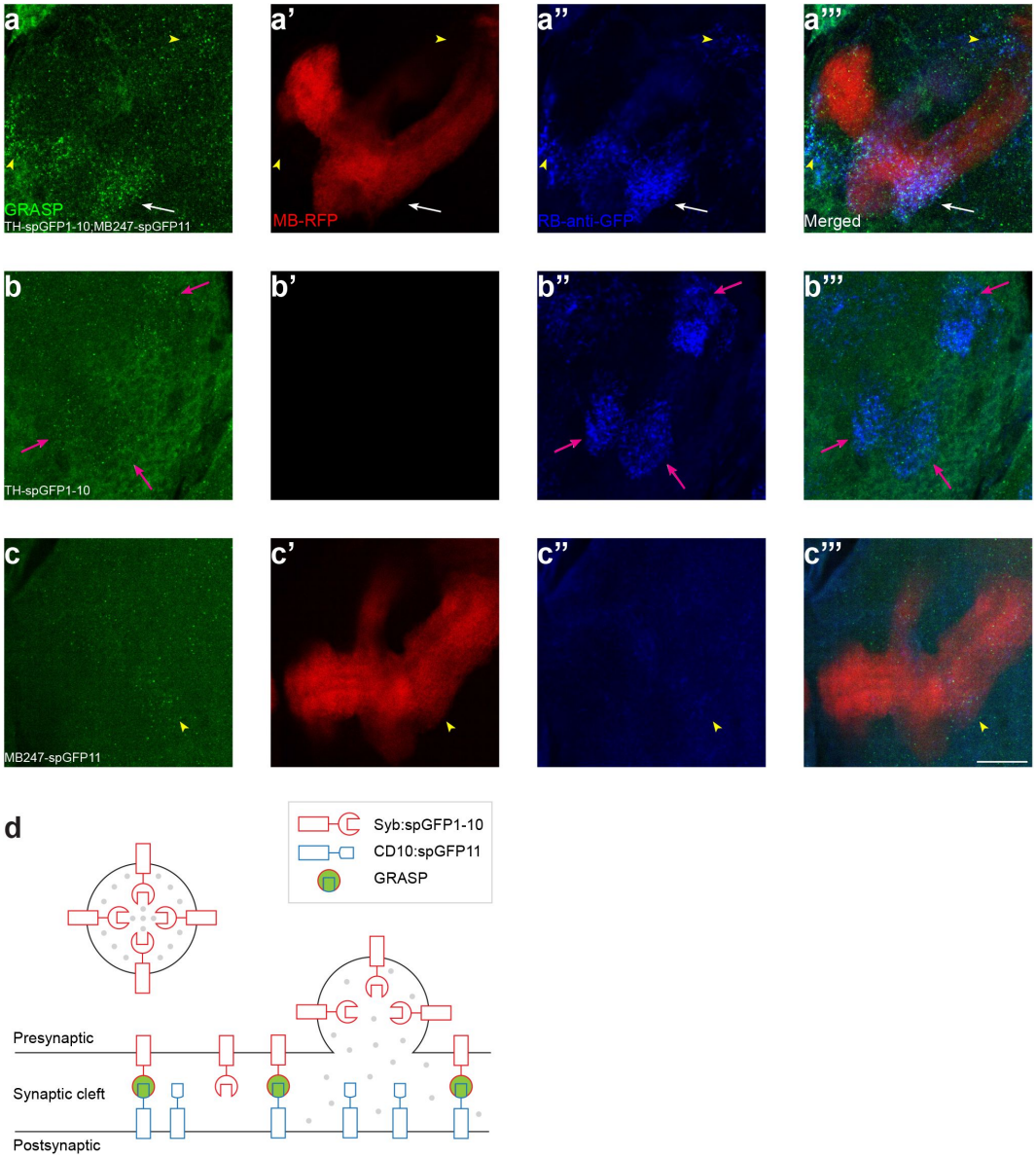

Figure S2.

Controls for GRASP experiments.

(a-c) The GRASP signals were not observed in control groups. The first column shows the mouse anti-GFP staining (green) for GRASP signals, the second column shows MB lobes by RFP (red), and the third column shows the rabbit anti-GFP staining (blue) which only recognizes the spGFP1-10. **(a)** Both GRASP (mouse antibody, green) and spGFP1-10 (rabbit antibody, blue) can be recognized in the lower peduncle (LP) compartment (white arrows), in a larval brain expressing spGFP1-10 under TH-Gal4 and spGFP11 under MB247-LexA. Some strong spGFP1-10 signals outside of MB are also colocalized with GRASP signals (yellow arrowheads). **(b)** GRASP signals are hardly observed in a larval brain only expressing spGFP1-10 under TH-Gal4 (no MB247-LexA in this brain), while anti-spGFP1-10 signals are still strong (magenta arrows). **(c)** Neither GRASP nor spGFP1-10 can be recognized in a larval brain only expressing spGFP11 under MB247-LexA (cyan arrowheads). Scale bars: 20 μm. **(d)** A Schematic diagram shows the mechanism of GRASP. Modified from Macpherson et. al.[89].

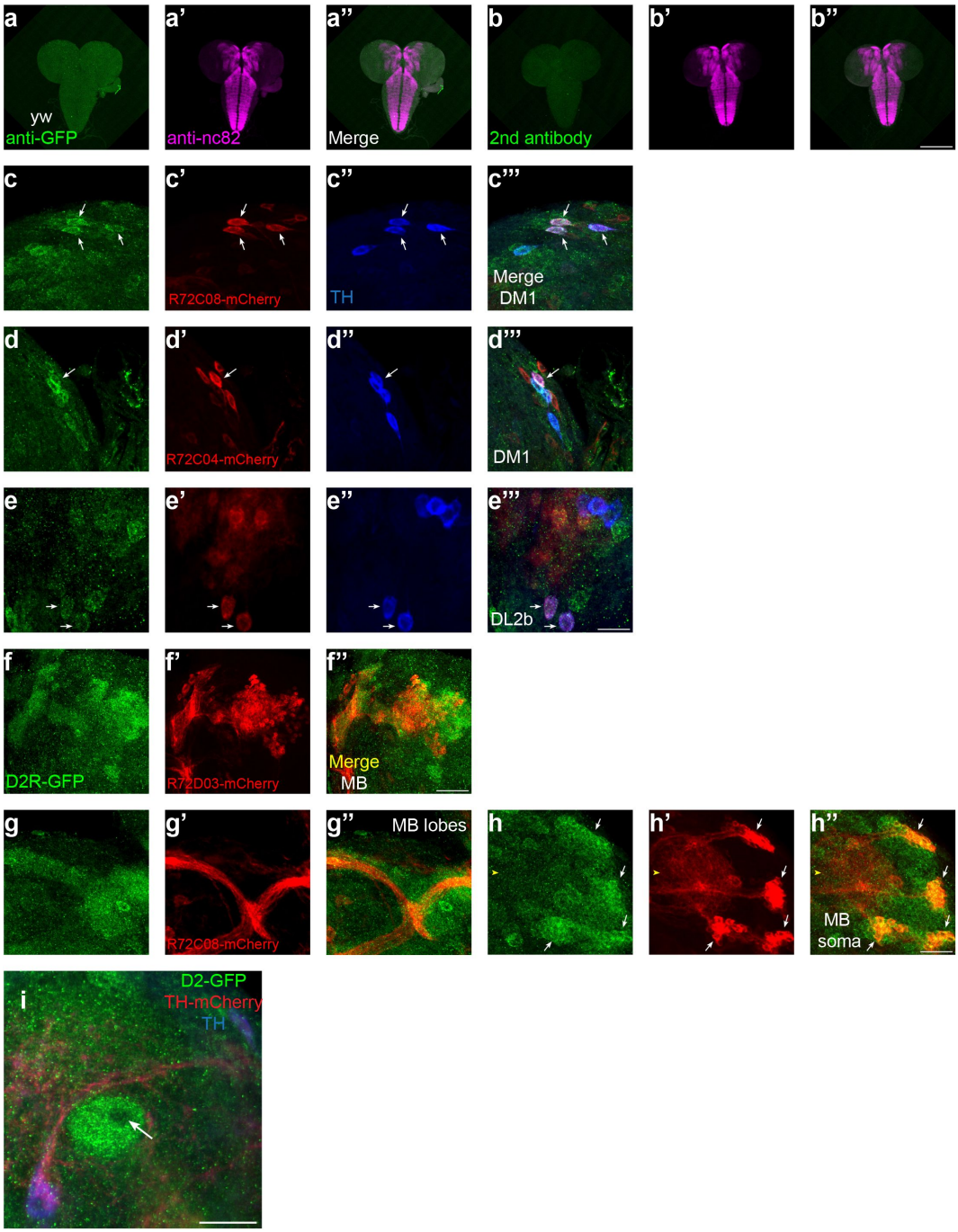

Figure S3.

