Neuroscience

A pair of dopaminergic neurons DAN-c1 mediate *Drosophila* larval aversive olfactory learning through D2-like receptors

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This study presents **valuable** findings 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** in the context of their behavioural paradigm. Controls using a reciprocal training protocol would have broadened the scope of their conclusions. The work will be of interest to researchers studying the role of dopamine during learning and memory.

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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 a pair of dopaminergic neurons (DAN) in the larval brain. 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.

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 (a center for *Drosophila* learning and memory) in both larval and adult brains[26,27]. Mutations of these genes lead to learning deficiencies, indicating their roles in larval[17,18] and adult 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], projection neurons (PNs)[30], mushroom body neurons (MBNs)[31], and dopaminergic neurons (DANs)[32], 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[33], 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[34] (or Dop1R1) and DAMB[35] (or Dop1R2), one D2-like receptor D2R[36] (or Dop2R), and one non-canonical receptor DopEcR[14,37]. dDA1 is mainly found in the mushroom body[38,39] and is necessary for appetitive and aversive olfactory learning in larvae[39] and adults[40]. D2R in the adult mushroom body is necessary for anesthesia-resistant memory[41]. In addition, D2R in GABAergic anterior paired lateral (APL) neurons is known to secure aversive conditioning in adult flies[42]. Although D2R expression has been reported in the ventral nerve cord[43], 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 a pair of DANs, 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 circuitries has been investigated in both first- and third-instar larvae[28,44]. The mushroom body serves as a primary learning center in *Drosophila*, which is composed by αβ, α'β', and γ neurons in adult brains[45]. In larvae, axons from γ neurons bifurcate and form the vertical and medial lobes, as αβ and α'β' neurons are not mature[31,46,47]. 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 third-instar brain hemisphere, and categorized into 4 clusters: DM1 (dorsomedial), pPAM (primary protocerebral anterior medial, or DM2)[48], DL1 (dorsolateral), and DL2[32]. DL1 neurons project to the vertical lobe[39], while pPAM neurons innervate the medial lobe[48] (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 a few dopaminergic neurons, which subsequently can be used to target genetic manipulations of corresponding neurons. A total of 57 driver strains identifying dopaminergic neurons were screened in this study (**Table 1**). These strains were chosen based on previous studies, either identifying a pair of dopaminergic neurons in larvae, or identifying only several in adult brains and indicating the potential of identifying a pair of DANs in larvae. TH-GAL4, a traditional dopaminergic neuronal driver[49], 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**)[50]. 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[44]. We found three DAN driver strains that identify a pair of dopaminergic neurons in the third-instar larval brain. DAN driver R76F02-AD;R55C10-DBD identifies a pair of dopaminergic neurons innervating the lower peduncle (LP), which would be DAN-c1 based on previous published nomenclature[28] (**Figure 1f**). MB296B driver identifies the dopaminergic neurons (DAN-d1) projecting to the lateral appendix (LA) (**Figure 1g**), as well as many non-dopaminergic neurons. SS1716 driver identifies a pair of dopaminergic neurons forming synapses in the lower vertical lobe (LVL), indicating it is DAN-g1 (**Figure 1h**).

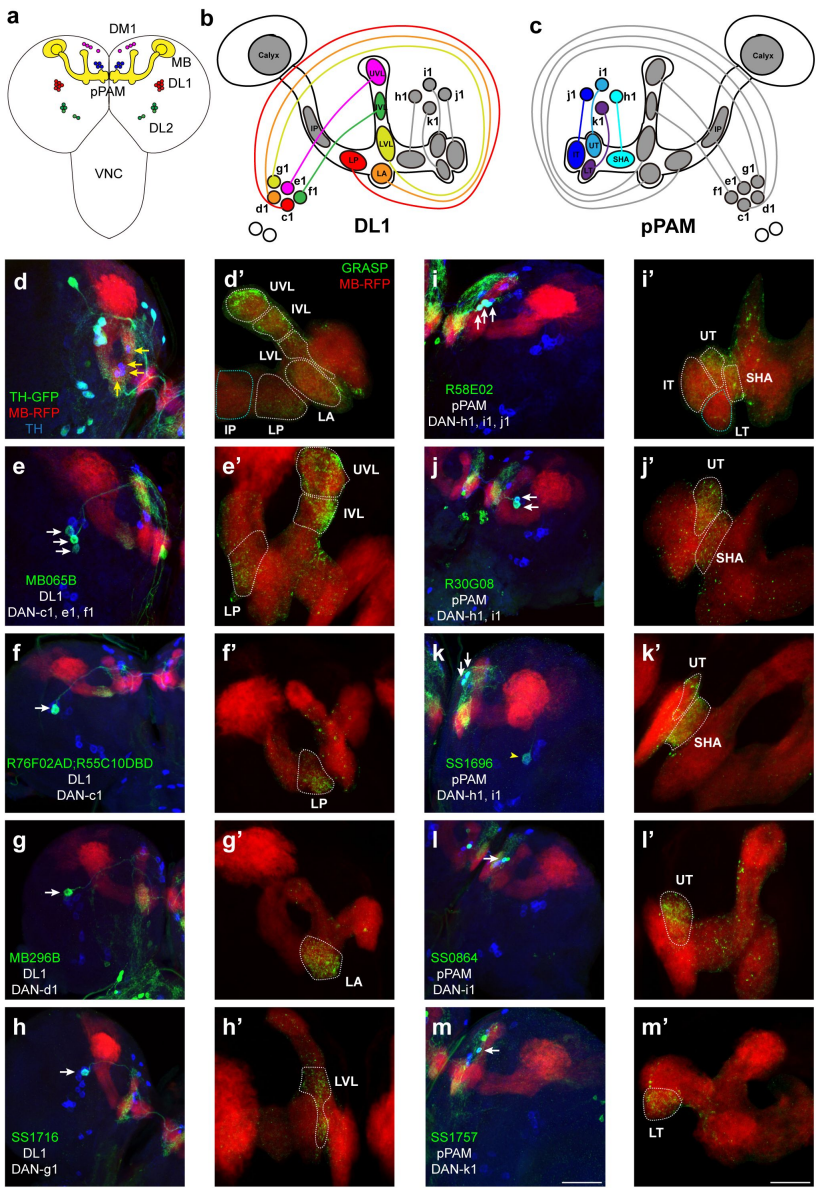

Figure 1.

Identification of driver strains for a pair of 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 (DANs) 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 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**. *N numbers for each strain can be found in* **Table S2**.

	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)	-	

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

15	MB109B	-	-	-	PAM-β'2a, PAM-γ5 (Adult)	-	Janelia Farm (Aso et al., 2014)
16	MB301B	-	-	-	PAM-β'2m, PAM-β2β'2a (Adult)	-	
17	MB056B	-	-	-	PAM-β'2m, PAM-β'2p (Adult)	-	
18	MB032B	-	-	-	PAM-β'2m, PAM-β'2p, PAM-β2β'2a, PAM-γ3 (Adult)	-	
19	MB312B	-	-	-	PAM-γ4, PAM-γ4<γ1γ2 (Adult)	-	
20	MB194B	-	-	-	PAM-α1, PAM-β'2a, PAM-β1, PAM-β1ped, PAM-β2 (Adult)	-	
21	MB063B	-	-	-	PAM-β1 (Adult)	-	
22	MB043B	-	h1, i1, j1	Weak, lots of MBN	PAM-α1, PAM-β'1 ap, PAM-β'1 m, PAM-β1 (Adult)	-	
23	MB441B	-	-	-	PAM-γ3 (Adult)	-	
24	MB025B	-	-	-	PAM-β'1ap/m (Adult)	-	
25	MB438B	-	-	-	PPL1-α'2α2, PPL1-α3, PPL1-γ1pedc (Adult)	-	
26	MB296B	DAN-d1	d1	Some in VNC	PPL1-γ2α'1 (Adult)	-	
27	MB304B	-	-	-	PPL1-α'3 (Adult)	-	

Table 1. (continued)

28	MB058B	-	-	-	PPL1-α'2α'2 (Adult)	-	
29	MB065B	-	c1, f1, e1	-	PPL1-α'2α2, PPL1-α'3, PPL1-α3, PPL1-γ2α'1 (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	
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)	-	

Table 1. (continued)

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	

Table 1. (continued)

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 neuronal driver strains screened can be used to investigate the roles of individually identified DANs in larval olfactory learning.

Dopamine release from DAN-c1 mediates larval aversive learning

Dopamine plays an important role during olfactory associative learning in both adults[51,52] and larvae[17,18]. In adults, dopaminergic neurons in PPL1 regulate aversive learning[53–56], while those in PAM mediate reward signals in appetitive learning[57–59]. In larvae, DL1 neurons innervating the vertical lobe and the peduncle are required for aversive learning[18,39], while those in pPAM projecting to the medial lobe are involved in appetitive learning[48].

In **Figure 1** and **Table 1**, three driver strains identifying distinct pairs of 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[60], which is a dopaminergic neuron involved in adult aversive learning[61]. Based on the analysis with 22 brain samples, we observed this driver strain labels one neuron per hemisphere in the third-instar larval brain (**Figure 2a-d**, **Figure S1c**, **Table S3**). 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 c**), with GRASP results supporting the existence of synapses in this compartment (**Figure 1f'**). All these characteristics are consistent with the previously published nomenclature[28], indicating that this pair of neurons are DAN-c1 (**Figure 2d**), 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[17,18,62]. Compared to those trained with distilled water (DW), control strains of 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 2e**; For further details, refer to the **Materials and Methods** section).

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 with QUI at 34°C, larvae with *Shibire^{ts1}* expression in DAN-c1 showed significantly increased R.I. compared to that at room temperature (22°C), while it is not significantly different from the DW at 34°C group (**Figure 2f**). The complete inactivation of dopamine release from DAN-c1 with *Shibire^{ts1}* impaired aversive learning, indicating that dopamine release from DAN-c1 is important for larval aversive learning to occur.