D2R-GAL4 strains support the expression pattern of D2R.

(a-b) Control staining for D2R-tagged GFP in **Figure 3**. The green channel represents GFP signals whereas magenta represents neuropils marked by nc82 antibody. GFP staining in WT (a). Secondary antibody only staining for the D2R-tagged GFP strain (b). **(c-e)** D2R-GAL4 strains support the expression of D2R in some DANs. The green channel represents GFP signals, red represents mCherry signals under D2R-GAL4 drivers, and blue marks DANs with TH antibodies. R72C08 labels three DM1 neurons (c). R72C04 labels one DM1 (d) and two DL2b neurons (e). **(f-h)** D2R-GAL4 strains support the expression of D2R in mushroom body neurons. The green channel represents GFP signals, and red represents mCherry signals under D2R-GAL4 drivers. R72D03 marks some MBNs in mushroom body (f). R72C08 labels some MBNs in axons (g), dendrites and soma (h). **(i)** D2R is absent in the core of mushroom body lobes, from a transection view of the lower peduncle. Scale bars: 200 μm (**a and b**); 20 μm (**c-e, g-i**), 50 μm (**f**).

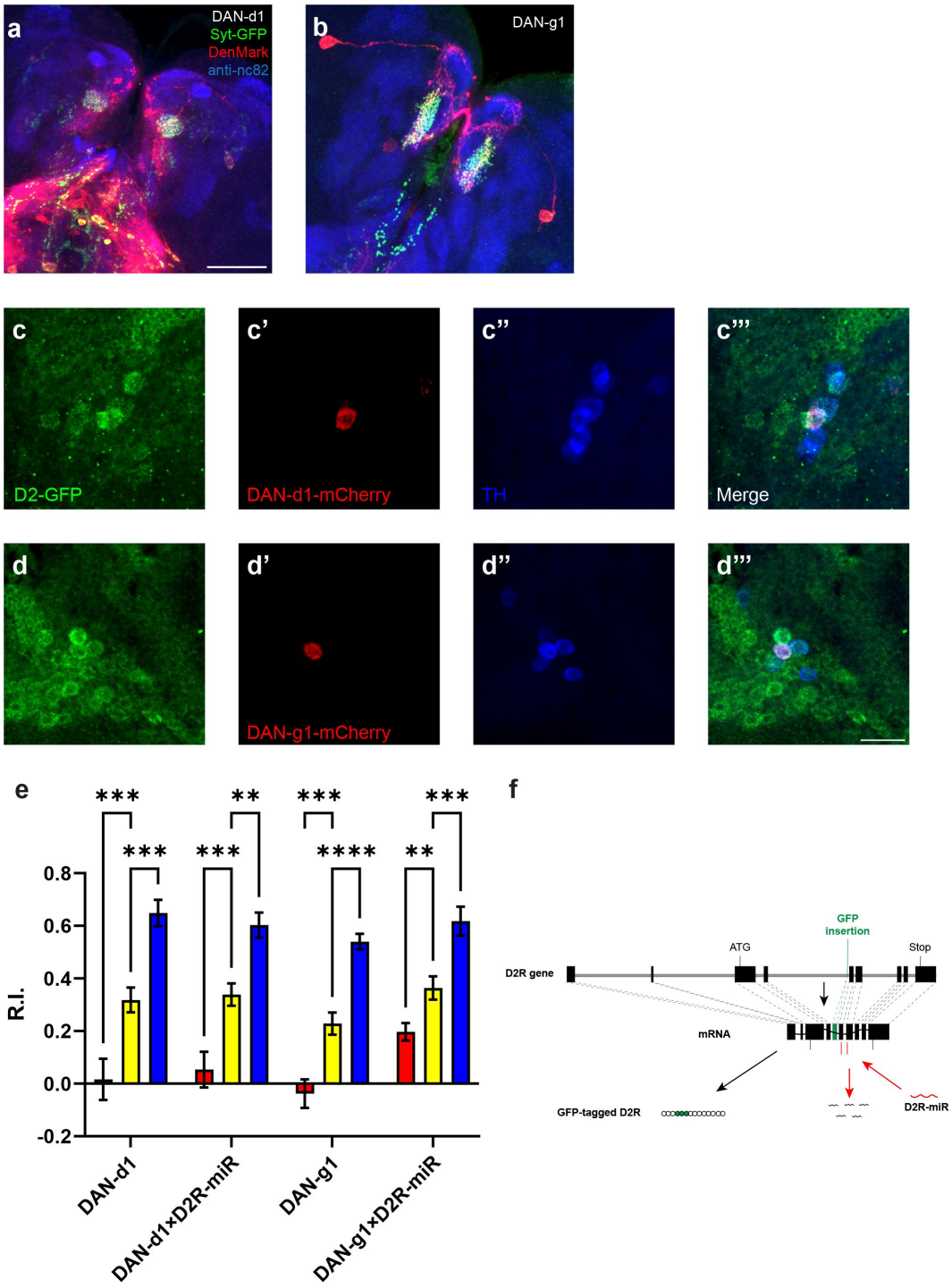

Figure S4.

D2Rs in DAN-d1 and DAN-g1 are not necessary for aversive learning.

(a-b) Dendrites and axons of DAN-d1 (a) and g1 (b) are labeled by DenMark (red) and sytGFP (green), correspondingly. **(c-d)** D2Rs are expressed in DAN-d1 (c) and DAN-g1 (d). The expression pattern of D2R is shown with tagged-GFP, DAN-g1 and d1 are marked by mCherry, and DANs are labeled by TH-antibody (blue). **(e)** Knockdown D2R in neither in DAN-g1 nor DAN-d1 impairs aversive learning. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, **p < 0.01, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S3**. **(f)** The gene structure of D2R, the insertion site of GFP, and the D2R-microRNA targeting sequence are shown in a schematic diagram. Modified from Xie and Ho[61]. Scale bars: 50 μm (a and b), 20 μm (c and d).