In the next experiments, a fly strain 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[57]. Compared to those at 22°C, activation of DAN-c1 with dTRPA1 at 34°C during training

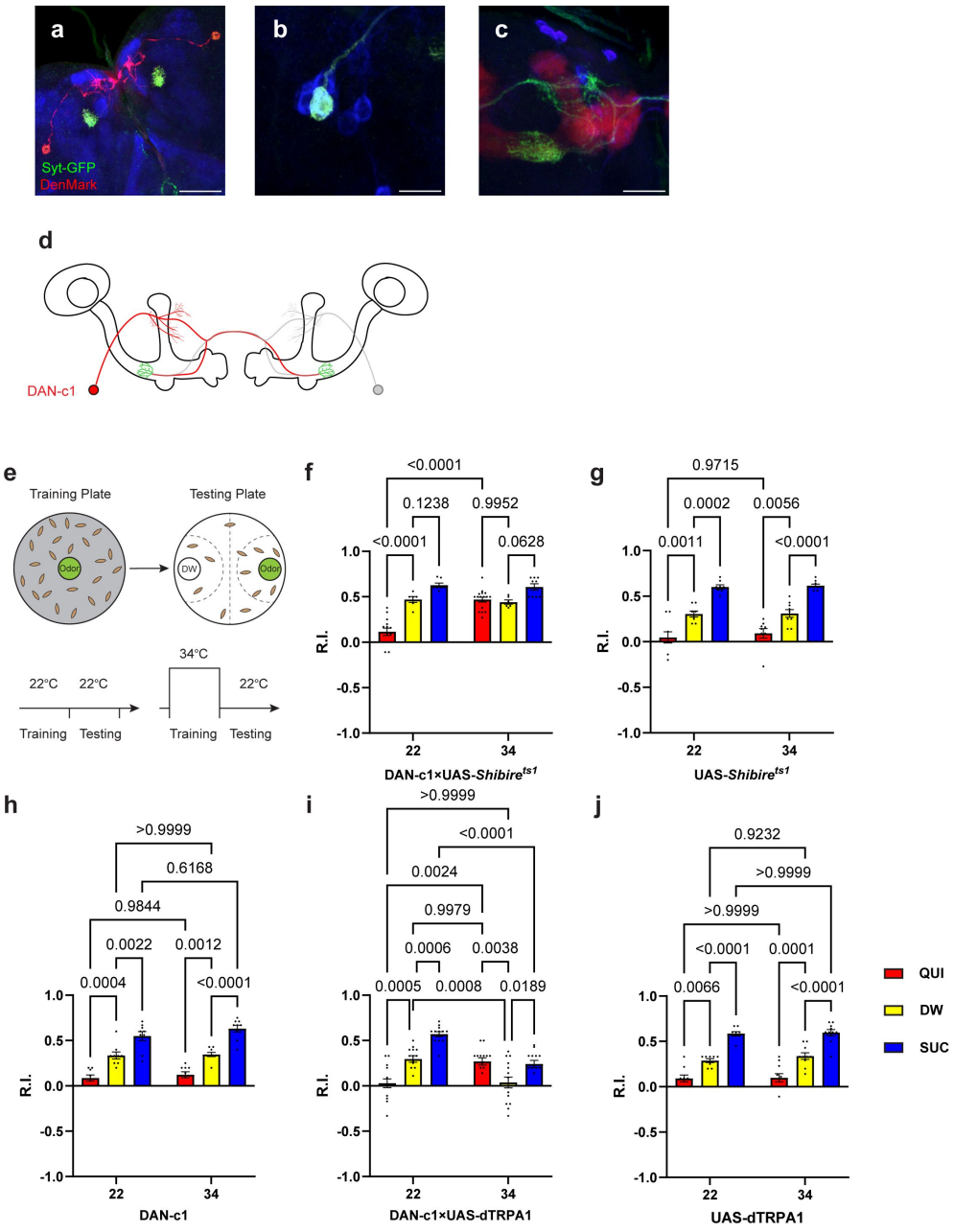

Figure 2.

Dopamine release from DAN-c1 mediates larval aversive learning.

(a-d) 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** and **c)** Soma and neurites from **Figure 1f** with higher magnification. DAN-c1 is labeled with GFP, the MB with RFP, and DANs with TH antibody (blue color). Only one DAN soma is identified (b), axons from DAN-c1 innervate the lower peduncle (LP) of the MB (c). A schematic diagram (**d**) shows the innervation patterns of DAN-c1. Modified from Eichler *et al.*[44], and Saumweber *et. al.*[28]. **(e)** A schematic paradigm for larval olfactory learning (top) and two different training paradigms for thermogenetics (bottom). **(f-h)** Blocking dopamine release from DAN-c1 during learning using *shibire*^{ts1}strain at 34°C impairs larval aversive learning. **(h-j)** Activation of DAN-c1 with dTRTPA1 at 34°C induces aversive learning. **QUI,** quinine. **DW,** distill water. **SUC,** sucrose. Data are shown as mean ± SEM. Two-way ANOVA, Tukey's multiple comparison test. For N numbers, interaction p-values, row factor p-values, and column factor p-values, see **Table S4**. Scale bars: 50 μm (a), 20 μm (b and c). *(Note) N numbers of immunostaining for each strain can be found in* **Table S2**.

induced repulsion to PA in the distilled water group, while it is not significantly different from the QUI group at 22°C(**Figure 2i**). These data suggested that DAN-c1 excitation and presumably increased dopamine release leads to 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 mediates 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 2i**). We suggest that these data indicate a critical role for the amount of dopamine release from DAN-c1 in larval associative learning, as dTRPA1 stimulation may result in excessive dopamine release (see the **Discussion** section).

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

Although dopamine D1-like receptors have been proven important for learning[40], 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)[47].

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[62]. Using a microRNA strain (UAS-D2R-miR)[60], a similar deficit was observed (**Figure S4f**). To further understand the roles of D2R in aversive learning, its expression in distinct DANs, as well as corresponding learning assays were 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 S4c**).

To investigate the roles of D2R in distinct dopaminergic neurons during larval associative learning, UAS-D2R-miR strain was crossed with distinct driver strains labeling different pairs of dopaminergic neurons. Among them, the knockdown of D2R in DAN-c1 impaired aversive learning with the odorant pentyl acetate, while appetitive learning was unaffected (**Figure 4e**). In

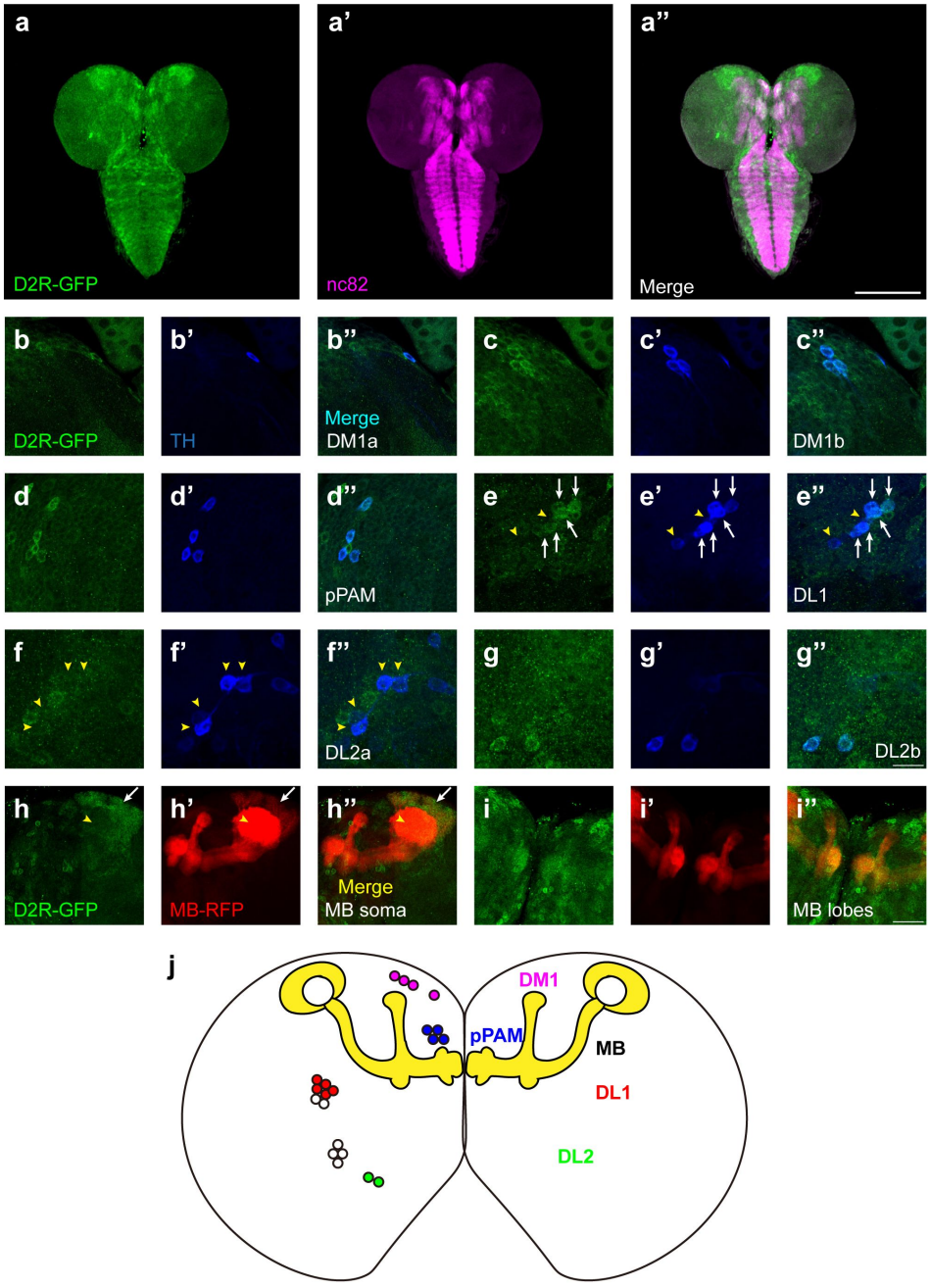

Figure 3.

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. *(Note) N numbers can be found in* **Table S2**.

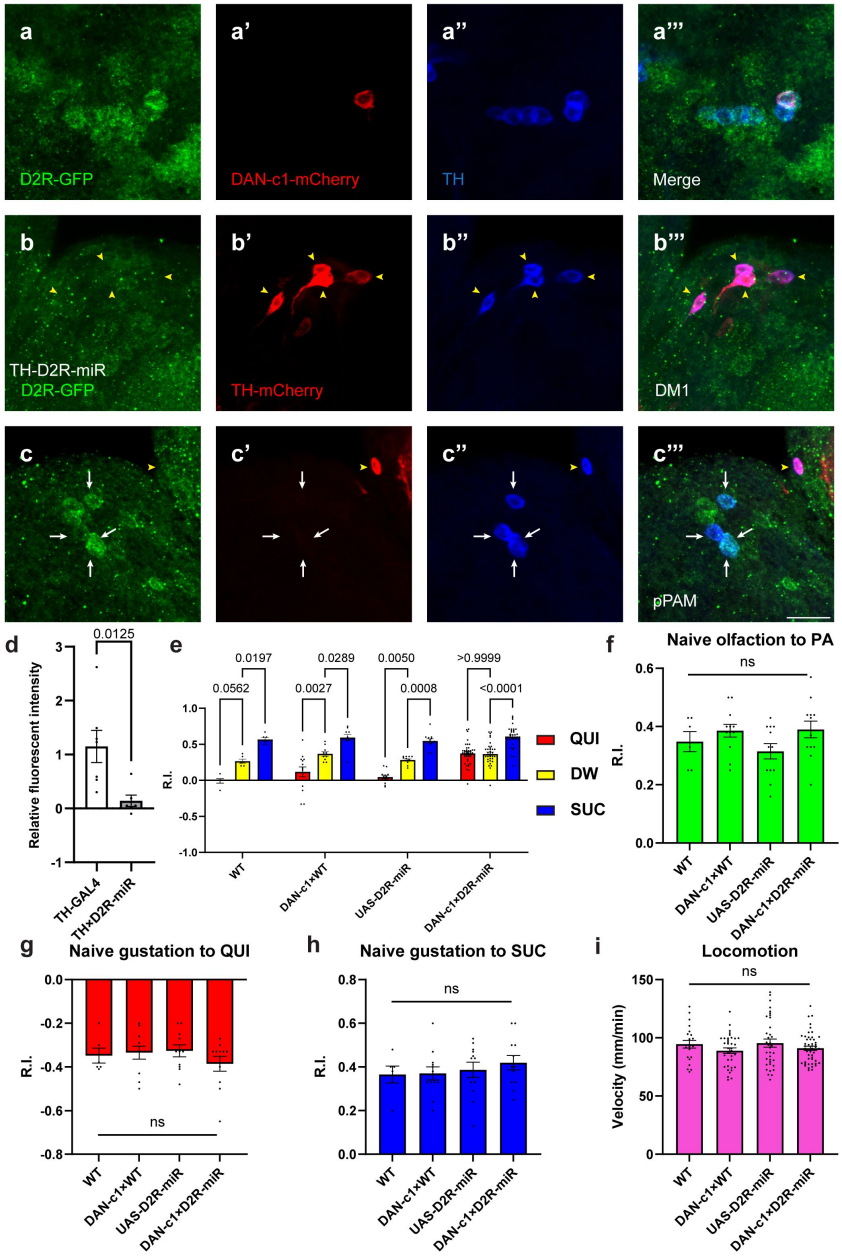

Figure 4.

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=7 brains; for the TH×D2R-miR group, N=6 brains. Unpaired t-test, p = 0.0125. **(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, Tukey's multiple comparison test, p < 0.0001 for interaction, row factor (genotype), and column factor (US) p-values. For N numbers, see **Table S5**. **(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's multiple comparison test. For N numbers, see **Table S5**. Scale bar: 20μm. *(Note)* **Figures S5** and **S6** *show additional information on naïve sensory and motor functions in larvae related to D2R-miR experiments. N numbers of immunostaining for each strain can be found in* **Table S2**.

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 S4d-f**, see the **Discussion** section). 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 2i**).

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[63,64]. 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, a 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 identifying a pair of DAN-c1 neurons in the third-instar larval brain was discovered and learning assays with thermogenetic tools (*Shibire*^{ts1}, dTRPA1) demonstrated 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 synaptic dopamine) mediates larval aversive learning to

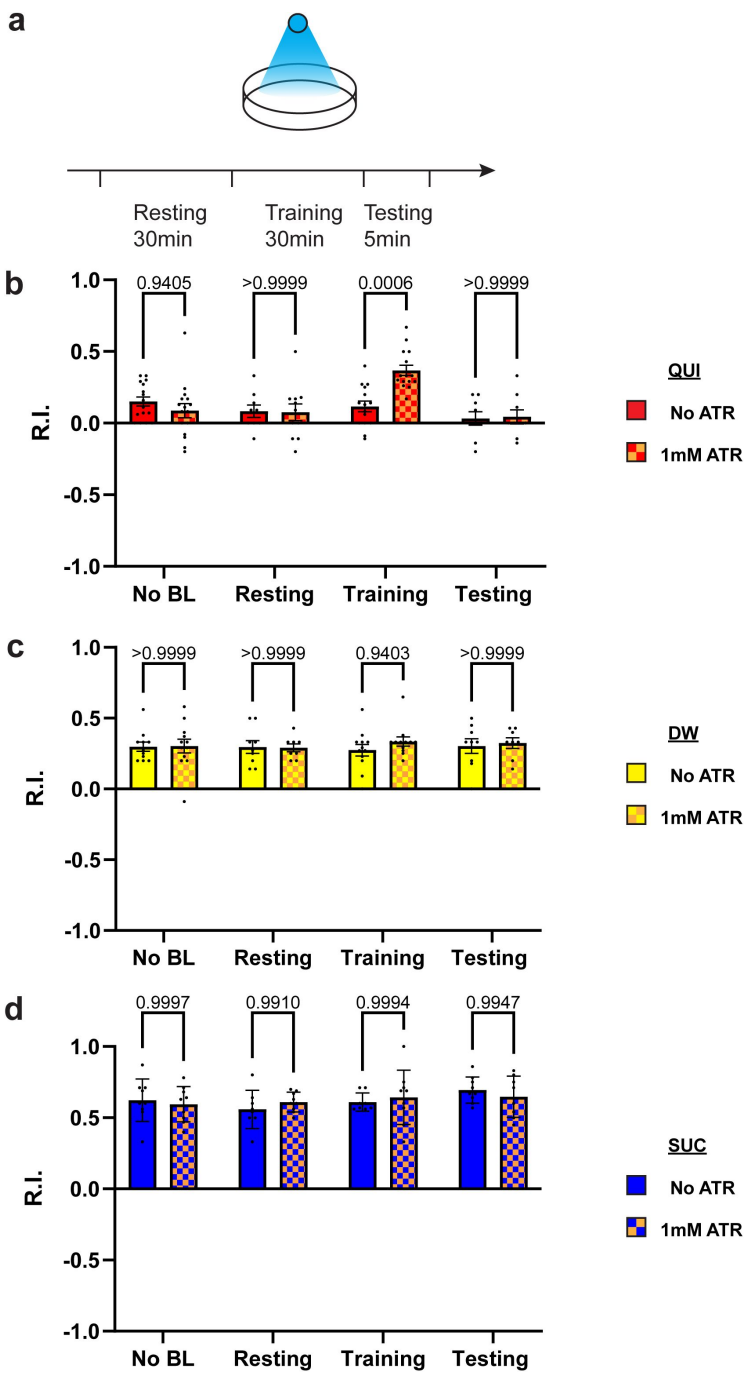

Figure 5.

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, Tukey's multiple comparison test. In QUI group p-values (b), p = 0.0009 for interaction, p < 0.0001 for row factor (training stages), and p = 0.1365 for column factor (whether with ATR); in DW group p-values (c), p = 0.8367 for interaction, p = 0.9750 for row factor (training stages), and p = 0.4872 for column factor (whether with ATR); in SUC group p-values (d), p = 0.6247 for interaction, p = 0.2550 for row factor (training stages), and p = 0.9437 for column factor (whether with ATR). For N numbers, see **Table S6**.