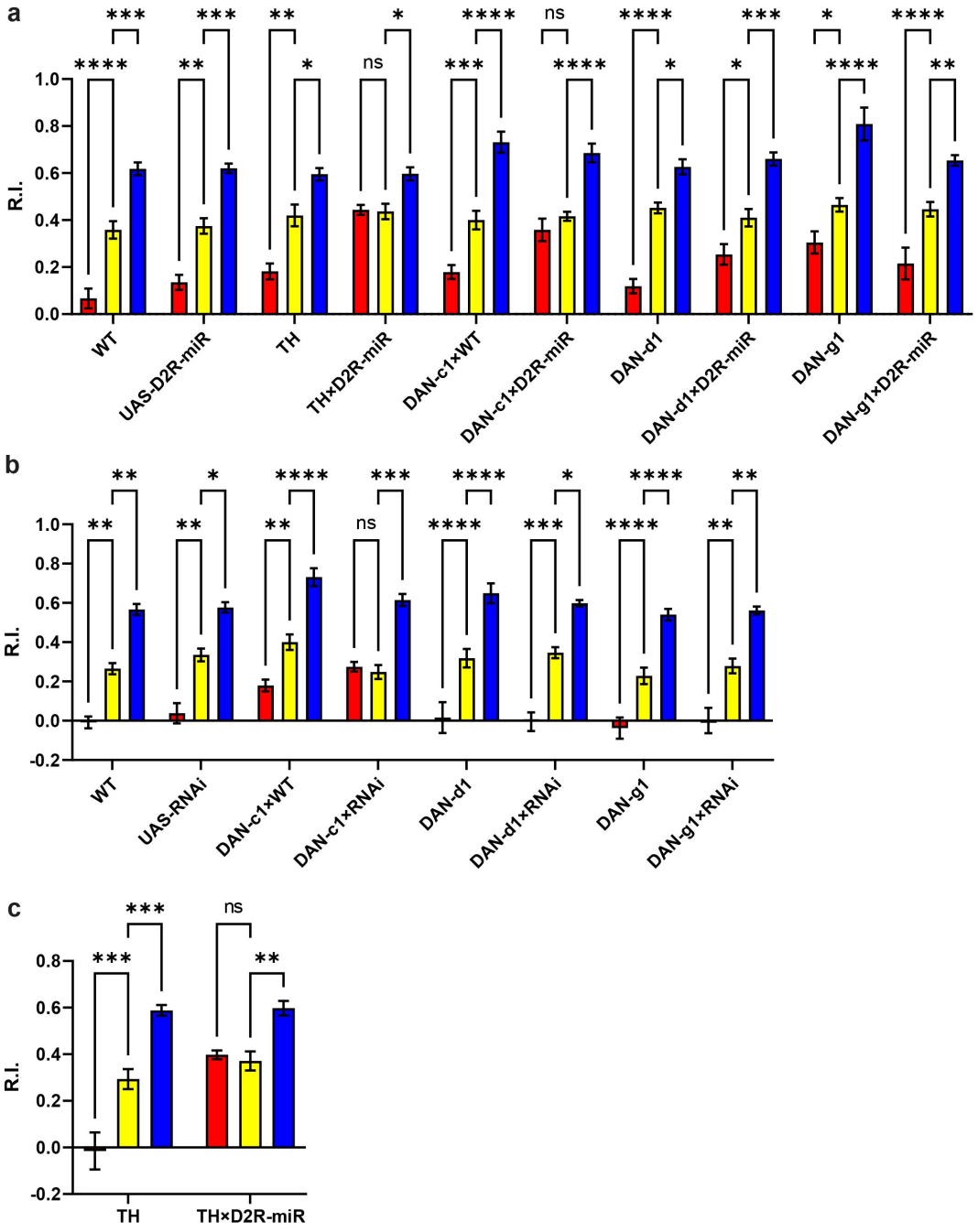

Figure S5.

Knockdown D2R in DAN-c1 with a RNAi strain also impairs aversive learning.

(a) Knockdown of D2R with D2R-miR in DAN-c1 impairs larval aversive learning when using propionic acid as the odorant. **(b)** Knockdown of D2R in DAN-c1 with a RNAi strain also impairs larval aversive learning when using pentyl acetate as the odorant, while knockdown of D2R in either DAN-d1 or g1 does not affect learning. **(c)** Knockdown of D2R with D2R-miR in DANs under TH-GAL4 impairs larval aversive learning when using pentyl acetate as the odorant. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, *p < 0.01, **p < 0.01, ***p < 0.001, ****p < 0.0001. For N numbers, see **Table S3**.

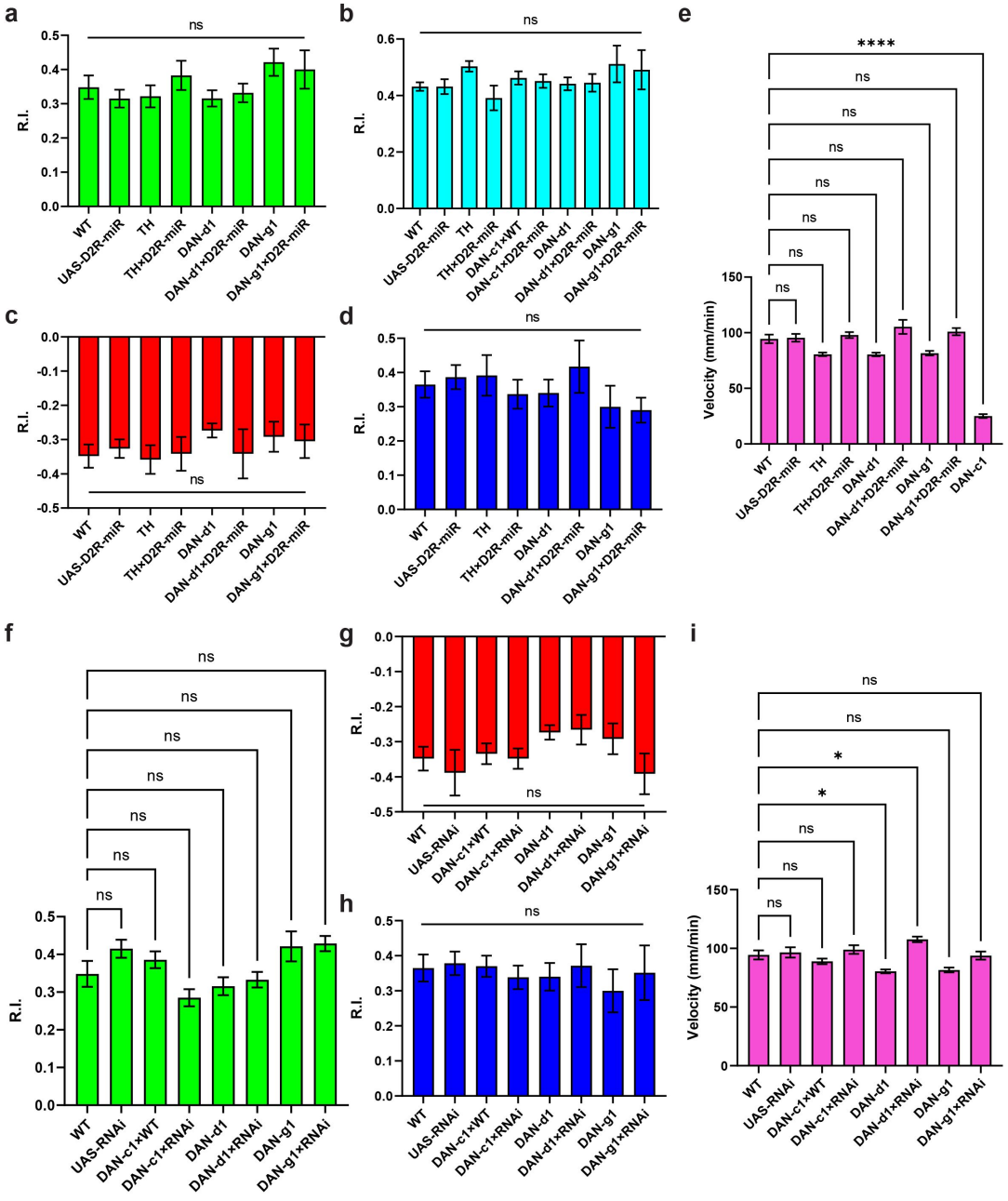

Figure S6.

Naïve sensory and motor functions of *Drosophila* larvae.

(a-e) The naïve sensory and motor functions in larvae related to D2R-miR experiments. **(a)** No significant difference exists between strains in naïve olfactory tests toward pentyl acetate (PA). **(b)** No significant difference exists between strains in naïve olfactory tests toward propionic acid (ProA). **(c)** No significant difference exists between strains in naïve gustatory tests toward quinine (QUI). **(d)** No significant difference exists between strains in naïve gustatory tests toward sucrose (SUC). **(e)** No significant difference is found between strains and WT, except DAN-c1. So, larvae from DAN-c1 × WT are used for all behavioral assays (refer to **i**). **(f-i)** The naïve sensory and motor functions in larvae related to RNAi experiments. **(f)** No significant difference exists between strains and WT. **(g)** No significant difference exists between strains in naïve gustatory tests toward QUI. **(h)** No significant difference exists between strains in naïve gustatory tests toward SUC. **(i)** There is a significant difference between strains in larval locomotion speed, and there is significant difference between DAN-d1, DAN-d1 × RNAi and WT, but these differences do not affect the learning ability of larvae. Data are shown as mean ± SEM. One-way ANOVA, Tukey multiple comparisons test. For N numbers, see **Table S3**.