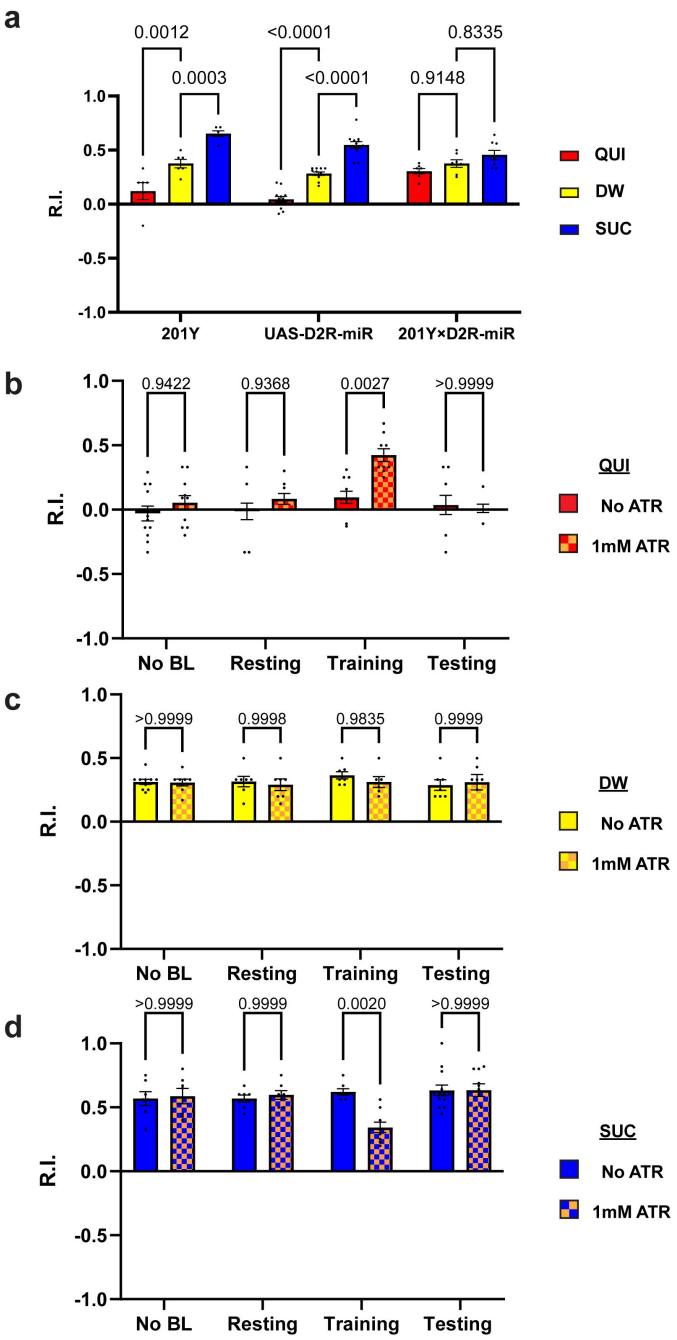

Figure 6.

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, Tukey's multiple comparison test. In D2R knockdown experiments (a), $p < 0.0001$ for interaction, $p = 0.0011$ for row factor (genotype), and $p < 0.0001$ for column factor (US). In optogenetic QUI group (b), $p = 0.0259$ for interaction, $p < 0.0001$ for row factor (training stages), and $p = 0.0040$ for column factor (whether with ATR); in DW group (c), $p = 0.8077$ for interaction, $p = 0.7623$ for row factor (training stages), and $p = 0.5846$ for column factor (whether with ATR); in SUC group p-values(d), $p = 0.0035$ for interaction, $p = 0.0086$ for row factor (training stages), and $p = 0.0865$ for column factor (whether with ATR). For N numbers, see **Table S5** and **S6**.

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 suggested that presynaptic D2Rs in a pair of dopaminergic neurons, 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.

The characteristics of the single odor larval learning paradigm

We adopted the single odor larval learning paradigm from previous publications[17,18]. To validate this paradigm induces associative learning responses, Honjo et al.[17,18] tested the paradigm from four aspects: First, the paradigm did not show obvious sensitization or habituation effects when larvae were tested. 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 for 30 minutes to either the odorant or the unconditioned stimulus alone did not show a different response to the odor compared to the naïve group. Second, the odor responses are associative. Honjo et al. showed only when the odorant was 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. Third, the odor responses are odor specific. When applied to a second odorant that was not used for training, larvae only showed learning responses to the odor paired with unconditioned stimulus. This result ruled out the explanation of a general olfactory suppression and indicated 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. Finally, well known learning deficit mutants did not show learned responses in this learning paradigm. Honjo et al. tested mutants (e.g., *rut* and *dnc*), which showed learning deficits in the adult stage with two odor reciprocal learning paradigm[21,25]. These mutant larvae also failed to show learning responses when tested with the single odor larval learning paradigm. Combining all the evidence above, we believe this single odor larval learning paradigm is a robust and reliable paradigm for larval associative learning assays, composing essential characteristics of classical conditioning. Previously, we applied this paradigm to investigate the roles of mushroom body serotonin receptors (5-HT7) in larval appetitive learning[65]. In this 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 using different odorants or D2R knockdown strains. 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 support this single odor learning paradigm is reliable to assess the learning abilities of *Drosophila* larvae. 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.

Insights into the neuronal circuits underlying larval olfactory associative learning

Mushroom body and dopaminergic neurons play important roles in *Drosophila* associative learning in both larvae[66] and adults[66–69]. Combining our results with previous learning circuitry research in adult flies and larvae, we hypothesized the mechanism underlying larval olfactory associative learning. 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 a primary 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) may be negatively modulated by dopamine, like those in adults[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.

When only the odorant appears, the subset of Kenyon cells representing this odor may be depolarized, inducing calcium influx as in adult flies[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. The co-existence of calcium and $G\alpha_s$ activates a Ca^{2+}-dependent adenylate cyclase (AC), rutabaga (rut, as in adults[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[28,53,66–69]. 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[80]. 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[60]. 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[61], and activation of its corresponding MBON-γ1pedc>α/β led to approach[81]. 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[82]. 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[83], 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

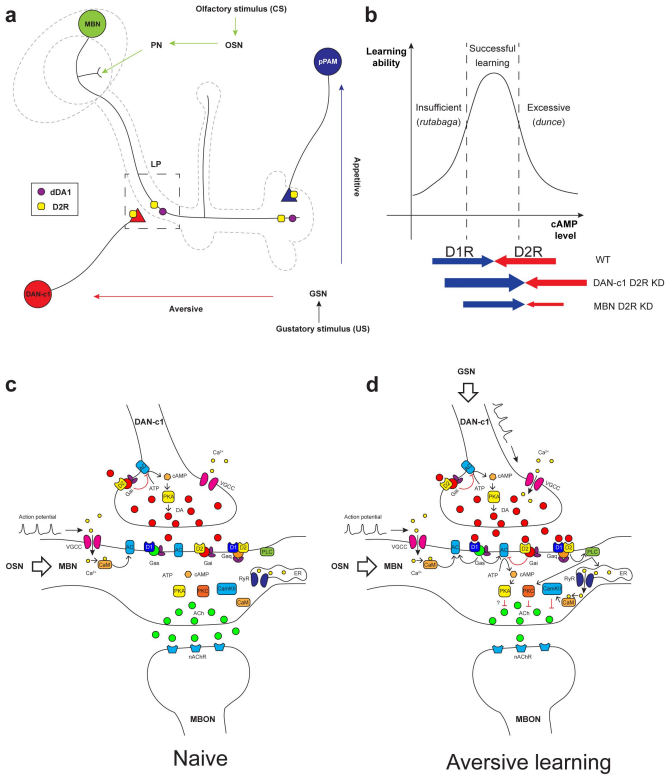

Figure 7.

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.

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 and d**).

Recent studies showed that the approach/repulsion in adult learning is achieved via inhibition of the repulsive/attractive representing compartments[67,74,75], which indicates dopamine inhibits acetylcholine release from MBN to MBON[84]. However, the PKA signaling pathway usually elevates neuronal excitability and increases neurotransmitter release[2,3], which is contradictory to the recent findings[85]. 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[86] (**Figure 7c and d**).

Explanation of 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 2i**). 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 limit (**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

Although D2Rs are also expressed in DAN-d1 and DAN-g1 (**Figure S4d** and **e**), the knockdown of D2R in these neurons did not impair larval aversive learning (**Figure S4f**). For DAN-g1, interestingly, the R.I. from D2R knockdown larvae trained with quinine (QUI, aversive learning)

showed significant difference when compared to the DW (control) group, but it was also significant different from the DAN-g1 genetic control group trained with QUI (two-way ANOVA, Tukey's multiple comparisons, p=0.0002), while not significant different from UAS-D2R-miR genetic control group trained with QUI (p=0.2724). Besides, D2R knockdown in DAN-g1 when trained with another odorant propionic acid (ProA) did not show aversive learning deficiency (**Figure S5a**). In addition, knockdown D2R in DAN-g1 using RNAi also did not show aversive learning deficiency when trained with odorant pentyl acetate (PA, **Figure S5b**). This discrepancy may be caused by the different stimulus intensity of distinct odorants, as well as the different efficiency of distinct knockdown methods (microRNA or RNAi strains we used). We supposed that D2Rs in DAN-g1 may partially affect larval aversive learning in a quantitative level; but not play an important role as those in DAN-c1, which will cause a qualitative change when knockdown.

Previous work reported that aversive olfactory learning was induced through the optogenetic activation of DAN-d1, f1, or g1, but not DAN-c1[87]. 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 S4f**). These data indicate D2Rs in DAN-d1 or g1 may not be important 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[87], indicating a functional differentiation from gustatory activated DAN-c1[61,88].

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[89,90]. The D2R-miR strain produces microRNA recognizing the sequence across the third and fourth exons, which is not affected by the GFP insertion (**Figure S4c**).

GRASP

Split-GFP reconstitution across synaptic partners (GRASP) was used to investigate whether neurons formed synapses[50] (**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 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

All control strains used in learning assays were homozygous except DAN-c1×WT, while all experimental groups (D2R knockdown, thermogenetics, and optogenetics) used were heterozygous by crossing the corresponding control strains. The 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 (100 mm 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 paper (0.25cm^2 square) 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 (0.25cm^2 square) 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 2e**):

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

Naïve Olfactory Test

Larvae were transferred into the midline of test plates. 2.5µL of odorant were added on a piece of filter paper (0.25cm^2 square) 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[91].

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[92]. 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 paper (0.25cm^2 square) 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*.[93,94] 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 paper (0.25cm^2 square) 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 (0.25cm^2 square) 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 in each brain, 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. In brief, two-way ANOVA and Tukey's multiple comparison test were used in **Figure 2f-j**, **Figure 4e**, **Figure 5b-d**, **Figure 6a-d**, and **Figure S7**; unpaired t-test was used in **Figure 4d**; one-way ANOVA and Tukey multiple's comparison test were used in **Figure 4f-i**; two-way ANOVA and Dunnett's multiple comparison test were used in **Figure S4f**, and **Figure S5a-b**; one-way ANOVA and Dunnett's multiple comparison test were used in **Figure S6a-i**.