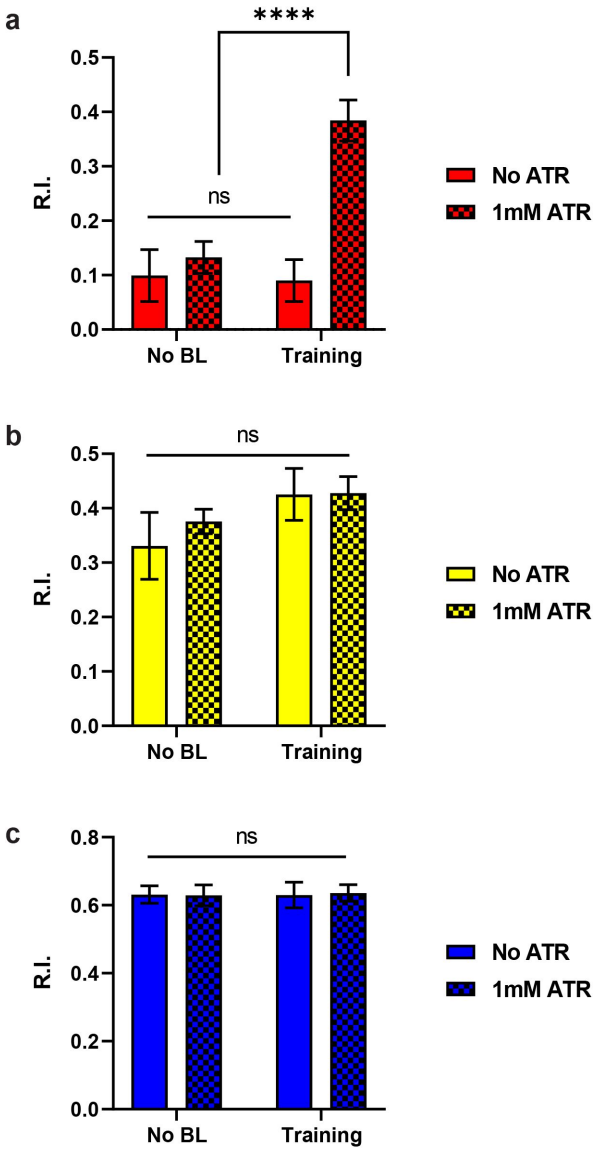

Figure S7.

Over-excitation of DANs during learning impairs larval aversive learning.

Third instar larvae with ChR2 expression in most DANs (TH-Gal4) were used. Activation of DANs during training impairs larval aversive learning (**a**), while keeps appetitive learning intact (**c**). No learning behaviors are observed in the control distilled water (DW) groups **(b)**. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett multiple comparisons test, ***p < 0.001. For N numbers, see **Table S4**.

Supplementary Tables

Table S1.

Other strains used in this study.

	Strains	Information	Source/Gift	Stock #
1	R72C04	D2R-GAL4 driver (verified)	BDSC	49416
2	R72C08	D2R-GAL4 driver (verified)	BDSC	49621
3	R72D03	D2R-GAL4 driver	BDSC	46676
4	D2R-EGFP	EGFP-tagged D2R	BDSC	60276
5	201Y-GFP	GFP expressed in Mushroom Body	BDSC	64296
6	MB247-LexA::Vp16/TM6B	MB-LexA, mushroom body driver	Dr. M. Gallio's Lab	Macpherson et al. 2015
7	UAS-nSyb-spGFP1-10,LexAop-CD4-spGFP11/CyO	GRASP strain	BDSC	64314
8	LexAop-nSyb-spGFP1-10,UAS-CD4-spGFP11;MKRS/TM6B	Reverse GRASP strain	BDSC	64315
9	WT(Canton-S)	Wild type	BDSC	64349 (1)
10	UAS-RNAi(D2R) (III) line 2	UAS-D2R-RNAi	Dr. A. Kopin's Lab	Draper et al. 2007
11	201Y-GAL4	MB-GAL4, mushroom body driver	BDSC	4440
12	CyO/Sco;TM2,Ubx/TM6,Hu.Tb	Ballancer Chromesome	Dr. S. Tanda's Lab	
13	1407-Gal4	Pan-neuronal driver	BDSC	8751
14	10XUAS-mCD8::GFP (I)	GFP strain	BDSC	32189
15	10XUAS-mCD8::GFP (II)	GFP strain	BDSC	32186
16	10XUAS-mCD8::GFP (III)	GFP strain	BDSC	32184
17	UAS-ChR2; UAS-ChrR2	Optogenetics strain	Dr. B. Condron's Lab	
18	LexAop-rCD2::RFP; UAS-CD4-spGFP1-10, LexAop-CD4-spGFP11	RFP expressed in MB; GRASP	BDSC	58755
19	UAS-CD8::mCherry (II)	mCherry strain	BDSC	27391
20	UAS-CD8::mCherry (III)	mCherry strain	BDSC	27392
21	yw1118	Wild type for D2R-EGFP	Dr. S. Tanda's Lab	
22	D2R-miR	UAS-DD2R-microRNA	Dr. M. Wu's Lab	Xie et al. 2018
23	UAS-*Shibirets1*	Thermogenetics strain	Dr. T. Kitamoto's Lab	
24	UAS-dTRPA1	Thermogenetics strain	BDSC	26263
25	UAS-nsyb::GFP; UAS-DenMark	Pre- and postsynaptic terminals	BDSC	33056

Table S2.

N numbers of learning assays with thermogenetics.

	22°C			34°C		
	QUI	**DW**	**SUC**	**QUI**	**DW**	**SUC**
shibire^{ts1}	9	8	8	9	9	9
DAN-c1×shibire^{ts1}	14	6	7	17	6	9
DAN-c1	8	9	9	9	9	9
DAN-c1×dTRPA1	15	14	13	14	15	13
dTRPA1	9	8	9	10	10	10

Table S3.

N numbers of DD2R knockdown experiments.

Driver	Cross	Pentyl acetate (PA)			Propionic acid (P-A)			Naïve olfactory		Naïve gustatory		Locomotion
		QUI	DW	SUC	QUI	DW	SUC	PA	P-A	QUI	SUC	
TH ×	miR	8	8	8	6	6	6	7	7	6	6	24
	RNAi	-	-	-	-	-	-	-	-	-	-	-
	CR	8	6	6	6	6	6	6	6	6	6	12
DAN-c1 ×	miR	38	38	37	14	13	14	12	8	11	12	50
	RNAi	6	6	6	-	-	-	8	-	6	6	24
	CR	14	10	10	11	8	8	12	9	11	12	36
DAN-d1 ×	miR	17	15	13	8	8	7	6	6	6	7	16
	RNAi	6	6	6	-	-	-	7	-	7	6	16
	CR	13	11	10	9	6	6	7	6	6	6	15
DAN-g1 ×	miR	31	22	16	13	8	8	8	8	6	7	22
	RNAi	8	8	6	-	-	-	8	-	6	7	24
	CR	23	19	18	8	8	7	8	6	6	6	15
201Y ×	miR	7	7	8	6	6	6	6	6	6	6	24
	RNAi	-	-	-	-	-	-	-	-	-	-	-
	CR	6	6	6	6	6	6	6	6	6	6	24
WT		6	6	6	6	6	6	6	6	6	6	24
D2R-miR		12	12	12	6	6	6	12	6	11	12	36
RNAi		6	6	6	-	-	-	6	-	6	6	20

Table S4.

N numbers of learning assays with optogenetics

Strains	Groups	BL									No BL		
		Resting			Learning			Testing					
		QUI	DW	SUC	QUI	DW	SUC	QUI	DW	SUC	QUI	DW	SUC
TH×ChR2	ATR	-	-	-	21	13	10	-	-	-	20	16	10
	No ATR	-	-	-	16	13	8	-	-	-	14	13	8
DAN-c1×ChR2	ATR	11	8	9	15	12	9	9	8	8	16	11	9
	No ATR	9	9	8	15	12	8	9	9	9	16	12	9
201Y×ChR2	ATR	8	7	7	9	6	9	7	7	10	11	8	7
	No ATR	10	7	7	10	7	7	9	7	13	12	10	7

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Editors

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Reviewer #1 (Public Review):

Summary:
Both flies and mammals have D1-like and D2-like dopamine receptors, yet the role of D2-like receptors in Drosophila learning and memory remains underexplored. The paper by Qi et al. investigates the role of the D2-like dopamine receptor D2R in single pairs of dopaminergic neurons (DANs) during single-odor aversive learning in the Drosophila larva. First, they use confocal imaging to screen driver strains with expression in only single pairs of dopaminergic neurons. Next, they use thermogenetic manipulations of one pair of DANs (DAN-c1) to implicate DAN-c1 activity during larval aversive learning. They then use confocal imaging to demonstrate expression of D2R in the DANs and mushroom body of the larval brain. Finally, they show that optogenetic activation during training phenocopies D2R knockdown in these neurons: aversive learning is impaired when DAN-c1 is targeted, while appetitive and aversive learning are impaired when the mushroom body is manipulated. Qi et al. thus propose a model in which D2R limits excessive dopamine release to facilitate successful olfactory learning.

Strengths:
The paper reproduces prior findings by Qi and Lee (2014), which demonstrated that D2R knockdown in DL1 DANs or the mushroom body impairs aversive olfactory learning in Drosophila larvae. The authors extended this previous work by screening 57 GAL4 drivers to identify tools that drive expression in individual DANs and used one of the tools, the R76F02-AD; R55C10-DBD driver, to manipulate DAN-c1 neurons with greater specificity. They also

show that GFP-tagged D2R is expressed in most DANs and the mushroom body. Although the authors only train larvae with a single odor, they demonstrate that driving D2R knockdown in DAN-c1 neurons impairs aversive learning, as do other loss-of-function manipulations of DAN-c1 neurons.

Weaknesses:
The authors claim to have identified drivers that label single DANs in Figure 1, but their confocal images in Figure S1 suggest that many of those drivers label additional neurons in the larval brain. It is also not clear why only some of the 57 drivers are displayed in Figure S1. Critically, R76F02-AD; R55C10-DBD labels more than one neuron per hemisphere in Figure S1c, and the authors cite Xie et al. (2018) to note that this driver labels two DANs in adult brains. Therefore, the authors cannot argue that the experiments throughout their paper using this driver exclusively target DAN-c1.
Missing from the screen of 57 drivers is the driver MB320C, which typically labels only PPL1-γ1pedc in the adult and should label DAN-c1 in the larva. If MB320C labels DAN-c1 exclusively in the larva, then the authors should repeat their key experiments with MB320C to provide more evidence for DAN-c1 involvement specifically.
The authors claim that the SS02160 driver used by Eschbach et al. (2020) labels other neurons in addition to DAN-c1. Could the authors use confocal imaging to show how many other neurons SS02160 labels? Given that both Eschbach et al. and Weber et al. (2023) found no evidence that DAN-c1 plays a role in larval aversive learning, it would be informative to see how SS02160 expression compares with the driver the authors use to label DAN-c1.
The claim that DAN-c1 is both necessary and sufficient in larval aversive learning should be reworded. Such a claim would logically exclude any other neuron or even the training stimuli from being involved in aversive learning (see Yoshihara and Yoshihara (2018) for a detailed discussion of the logic), which is presumably not what the authors intended because they describe the possible roles of other DANs during aversive learning in the discussion.
Moreover, if DAN-c1 artificial activation conveyed an aversive teaching signal irrespective of the gustatory stimulus, then it should not impair aversive learning after quinine training (Figure 2k). While the authors interpret Figure 2k (and Figure 5) to indicate that artificial activation causes excessive DAN-c1 dopamine release, an alternative explanation is that artificial activation compromises aversive learning by overriding DAN-c1 activity that could be evoked by quinine.
The authors should not necessarily expect that D2R enhancer driver strains would reflect D2R endogenous expression, since it is known that TH-GAL4 does not label p(PAM) dopaminergic neurons. Their observations of GFP-tagged D2R expression could be strengthened with an anti-D2R antibody such as that used by Lam et al., (1999) or Love et al., (2023).
Finally, the authors could consider the possibility other DANs may also mediate aversive learning via D2R. Knockdown of D2R in DAN-g1 appears to cause a defect in aversive quinine learning compared with its genetic control (Figure S4e). It is unclear why the same genetic control has unexpectedly poor aversive quinine learning after training with propionic acid (Figure S5a). The authors could comment on why RNAi knockdown of D2R in DAN-g1 does not similarly impair aversive quinine learning (Figure S5b).

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Reviewer #2 (Public Review):

Summary:
The study wanted to functionally identify individual DANs that mediate larval olfactory learning. Then search for DAN-specific driver strains that mark single dopaminergic neurons, which subsequently can be used to target genetic manipulations of those neurons. 56 GAL4 drivers identifying dopaminergic neurons were found (Table 1) and three of them drive the

expression of GFP to a single dopaminergic neuron in the third-instar larval brain hemisphere. The DAN driver R76F02-AD;R55C10-DBD appears to drive the expression to a dopaminergic neuron innervating the lower peduncle (LP), which would be DAN-c1.
Split-GFP reconstitution across synaptic partners (GRASP) technique was used to investigate the "direct" synaptic connections from DANs to the mushroom body. Potential synaptic contact between DAN-c1 and MB neurons (at the lower peduncle) were detected.
Then single odor associative learning was performed and thermogenetic tools were used (Shi-ts1 and TrpA1). When trained at 34{degree sign}C, the complete inactivation of dopamine release from DAN-c1 with Shibirets1 impaired aversive learning (Figure 2h), while Shibirets1 did not affect learning when trained at room temperature (22{degree sign}C). When paired with a gustatory stimulus (QUI or SUC), activation of DAN-c1 during training impairs both aversive and appetitive learning (Figure 2k).
They examined the expression pattern of D2R in fly brains and were found in dopaminergic neurons and the mushroom body (Figure 3). To inspect whether the pattern of GFP signals indeed reflected the expression of D2R, three D2R enhancer driver strains (R72C04, R72C08, and R72D03-GAL4) were crossed with the GFP-tagged D2R strain.
D2R knockdown (UAS-RNAi) in dopaminergic neurons driven by TH-GAL4 impaired larval aversive learning. Using a microRNA strain (UAS-D2R-miR), a similar deficit was observed.
Crossing the GFP-tagged D2R strain with a DAN-c1-mCherry strain demonstrated the expression of D2R in DAN-c1 (Figure 4a). Knockdown of D2R in DAN-c1 impaired aversive learning with the odorant pentyl acetate, while appetitive learning was unaffected (Figure 4e). Sensory and motor functions appear not affected by D2R suppression.
To exclude possible chronic effects of D2R knockdown during development, optogenetics was applied at distinct stages of the learning protocol. ChR2 was expressed in DAN-c1, and blue light was applied at distinct stages of the learning protocol. Optogenetic activation of DAN-c1 during training impaired aversive learning, not appetitive learning (Figure 5b-d).
Knockdown of D2Rs in MB neurons by D2R-miR impaired both appetitive and aversive learning (Figure 6a). Activation of MBNs during training impairs both larval aversive and appetitive learning.
Finally, based on the data the authors propose a model where the effective learning requires a balanced level of activity between D1R and D2R (Figure 7).

Strengths:
The work is well written, clear, and concise. They use well documented strategies to examine GAL4 drivers with expression in a single DAN, behavioral performance in larvae with distinct genetic tools including those to do thermo and optogenetics in behaving flies. Altogether, the study was able to expand our understanding of the role of D2R in DAN-c1 and MB neurons in the larva brain.

Weaknesses:
Is not completely clear how the system DAN-c1, MB neurons and Behavioral performance work. We can be quite sure that DAN-c1;Shits1 were reducing dopamine release and impairing aversive memory (Figure 2h). Similarly, DAN-c1;ChR2 were increasing dopamine release and also impaired aversive memory (Figure 5b). However, is not clear what is happening with DAN-c1;TrpA1 (Figure 2K). In this case the thermos-induction appears to impair the behavioral performance of all three conditions (QUI, DW and SUC) and the behavior is quite distinct from the increase and decrease of dopamine tone (Figure 2h and 5b).

The study successfully examined the role of D2R in DAN-c1 and MB neurons in olfactory conditioning. The conclusions are well supported by the data, with the exception of the claim that dopamine release from DAN-c1 is sufficient for aversive learning in the absence of unconditional stimulus (Figure 2K). Alternatively, the authors need to provide a better explanation of this point.
The study provides insight into the role of D2R in associative learning expanding our

understanding and might be a reference similar to previous key findings (Qi and Lee, 2014, https://doi.org/10.3390/biology3040831).

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Reviewer #3 (Public Review):

It is a strength of the paper that it analyses the function of dopamine neurons (DANs) at the level of single, identified neurons, and uses tools to address specific dopamine receptors (DopRs), exploiting the unique experimental possibilities available in larval Drosophila as a model system. Indeed, the result of their screening for transgenic drivers covering single or small groups of DANs and their histological characterization provides the community with a very valuable resource. In particular the transgenic driver to cover the DANc1 neuron might turn out useful. However, I wonder in which fraction of the preparations an expression pattern as in Figure 1f/ S1c is observed, and how many preparations the authors have analyzed. Also, given the function of DANs throughout the body, in addition to the expression pattern in the mushroom body region (Figure 1f) and in the central nervous system (Figure S1c) maybe attempts can be made to assess expression from this driver throughout the larval body (same for Dop2R distribution).