Supplemental information

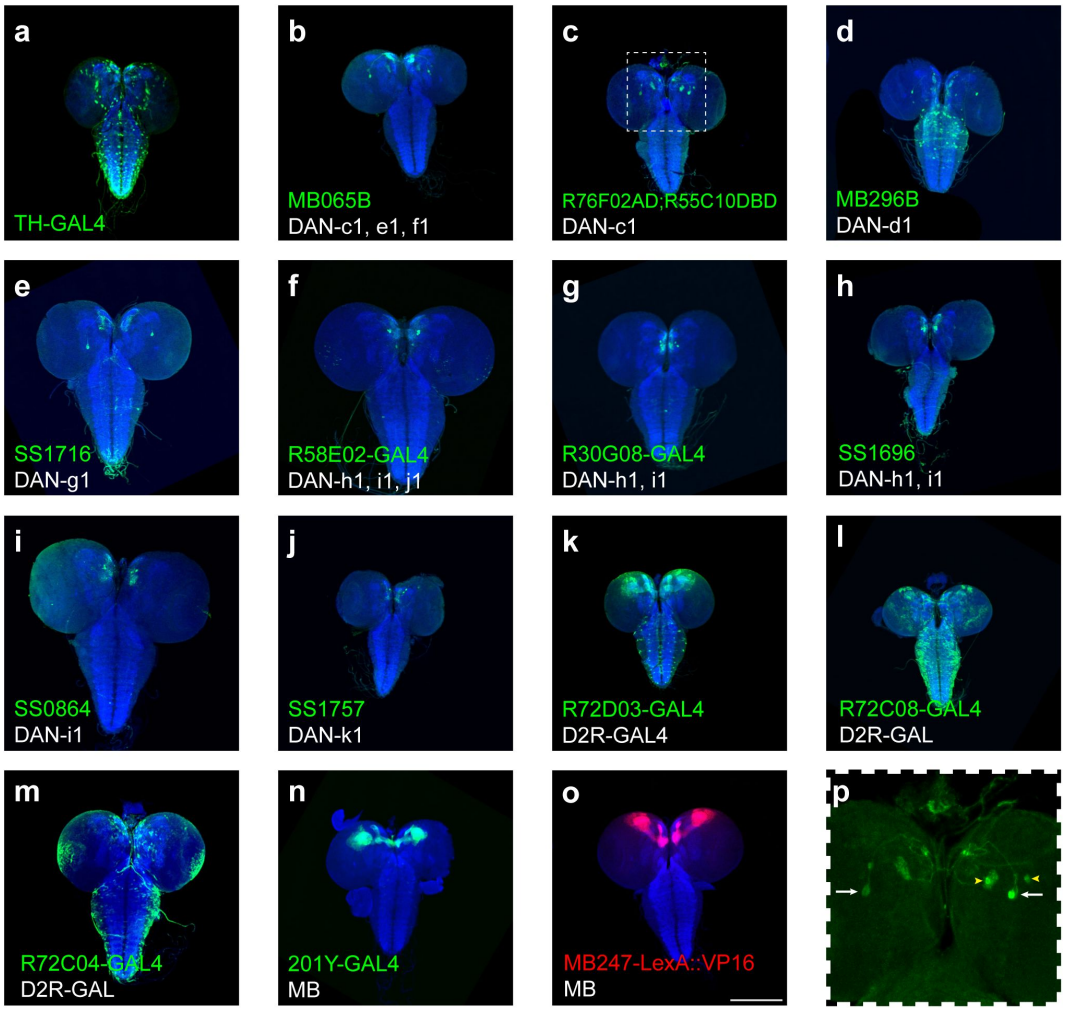

Figure S1.

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**. Square region is enlarged in **(p)** to show the soma and neurites. White arrows label the soma of DAN-c1, while yellow arrow heads label other neurons. Summary of analysis can be found in **Table S3**. Scale bar: 200 μm. ***(Note)*** *N numbers can be found in* **Table S2**. *In* **Figure 1** *and* **S1**, *we mainly showed the strains labeling distinct pairs of dopaminergic neurons. The labeling patterns of the rest 47 strains screened were summarized in* **Table 1**, *whose brain images are available and can be provided upon request*.

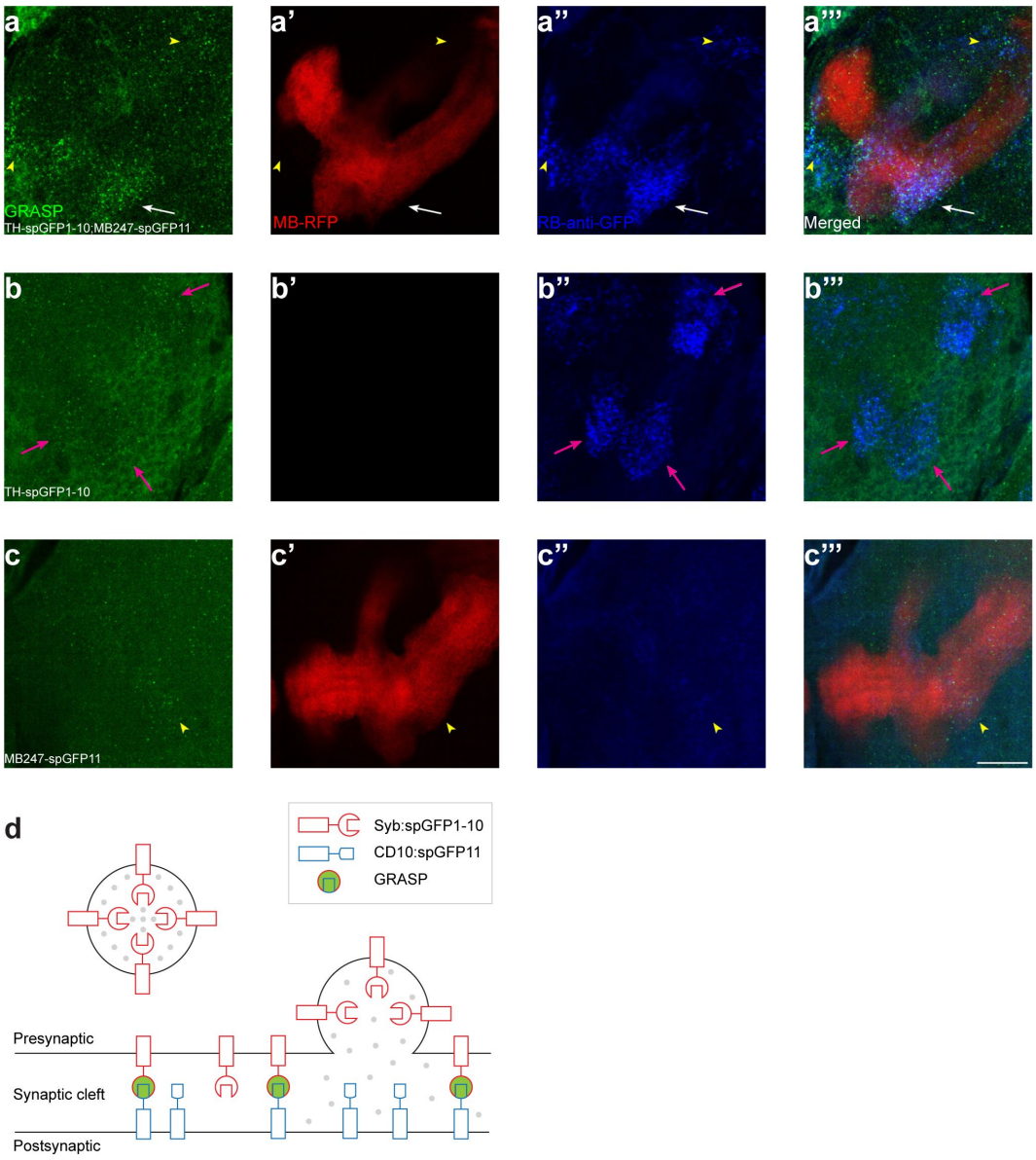

Figure S2.

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.[50]. *(Note) N numbers can be found in* **Table S2**.