A first major weakness is that the main conclusion of the paper, which pertains to associative memory (last sentence of the abstract, and throughout the manuscript), is not justified by their evidence. Why so? Consider the paradigm in Figure 2g, and the data in Figure 2h (22 degrees, the control condition), where the assay and the experimental rationale used throughout the manuscript are introduced. Different groups of larvae are exposed, for 30min, to an odour paired with either i) quinine solution (red bar), ii) distilled water (yellow bar), or iii) sucrose solution (blue bar); in all cases this is followed by a choice test for the odour on one side and a distilled-water blank on the other side of a testing Petri dish. The authors observe that odour preference is low after odour-quinine pairing, intermediate after odour-water pairing and high after odour-sucrose pairing. The differences in odour preference relative to the odour-water case are interpreted as reflecting odour-quinine aversive associations and odour-sucrose appetitive associations, respectively. However, these differences could just as well reflect non-associative effects of the 30-min quinine or sucrose exposure per se (for a classical discussion of such types of issues see Rescorla 1988, Annu Rev Neurosci, or regarding Drosophila Tully 1988, Behav Genetics, or with some reference to the original paper by Honjo & Furukubo-Tokunaga 2005, J Neurosci that the authors reference, also Gerber & Stocker 2007, Chem Sens).
As it stands, therefore, the current 3-group type of comparison does not allow conclusions about associative learning.

A second major weakness is apparent when considering the sketch in Figure 2g and the equation defining the response index (R.I.) (line 480). The point is that the larvae that are located in the middle zone are not included in the denominator. This can inflate scores and is not appropriate. That is, suppose from a group of 30 animals (line 471) only 1 chooses the odour side and 29, bedazzled after 30-min quinine or sucrose exposure or otherwise confused by a given opto- or thermogenetic treatment, stay in the middle zone... a P.I. of 1.0 would result.

Unless experimentally demonstrated, claims that the thermogenetic effector shibire/ts reduces dopamine release from DANs are questionable. This is because firstly, there might be shibire/ts-insensitive ways of dopamine release, and secondly because shibire/ts may affect co-transmitter release from DANs.
To implicate a role of dopamine in DANs, previous work used e.g. RNAi against the dopamine-synthesizing TH enzyme (Rohwedder et al, cited).

It is not clear whether the genetic controls when using the Gal4/ UAS system are the homozygous, parental strains (XY-Gal4/ XY-Gal4 and UAS-effector/ UAS-effector), or as is standard in the field the heterozygous driver (XY-Gal4/ wildtype) and effector controls (UAS-effector/ wildtype) (in some cases effector controls appear to be missing, e.g. Figure 4d, Figure S4e, Figure S5c).

As recently suggested by Yamada et al 2024, bioRxiv, high cAMP can lead to synaptic depression (sic). That would call into question the interpretation of low-Dop2R leading to high-cAMP, leading to high-dopamine release, and thus the authors interpretation of the matching effects of low-Dop2R and driving DANs.

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Author response:

> ***Reviewer #1 (Public Review):***
>
> *Weakness #1: The authors claim to have identified drivers that label single DANs in Figure 1, but their confocal images in Figure S1 suggest that many of those drivers label additional neurons in the larval brain. It is also not clear why only some of the 57 drivers are displayed in Figure S1.*

As introduced in the results section, we screened 57 driver strains based on previous studies, either they were reported identifying a single (a pair of) dopaminergic neuron (DAN) in larvae or identifying only several DANs in the adult brain indicating the potential of identifying single dopaminergic neuron in larvae. In Figure 1, TH-GAL4 was used to cover all neurons in the DL1 cluster, while R58E02 and R30G08 were well known drivers for pPAM. Fly strains in Figure 1h, k, l, and m were reported as single DAN strains in larvae4, while strains in Figure 1e, f, g were reported identifying only several DANs in adult brains5,6. We examined these strains and only some of them labeled single DANs in 3rd instar larval brains (Figure 1f, g, h, l and m). Among them, only strains in Figure 1f and h labeled single DAN in the brain hemisphere, without labeling other non-DANs. Other strains labeled non-DANs in addition to single DANs (Figure 1g, l and m). Taking ventral nerve cord (VNC) into consideration, strain in Figure 1h also labeled neurons in VNC (Figure S1e), while strain in Figure 1f did not (Figure S1c).

In summary, the strain in Figure 1f (R76F02AD;R55C10DBD, labeling DAN-c1) is a strain we screened labeling only a single DAN in the 3rd instar larval brains. Others (Figure 1g, h, l, and m) we still describe them as strains labeling single DANs, but they also label one to several non-DANs. In Figure 1, we mainly showed the strains labeling single DANs. The labeling patterns of other screened driver strains were summarized in Table1. Since all brain images of the rest 47 strains are available, we will state in Fig S1 that additional brain images can be provided upon request.

> *Weakness #2: Critically, R76F02-AD; R55C10-DBD labels more than one neuron per hemisphere in Figure S1c, and the authors cite Xie et al. (2018) to note that this driver labels two DANs in adult brains. Therefore, the authors cannot argue that the experiments throughout their paper using this driver exclusively target DAN-c1.*

Figure S1c shows single DA neuron in each brain hemisphere. Additional GFP (+) signals were often observed, but not from cell bodies of DANs because they were not stained by a TH antibody. These additional GFP (+) signals were mainly neurites, including axonal terminals, but could be false positive signals or weakly stained non-neuronal cell bodies. This

conclusion was based on analysis of a total of 22 larval brains. We will add this in the text or Fig S1 caption. Enlarged insert of GFP (+) signals will be added also to Figure S1c.

> *Weakness #3: Missing from the screen of 57 drivers is the driver MB320C, which typically labels only PPL1-γ1pedc in the adult and should label DAN-c1 in the larva. If MB320C labels DAN-c1 exclusively in the larva, then the authors should repeat their key experiments with MB320C to provide more evidence for DAN-c1 involvement specifically.*

We thank the reviewer for the suggestion. MB320C mainly labels PPL1-γ1pedc in the adult brain, with one or two other weakly labeled cells. It will be interesting to investigate the pattern of this driver in 3rd instar larval brains. If it only covers DAN-c1, we can try to knock-down D2R in this strain to check whether it can repeat our results. This will be an interesting fly strain to test, but we believe that it will not be necessary for our current manuscript as DAN-c1 driver is very specific (for details, refer to our response to Reviewer#3). However, this line will be very useful for future experiments.

> *Weakness #4: The authors claim that the SS02160 driver used by Eschbach et al. (2020) labels other neurons in addition to DAN-c1. Could the authors use confocal imaging to show how many other neurons SS02160 labels? Given that both Eschbach et al. and Weber et al. (2023) found no evidence that DAN-c1 plays a role in larval aversive learning, it would be informative to see how SS02160 expression compares with the driver the authors use to label DAN-c1.*

We did not have our own images showing DANs in brains of SS02160 driver cross line. However, Extended Data Figure 1 in the paper of Eschbach et al. (2020) shows strongly labeled four neurons on each brain hemisphere9, indicating that this driver is not a strain only labeling one neuron, DAN-c1.

> *Weakness #5: The claim that DAN-c1 is both necessary and sufficient in larval aversive learning should be reworded. Such a claim would logically exclude any other neuron or even the training stimuli from being involved in aversive learning (see Yoshihara and Yoshihara (2018) for a detailed discussion of the logic), which is presumably not what the authors intended because they describe the possible roles of other DANs during aversive learning in the discussion.*

We agree that the words ‘necessary’ and ‘sufficient’ are too exclusive for other neurons. As mentioned in the Discussion part, we do think other dopaminergic neurons may also be involved in larval aversive learning. We are going to re-phrase these words by replacing them with more logically appropriate words, such as ‘important’, ‘essential’, or ‘mediating’.

> *Weakness #6: Moreover, if DAN-c1 artificial activation conveyed an aversive teaching signal irrespective of the gustatory stimulus, then it should not impair aversive learning after quinine training (Figure 2k). While the authors interpret Figure 2k (and Figure 5) to indicate that artificial activation causes excessive DAN-c1 dopamine release, an alternative explanation is that artificial activation compromises aversive learning by overriding DAN-c1 activity that could be evoked by quinine.*

This is a great point! Yes, we cannot rule out the possibility that artificial activation compromises aversive learning by overriding DAN-c1 activity that could be evoked by quinine. The experimental results with TRPA1 could be caused by depletion of dopamine, or DA inactivation due to prolonged depolarization or adaptation. However, we still think that our hypothesis on the over-excitation of DAN-c1 is more consistent with our experimental results and other published data. Our justification is as follows:

(1) Associative learning occurs only when the CS and US are paired. In wild type larvae, a specific odor (conditioned stimulus, CS, such as pentyl acetate) depolarizes a subset of Kenyon cells in the mushroom body, while gustatory unconditioned stimulus (US, quinine) induces dopamine release from DAN-c1 to the lower peduncle (LP) compartment in the mushroom body (Figure 7a). Only when the CS and US are paired, calcium influx caused by CS and Gas activated by D1R binding to dopamine will turn on a mushroom body specific version of adenylyl cyclase, rutabaga, which is the co-incidence detector in associative learning (Figure 7d).