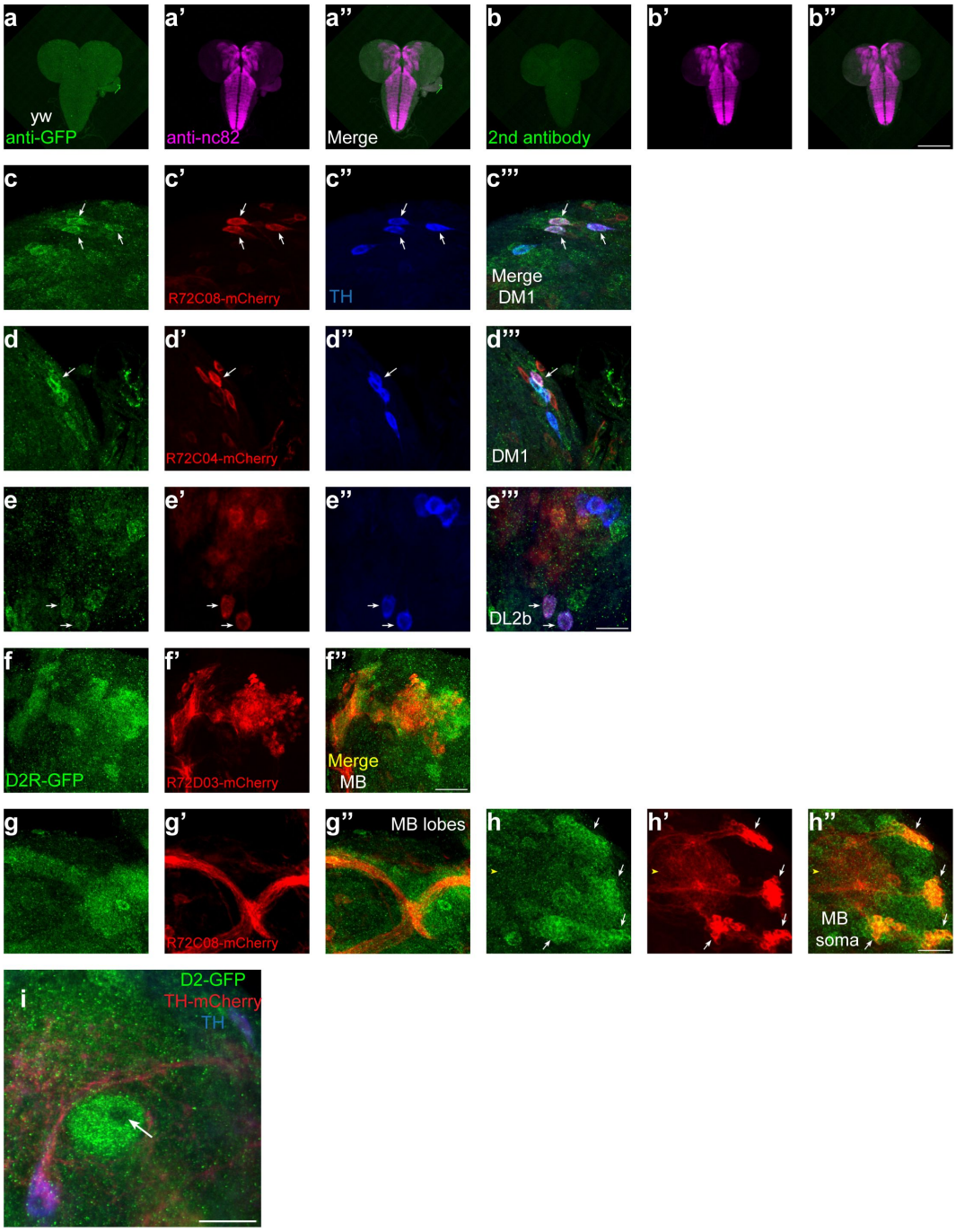

Figure S3.

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). *(Note) N numbers can be found in* **Table S2**.

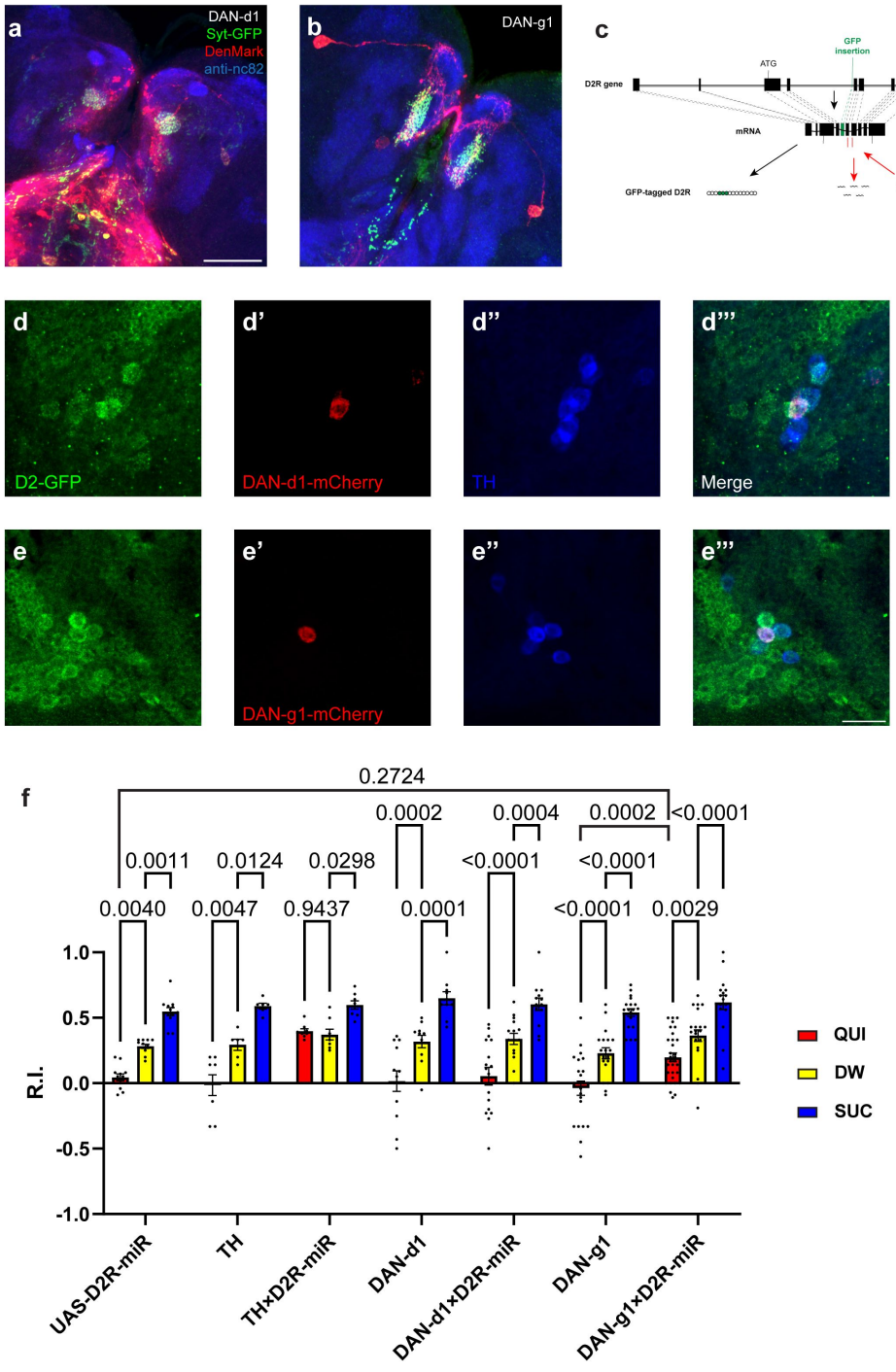

Figure S4.

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)** 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[60]. **(d-e)** D2Rs are expressed in DAN-d1 (d) and DAN-g1 (e). 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). **(f)** Knockdown D2R in neither in DAN-g1 nor DAN-d1 impairs aversive learning. Data are shown as mean ± SEM. UAS-D2R-miR data is from **Figure 4e**. Two-way ANOVA, Dunnett's multiple comparison test. $p = 0.0544$ for interaction, $p < 0.0001$ for row factor (genotype), and $p < 0.0001$ for column factor (US). For N numbers, see **Table S5**. Scale bars: 50 μm (a and b), 20 μm (d and e). *(Note) N numbers of immunostaining for each strain can be found in* **Table S2**.

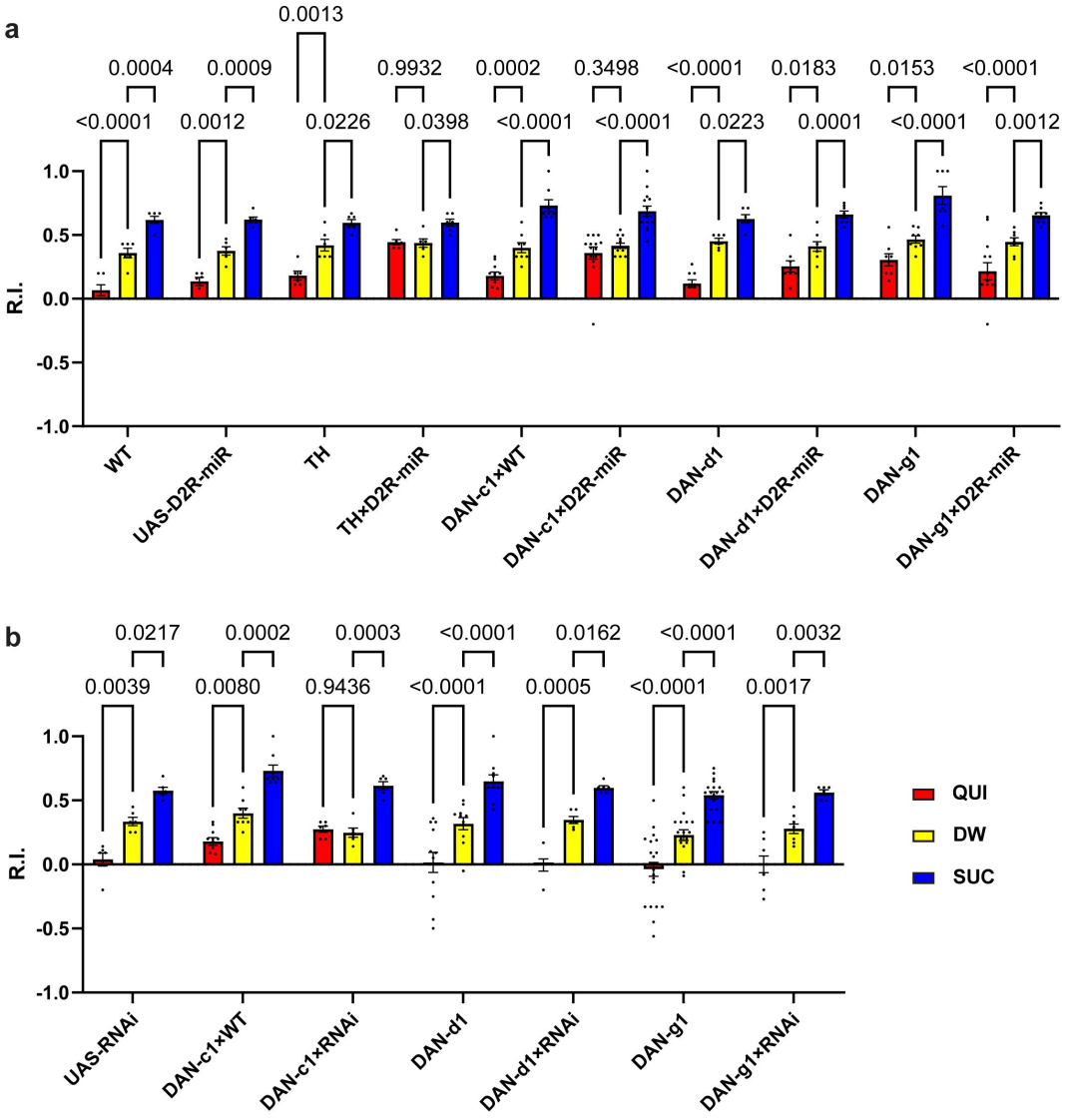

Figure S5.

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. Data are shown as mean ± SEM. Two-way ANOVA, Dunnett's multiple comparison test. In D2R-miR experiments (a), $p = 0.0009$ for interaction, $p < 0.0001$ for row factor (genotype), and $p < 0.0001$ for column factor (US); in D2R-RNAi experiments (b), $p = 0.3802$ for interaction, $p = 0.0001$ for row factor (genotype), and $p < 0.0001$ for column factor (US). For N numbers, see **Table S5**.

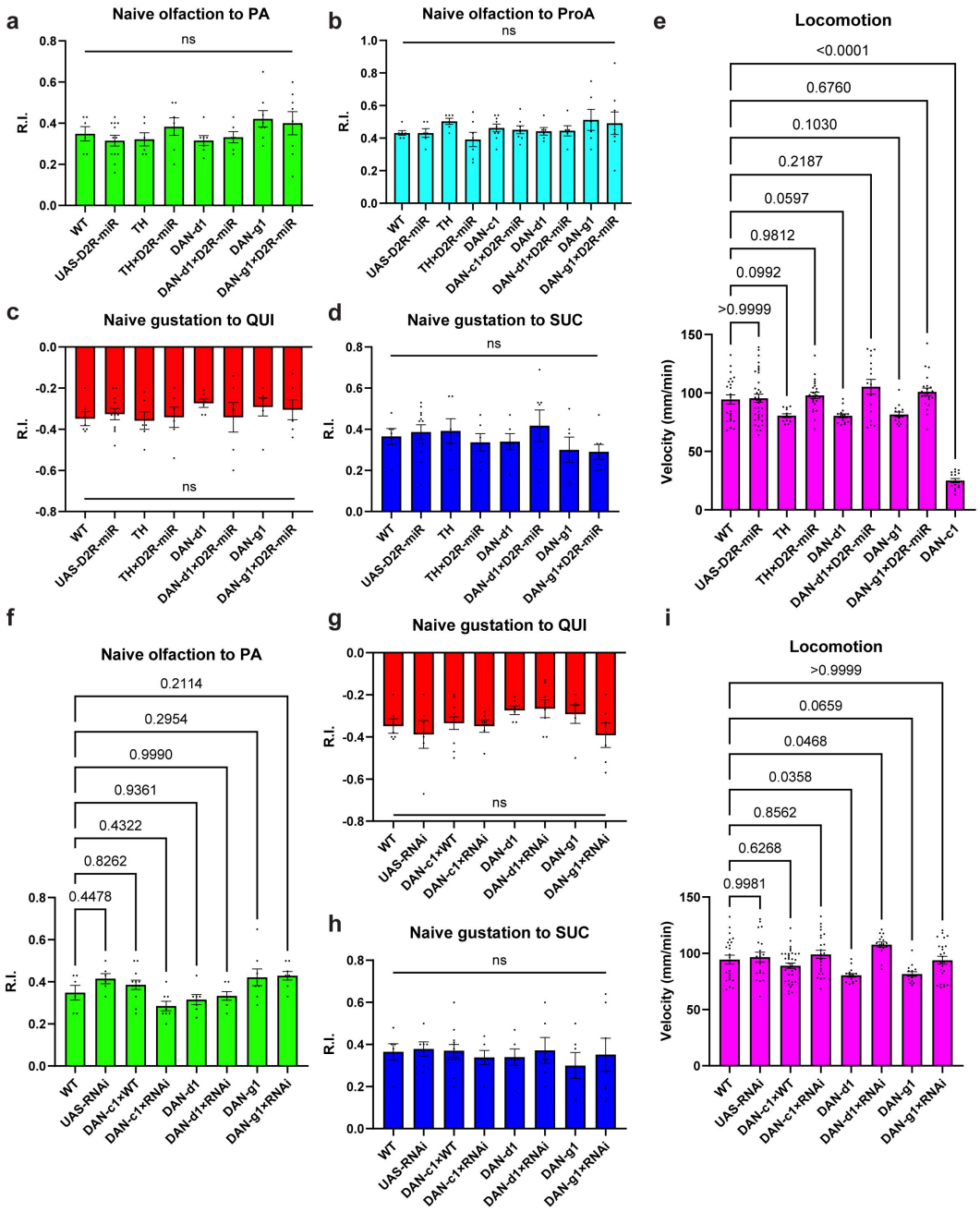

Figure S6.

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, Dunnett's multiple comparison test. For N numbers, see **Table S5**.

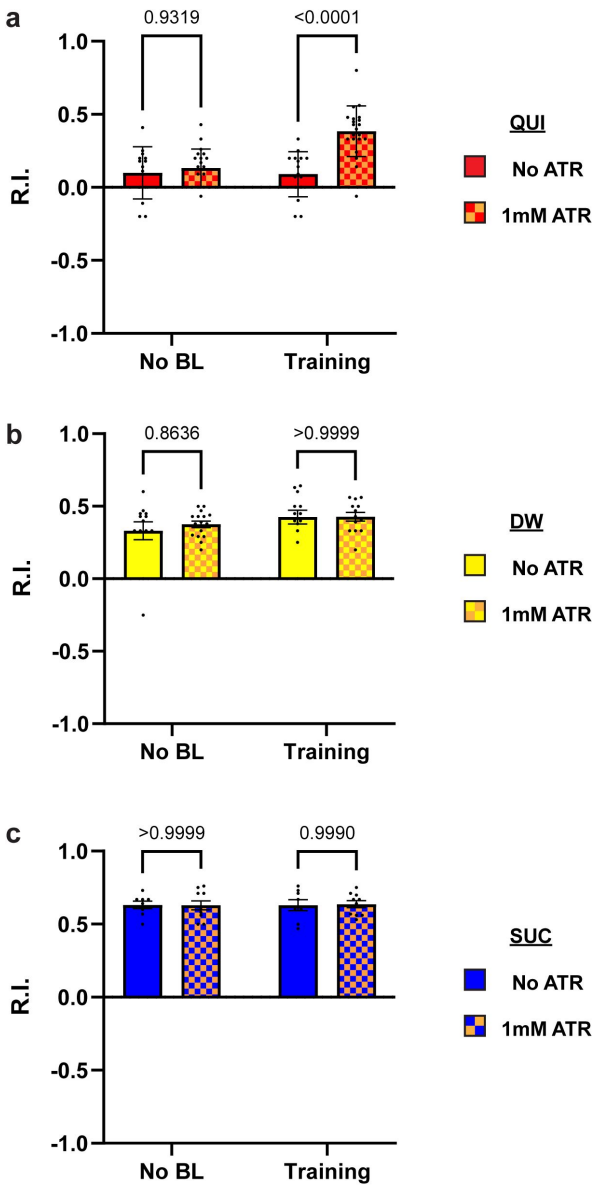

Figure S7.

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 keeping 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, Tukey's multiple comparison test. In QUI group (a), $p = 0.0011$ for interaction, $p = 0.0023$ for row factor (training stages), and $p < 0.0001$ for column factor (whether with ATR); in DW group (b), $p = 0.6126$ for interaction, $p = 0.0850$ for row factor (training stages), and $p = 0.5748$ for column factor (whether with ATR); in SUC group (c), $p = 0.8910$ for interaction, $p = 0.9239$ for row factor (training stages), and $p = 0.9503$ for column factor (whether with ATR). For N numbers, see **Table S6**.

Supplementary Tables

	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 S1.

Other strains used in this study.

Figure 1	N			Figure S1	N
Strains	**IHC**	**GRASP**		TH-GAL4	4
TH-GAL4	8	16		MB065B	4
MB065B	14	15		R76F02AD;R55C10DBD	5
R76F02AD;R55C10DBD	13	13		MB296B	3
MB296B	14	4		SS1716	2
SS1716	10	21		R58E02	3
R58E02	5	10		R30G08	2
R30G08	13	6		SS1696	4
SS1696	16	4		SS0864	4
SS0864	7	8		SS1757	4
SS1757	6	13		R72D03-GAL4	7
				R72C08-GAL4	5
Figure 2	**N**			R72C04-GAL4	3
DAN-c1 x Syt-GFP;DenMark	4			201Y-GAL4	4
				MB247-LexA::VP16	3
Figure 3	**N**				
DM1a	11			**Figure S2**	**N**
DM1b	9			TH-spGFP1-10	3
pPAM	11			MB247-spGFP11	4
DL1	10				
DL2a	7			**Figure S3**	**N**
DL2b	5			R72D03	5
MB Soma	6			R72C08	4
MB Lobe	8			R72C04	5
Figure 4	**N**			**Figure S4**	**N**
DAN-c1 x D2-GFP	9			DAN-d1 x Syt-GFP;DenMark	6
TH-Gal4	7			DAN-g1 x Syt-GFP;DenMark	3
TH-miR	6			DAN-d1 x D2-GFP	13
				DAN-g1 x D2-GFP	7

Table S2.

N numbers for brain samples used in Figures.

Table S3.

Summary of R76F02AD;R55C10DBD (DAN-c1) identifying patterns in larval brains.

For the strain R76F02AD; R55C10DBD, 22 third-instar larval brains expressing GFP or SytGFP and DenMark were examined, and all of them clearly identified DAN-c1. Half of them only identified DAN-c1, the rest had 1 to 5 weak identified cells without neurites, and barely 1 or 2 strong identified cells appeared. These non-DAN-c1 neurons were seldom dopaminergic neurons. In ventral nerve cord (VNC), 8 out of 12 did not have any identified cells, 3 had 2-4 strong identified cells. These data supported that R76F02AD;R55C10DBD exclusively labels DAN-c1 in third-instar larval brains.