(2) Rutabaga transforms ATP into cAMP, activating PKA signaling pathway and modifying the synaptic strength from mushroom body neurons (MBN, also called Kenyan cells) to the mushroom body output neurons (MBON, Figure 7d). This change in synaptic strength will lead to learned responses when the same odor appears again.

(3) In our work, we found D2R is expressed in DAN-c1, and knockdown D2R in DAN-c1 impairs larval aversive learning. As D2R reduces cAMP level and neuronal excitability3, we hypothesized that knockdown of D2R in DAN-c1 would remove the inhibition of D2R auto-receptor, and lead to more dopamine (DA) release when US (quinine) was delivered compared to the wild type larvae. The elevated DA release along with calcium influx caused by CS increases the cAMP level in MBN, which leads to the learning deficit (over-excitation, Figure 7b). Mutant larvae with excessive cAMP, *dunce*, showed aversive learning deficiency, supporting our hypothesis2.

(4) Our results of TRPA1 can be explained by this over-excitation hypothesis. When DAN-c1 is activated (34C) in distilled water group, the artificial activation mimicked the gustatory activation of quinine. The larvae showed the aversive learning responses towards the odor (Figure 2k DW group). When DAN-c1 is activated (34C) in sucrose group, the artificial activation mimicked the gustatory activation of quinine, so the larvae showed a learning response combining both appetitive and aversive learning (Figure 2k SUC group).

(5) When DAN-c1 is activated (34C) in quinine group, the artificial activation and the gustatory activation of quinine lead to elevated DA release from DAN-c1. During training, this elevated DA caused over-excitation of MBN, leading to failure of aversive learning (Figure 2k QUI group), which had a similar phenotype compared to larvae with D2R knockdown in DAN-c1.

(6) Similarly, optogenetic activation of DAN-c1 during aversive training, leads to elevated DA release from DAN-c1 (both gustatory activation of quinine and artificial activation). This would also cause over-excitation of MBN, and lead to failure of aversive learning. Artificial activation in other stages (resting or testing) won't cause elevated DA release during training, so the aversive learning was not affected (Figure 5b).

(7) However, when optogenetic activation was applied during training, we did not observe aversive learning responses in the distilled water group, or a reduction in the sucrose group (Figure 5c, Figure 5d). Our explanation is that the optogenetic stimulus we applied is too strong, DAN-c1 has already released elevated DA in both groups. So, the aversive learning in these groups has already been impaired, they just showed the corresponding learning responses to distilled water or sucrose.

(8) We also applied this over-excitation to activate MBNs. As MBN takes over both appetitive and aversive learnings, over-excitation of MBNs led to deficit in both types of learning, which follows our hypothesis (Figure 6).

In summary, we hypothesized that DAN-c1 restricts DA release via activation of D2R, which is important for larval aversive learning. D2R knockdown or artificial activation of DAN-c1

during training would induce elevated DA release, leading to over-excitation of MBNs and failure of aversive learning.

> *Weakness #7: The authors should not necessarily expect that D2R enhancer driver strains would reflect D2R endogenous expression, since it is known that TH-GAL4 does not label p(PAM) dopaminergic neurons.*

Just like the example of TH-GAL4, it is possible that the D2R driver strains may partially reflect the expression pattern of endogenous D2R in larval brains. When we crossed the D2R driver strains with the GFP-tagged D2R strain, however, we observed co-localization in DM1 and DL2b dopaminergic neurons, as well as in mushroom body neurons (Figure S3 c to h). In addition, D2R knockdown with D2R-miR directly supported that the GFP-tagged D2R strain reflected the expression pattern of endogenous D2R (Figure 4b to d, signals were reduced in DM1). In summary, we think the D2R driver strains supported the expression pattern we observed from the GFP-tagged D2R strain, especially in DM1 DANs.

> *Weakness #8: Their observations of GFP-tagged D2R expression could be strengthened with an anti-D2R antibody such as that used by Lam et al., (1999) or Love et al., (2023).*

Love et al., (2023) used the antibody from Draper et al.10. We have tried the same antibody, but we were not able to observe clear signals after staining. Maybe it is not specific for the neurons in the fly larval brain, or our staining protocol did not fit with this antibody.

Unfortunately, we were not able to find Lam (1999) paper.

> *Weakness #9: Finally, the authors could consider the possibility other DANs may also mediate aversive learning via D2R. Knockdown of D2R in DAN-g1 appears to cause a defect in aversive quinine learning compared with its genetic control (Figure S4e). It is unclear why the same genetic control has unexpectedly poor aversive quinine learning after training with propionic acid (Figure S5a). The authors could comment on why RNAi knockdown of D2R in DAN-g1 does not similarly impair aversive quinine learning (Figure S5b).*

We also think that other DANs may be involved in aversive learning. We re-analyzed the learning assay data, seemingly D2R knockdown in DAN-g1 with miR partially affected aversive learning when trained with pentyl acetate (Figure S4e). We are going to build single statistic panels for DAN-g1 and DAN-d1. However, neither larvae with D2R knockdown in DAN-g1 using miR trained with propionic acid (Figure S5a), nor larvae with D2R knockdown in DAN-g1 using RNAi trained with pentyl acetate (Figure S5b) showing aversive learning deficit. We will add paragraphs about this in both Results and Discussion sections.

> ***Reviewer #2 (Public Review):***
>
> *Weakness#1: Is not completely clear how the system DAN-c1, MB neurons and Behavioral performance work. We can be quite sure that DAN-c1;Shits1 were reducing dopamine release and impairing aversive memory (Figure 2h). Similarly, DAN-c1;ChR2 were increasing dopamine release and also impaired aversive memory (Figure 5b). However, is not clear what is happening with DAN-c1;TrpA1 (Figure 2K). In this case the thermos-induction appears to impair the behavioral performance of all three conditions (QUI, DW and SUC) and the behavior is quite distinct from the increase and decrease of dopamine tone (Figure 2h and 5b).*
>
> *The study successfully examined the role of D2R in DAN-c1 and MB neurons in olfactory conditioning. The conclusions are well supported by the data, with the exception of the claim that dopamine release from DAN-c1 is sufficient for aversive learning in the*

> *absence of unconditional stimulus (Figure 2K). Alternatively, the authors need to provide a better explanation of this point.*

Please refer to our response to Weakness #6 of Public Reviewer #1.

> ***Reviewer #3 (Public Review):***
>
> *Weakness #1: It is a strength of the paper that it analyses the function of dopamine neurons (DANs) at the level of single, identified neurons, and uses tools to address specific dopamine receptors (DopRs), exploiting the unique experimental possibilities available in larval Drosophila as a model system. Indeed, the result of their screening for transgenic drivers covering single or small groups of DANs and their histological characterization provides the community with a very valuable resource. In particular the transgenic driver to cover the DANc1 neuron might turn out useful. However, I wonder in which fraction of the preparations an expression pattern as in Figure 1f/ S1c is observed, and how many preparations the authors have analyzed. Also, given the function of DANs throughout the body, in addition to the expression pattern in the mushroom body region (Figure 1f) and in the central nervous system (Figure S1c) maybe attempts can be made to assess expression from this driver throughout the larval body (same for Dop2R distribution).*

We thank the reviewer for the positive comments and the suggestions. For the strain R76F02AD; R55C10DBD, we examined 22 third instar larval brains expressing GFP or Syt-GFP and Den-mCherry, all of them clearly labeled DAN-c1. Half of them only labeled DAN-c1, the rest have 1 to 5 weak labeled soma without neurites. Barely 1 or 2 strong labeled cells appear. These non-DAN-c1 neurons are seldom dopaminergic neurons. In VNC, 8 out of 12 do not label cells, 3 have 2-4 strong labeled cells. These data supported that R76F02AD;R55C10DBD exclusively labeled DAN-c1 in 3rd instar larval brains.

For the question about the pattern of R76F02AD; R55C10DBD and the expression pattern of D2R in larval body, it is an interesting question. However, our main focus was on the central nervous system and the learning behaviors in fruit fly larvae, we may investigate this question in the future.

> *Weakness #2: A first major weakness is that the main conclusion of the paper, which pertains to associative memory (last sentence of the abstract, and throughout the manuscript), is not justified by their evidence. Why so? Consider the paradigm in Figure 2g, and the data in Figure 2h (22 degrees, the control condition), where the assay and the experimental rationale used throughout the manuscript are introduced. Different groups of larvae are exposed, for 30min, to an odour paired with either i) quinine solution (red bar), ii) distilled water (yellow bar), or iii) sucrose solution (blue bar); in all cases this is followed by a choice test for the odour on one side and a distilled-water blank on the other side of a testing Petri dish. The authors observe that odour preference is low after odour-quinine pairing, intermediate after odour-water pairing and high after odour-sucrose pairing. The differences in odour preference relative to the odour-water case are interpreted as reflecting odour-quinine aversive associations and odour-sucrose appetitive associations, respectively. However, these differences could just as well reflect non-associative effects of the 30-min quinine or sucrose exposure per se (for a classical discussion of such types of issues see Rescorla 1988, Annu Rev Neurosci, or regarding Drosophila Tully 1988, Behav Genetics, or with some reference to the original paper by Honjo & Furukubo-Tokunaga 2005, J Neurosci that the authors reference, also Gerber & Stocker 2007, Chem Sens).*
> *As it stands, therefore, the current 3-group type of comparison does not allow conclusions about associative learning.*

We adopted this single odor larval learning paradigm from Honjo's papers1,2. In these works, Honjo et al. first designed and performed this single odor paradigm for larval olfactory associative learning. To address the reviewer's question about the potential non-associative effects of the 30-min quinine or sucrose exposure, we would like to defend it primarily based on results from Honjo et al. (2005 and 2009). They applied the odorant to the larvae after training, only the ones had paired training with both odor and unconditioned stimulus (quinine or sucrose) showed learning responses. Larvae exposed 30 min in only odorant or unconditioned stimulus did not show different response to the odor compared to the naïve group1,2. To validate this paradigm induces associative learning responses, they also tested the paradigm from three aspects:

(1) The odor responses are associative. Honjo et al. showed only when the odorant paired with unconditioned stimulus would induce corresponding attraction or repulsion of larvae to the odor. Neither odorant alone, unconditioned stimulus alone, nor temporal dissociation of odorant and unconditioned stimulus would induce learning responses.

(2) The odor responses are odor specific. When applied a second odorant that was not used for training, larvae only showed learning responses to the unconditioned stimulus paired odor. This result ruled out the explanation of a general olfactory suppression and indicates larvae can discriminate and specifically alter the responses to the odor paired with unconditioned stimulus. Although the two-odor reciprocal training is not used, these results can show the association of unconditioned stimulus and the corresponding paired odor.

(3) Well known learning deficit mutants did not show learned responses in this learning paradigm. Honjo et al. tested mutants (e.g., *rut* and *dnc*) showing learning deficits in the adult stage with two odor reciprocal learning paradigm. These mutant larvae also failed to show learning responses tested with the single odor larval learning paradigm.

(4) In our study, we used two distinct odorants (pentyl acetate and propionic acid), as well as two D2R knockdown strains (UAS-miR and UAS-RNAi for D2R). We obtained similar results for larvae with D2R knockdown in DAN-c1. In addition, our naïve olfactory, naïve gustatory, and locomotion data ruled out the possibilities that the responses were caused by impaired sensory or motor functions. Comparison with the control group (odor paired with distilled water) ruled out the potential effects if habituation existed. All these results supported this single odor learning paradigm is reliable to assess the learning abilities of *Drosophila* larvae. And the failure of reduction in R.I when larvae with D2R knockdown in DAN-c1 were trained in quinine paired with the odorant is caused by deficit in aversive learning ability. We will add a paragraph to address this in the Discussion part.

> *Weakness #3: A second major weakness is apparent when considering the sketch in Figure 2g and the equation defining the response index (R.I.) (line 480). The point is that the larvae that are located in the middle zone are not included in the denominator. This can inflate scores and is not appropriate. That is, suppose from a group of 30 animals (line 471) only 1 chooses the odor side and 29, bedazzled after 30-min quinine or sucrose exposure or otherwise confused by a given opto- or thermogenetic treatment, stay in the middle zone... a P.I. of 1.0 would result.*

It is a good question. We gave 5 min during the testing stage to allow the larvae to wander in the testing plate. Under most conditions, more than half of larvae (>50%) will explore around, and the rest may stay in the middle zone (will not be calculated). We used 25-50 larvae in each learning assay, so finally around 10-30 larvae will locate in two semicircular areas. Indeed, based on our raw data, a R.I. of 1 seldom appears. Most of the R.I.s fall into a region from -0.2 to 0.8. We should admit that the calculation equation of R. I. is not linear, so it would be sharper (change steeply) when it approaching to -1 and 1. However, as most of the values

fall into the region from -0.2 to 0.8, we think 'border effects' can be neglected if we have enough numbers of larvae in the calculation (10-30).

> *Weakness #4: Unless experimentally demonstrated, claims that the thermogenetic effector shibire/ts reduces dopamine release from DANs are questionable. This is because firstly, there might be shibire/ts-insensitive ways of dopamine release, and secondly because shibire/ts may affect co-transmitter release from DANs.*

Shibirets1 gene encodes a thermosensitive mutant of dynamin, expressing this mutant version in target neurons will block neurotransmitter release at the ambient temperature higher than 30C, as it represses vesicle recycling1. It is a widely used tool to examine whether the target neuron is involved in a specific physiological function. We cannot rule out that there might be *Shibirets1* insensitive ways of dopamine release exist. However, blocking dopamine release from DAN-c1 with *Shibirets1* has already led to learning responses changing (Figure 2h). This result indicated that the dopamine release from DAN-c1 during training is important for larval aversive learning, which has already supported our hypothesis.

For the second question about the potential co-transmitter release, we think it is a great question. Recently Yamazaki et al. reported co-neurotransmitters in dopaminergic system modulate adult olfactory memories in _Drosophila_11, and we cannot rule out the roles of co-released neurotransmitters/neuropeptides in larval learning. Ideally, if we could observe the real time changes of dopamine release from DAN-c1 in wild type and TH knockdown larvae would answer this question. However, live imaging of dopamine release from one dopaminergic neuron is not practical for us at this time. On the other hand, the roles of dopamine receptors in olfactory associative learning support that dopamine is important for *Drosophila* learning. D1 receptor, dDA1, has been proven to be involved in both adult and larval appetitive and aversive learning12,13. In our work, D2R in the mushroom body showed important roles in both larval appetitive and aversive learning (Figure 6a). All this evidence reveals the importance of dopamine in *Drosophila* olfactory associative learning. In addition, there is too much unknow information about the co-release neurotransmitter/neuropeptides, as well as their potential complex 'interaction/crosstalk' relations. We believe that investigation of co-released neurotransmitter/neuropeptides is beyond the scope of this study at this time.

> *Weakness #5: It is not clear whether the genetic controls when using the Gal4/ UAS system are the homozygous, parental strains (XY-Gal4/ XY-Gal4 and UAS-effector/ UAS-effector), or as is standard in the field the heterozygous driver (XY-Gal4/ wildtype) and effector controls (UAS-effector/ wildtype) (in some cases effector controls appear to be missing, e.g. Figure 4d, Figure S4e, Figure S5c).*

Almost all controls we used were homozygous parental strains. They did not show abnormal behaviors in either learnings or naïve sensory or locomotion assays. The only exception is the control for DAN-c1, the larvae from homozygous R76F02AD; R55C10DBD strain showed much reduced locomotion speed (Figure S6). To prevent this reduced locomotion speed affecting the learning ability, we used heterozygous R76F02AD; R55C10DBD/wildtype as control, which showed normal learning, naïve sensory and locomotion abilities (Figure 4e to i).

For Figure 4d, it is a column graph to quantify the efficiency of D2R knockdown with miR. Because we need to induce and quantify the knockdown effect in specific DANs (DM1), only TH-GAL4 can be used as the control group, rather than UAS-D2R-miR.

For the missing control groups in Figure S4e and S5c, we have shown them in other Figures (Figure 4e). We will re-organize the figures to make them easier to understand.

> *Weakness #6: As recently suggested by Yamada et al 2024, bioRxiv, high cAMP can lead to synaptic depression (sic). That would call into question the interpretation of low-Dop2R leading to high-cAMP, leading to high-dopamine release, and thus the authors interpretation of the matching effects of low-Dop2R and driving DANs.*

We will read through this paper and try to add it as possible explanations for the learning mechanisms. As we introduced in the Discussion section, the learning mechanism is quite complex, mixing both non-linear neuronal circuits and multiple signaling pathways, in responding to complex environmental learning contexts. We will try to develop a better hypothesis with the best compatibility to accommodate our results with published data.

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