R76F02AD;R55C10DBD (DAN-c1) brains	other DAN	None DAN	VNC
1	0	1 strong, 4 weak	NA
2	1 weak no neurites	0	3 weak
3	0	7 weak	NA
4	0	0	2 strong
5	0	0	2 strong
6	0	2 strong, 3 weak	NA
7	0	0	NA
8	0	0	4 strong
9	0	5 weak	NA
10	0	1 weak	NA
11	0	8 weak	NA
12	0	0	NA
13	0	4 strong 1 weak	NA
14	0	0	0
15	0	0	0
16	0	0	NA
17	0	0	0
18	NA	1 strong, 1 weak	0
19	NA	7 weak	0
20	NA	5 weak	0
21	NA	0	0
22	NA	1 weak	0

Table S4.

N numbers and p-values for learning assays with thermogenetics.

	22°C			34°C			Interaction p-value	Row Factor p-value (Temperature)	Column Factor p-value (US)
	QUI	DW	SUC	QUI	DW	SUC			
shibire^{ts1}	9	8	8	9	9	9	0.8880	0.5290	<0.0001
DAN-c1×shibire^{ts1}	14	6	7	17	6	9	<0.0001	0.0018	<0.0001
DAN-c1	8	9	9	9	9	9	0.6143	0.1664	<0.0001
DAN-c1×dTRPA1	15	14	13	14	15	13	<0.0001	0.0019	<0.0001
dTRPA1	9	8	9	10	10	10	0.8001	0.4228	<0.0001

Table S5.

N numbers for D2R 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 S6.

N numbers for 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

Data Availability

Data will be made available on request.

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Additional information

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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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. This paper 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, confocal imaging is used to screen GAL4 driver strains that drive GFP expression in just single pairs of dopaminergic neurons. Next, thermogenetic manipulations of one pair of DANs (DAN-c1) suggest that DAN-c1 activity during larval aversive learning is important. Confocal imaging is then used to reveal expression of D2R in the DANs and mushroom body of the larval brain. Finally, 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. Finally, a model is proposed in which D2R limits excessive dopamine release to facilitate successful olfactory learning.

Strengths:

The paper convincingly reproduces prior findings that demonstrated D2R knockdown in DL1 DANs or the mushroom body impairs aversive olfactory learning in Drosophila larvae (Qi and Lee, 2014; doi:10.3390/biology3040831). These previous findings were built upon and extended with a comprehensive confocal imaging screen of 57 GAL4 drivers that identified tools driving GFP expression in individual DANs. One of the drivers, R76F02-AD; R55C10-DBD, was consistently shown to label DAN-c1 neurons and no other DANs in the larval brain. Confocal imaging is also used to demonstrate that GFP-tagged D2R is expressed in most DANs and the mushroom body. Behavioral experiments demonstrate that driving D2R knockdown in DAN-c1 neurons impairs aversive learning, as do other loss-of-function manipulations of DAN-c1 neurons.

Limitations:

(1) The single-odor paradigm used to train larvae does not have the advantages of a more conventional balanced or reciprocal training paradigm. The paper describes how the single-odor experimental design could be controlled for non-associative effects, but does not provide an independent validation of the control experiments performed by a different research group using different odors and genotypes 15 to 20 years ago (see Honjo and Furukubo-Tokunaga, 2005; doi:10.1523/jneurosci.2135-05.2005 and Honjo and Furukubo-Tokunaga, 2009; doi:10.1523/jneurosci.1315-08.2009). Whether the involvement of DAN-c1 for aversive learning generalizes to standard paradigms remains unclear (see Eschbach et al., 2020; doi:10.1038/s41593-020-0607-9 and Weber et al., 2023; doi:10.7554/elife.91387.1).

(2) In 11 of 22 larval brains examined in the paper, R76F02-AD; R55C10-DBD appears to drive GFP expression in 1 to 8 additional non-dopaminergic neurons (Figure S1P and Table S3). Of the remaining 11 brains, 4 of their corresponding ventral nerve cords also have expression in 2 to 4 neurons (Table S3). Therefore, experiments involving with the R76F02-AD; R55C10-DBD driver could be manipulating the activity of additional neurons in around 60% of larvae. The conclusions of the paper would be strengthened if key experiments were repeated with other GAL4 drivers that may label DAN-c1 with even greater specificity, such as SS03066 (Truman et al., 2023; doi:10.7554/elife.80594) or MB320C (Hige et al., 2015; doi:10.1016/j.neuron.2015.11.003).

(3) Successful immunostaining with an anti-D2R antibody (Draper et al., 2007; doi:10.1002/dneu.20355 and Love et al., 2023; doi:10.1111/gbb.12836) could validate GFP-tagged D2R expression (Figure 3) in the same way that TH immunostaining was used throughout the paper to determine whether neurons were dopaminergic.

(4) The paper proposes a model in which DAN-c1 activity conveys an aversive teaching signal (Figure 2f) but excessive artificial DAN-c1 activation causes excessive dopamine release that impairs aversive learning (Figures 2i and 5b). According to this model, thermogenetic DAN-c1 activation during training with water or sucrose conveys an aversive teaching signal that reduces performance (Figure 2i) whereas optogenetic DAN-c1 activation does not due to excessive dopamine release (Figures 5c and 5d). The model suggests that optogenetic DAN-c1 activation is strong enough to cause excessive dopamine release by itself whereas thermogenetic DAN-c1 activation can only achieve the same outcome when it occurs in conjunction with natural DAN-c1 activation evoked by quinine. Therefore, an experiment with weaker optogenetic DAN-c1 activation (with lower intensity light or pulsed at a lower frequency) during water or sucrose training would be expected to convey an aversive teaching signal rather than excessive dopamine release, reducing performance. Such an experiment could reconcile the differing thermogenetic and optogenetic results of the paper.

https://doi.org/10.7554/eLife.100890.2.sa2

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).
Then 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.

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 and the model of adequate levels of cAMP (Figure 7b) appears to be able to explain a poor memory after insufficient or excessive cAMP signaling. The study provides insight into the role of D2R in associative learning expanding our understanding and might be a reference similarly to previous key findings (Qi and Lee, 2014, https://doi.org/10.3390/biology3040831).

https://doi.org/10.7554/eLife.100890.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
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> ***Reviewer #1 (Public review):***
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> *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 described in the Results section, we screened 57 GAL4 driver lines based on previous reports. These included drivers that had been shown to label a single dopaminergic neuron (DAN) or a small subset of DANs in the larval or adult brain hemisphere, suggesting potential for specific DAN labeling 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 larvae[1], while strains in Figure 1e, f, g were reported identifying only several DANs in adult brains[2,3]. We examined these strains and only some of them labeled single DANs in 3rd instar larval brain hemisphere (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 driver shown in Figure 1f (R76F02AD;R55C10DBD, labeling DAN-c1) is the only line we identified that labels a single DAN in the 3rd instar larval brain hemisphere without additional labeling. The other lines shown in Figure 1 (g, h, l, m) label a single DAN but also include some non-DANs. Figure 1 focuses on strains that label a single or a pair of DANs.

Labeling patterns for all 57 driver lines are summarized in Table 1. Figure S1 includes representative examples; full confocal images for all screened strains are available upon request, as stated in the figure legend.

> *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 a single dopaminergic (DA) neuron in each brain hemisphere. While additional GFP-positive signals were occasionally observed, they did not originate from the cell bodies of DA neurons, as these were not labeled by the tyrosine hydroxylase (TH) antibody. These additional GFP signals primarily appeared to be neurites, including axonal terminals, although we cannot rule out the possibility that some represent false-positive signals or weakly stained non-neuronal cell bodies. This interpretation is based on the analysis of 22 third-instar larval brains.

To clarify this point in the manuscript, we added the following sentence to the Results section: “Based on the analysis of 22 brain samples, we observed this driver strain labels one neuron per hemisphere in the third-instar larval brain (Figure 2a–d, Figure S1c, Table S3).” Additionally, Table S3 was included to summarize the DAN-c1 labeling pattern across all 22 samples. An enlarged inset highlighting GFP-positive signals was also added 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 this insightful suggestion. The MB320C driver primarily labels the PPL1-γ1pedc neuron in the adult brain, along with one or two additional weakly labeled cells. It would indeed be interesting to examine the expression pattern of this driver in third-instar larval brains. If it is found to label only DAN-c1 at this stage, we could consider using it to knock down D2R and assess whether this recapitulates our current findings.

While we agree that this is a promising direction for future studies, we believe it is not essential for the current manuscript, given the specificity of the DAN-c1 driver (please see our response to Reviewer #3 for details). Nonetheless, we appreciate the reviewer's suggestion, and we recognize that MB320C could be a valuable tool 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. shows strongly labeled four neurons on each brain hemisphere[4], 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 with the reviewer that the terms "necessary" and "sufficient" may be too exclusive and could unintentionally exclude contributions from other neurons. As noted in the Discussion section, we acknowledge that additional dopaminergic neurons may also play roles in larval aversive learning. To reflect this, we have revised our wording to use "important" and "mediates" instead of the more definitive terms "necessary" and "sufficient," making our conclusions more accurate and appropriately measured.

> *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 an excellent point, and we agree that we cannot rule out the possibility that artificial activation interferes with aversive learning by overriding the natural activity of DAN-c1 that would normally be evoked by quinine. The observed results with TRPA1 could potentially be attributed to dopamine depletion, inactivation due to prolonged depolarization, or neural adaptation. However, we believe that our hypothesis - that over-excitation of DAN-c1 impairs learning - is more consistent with our experimental findings and with previously published data. Our rationale is as follows: (1) Associative learning in larvae occurs only when the

conditioned stimulus (CS, e.g., an odor such as pentyl acetate) and unconditioned stimulus (US, e.g., quinine) are paired. In wild-type larvae, the CS depolarizes a subset of Kenyon cells in the mushroom body (MB), while the US induces dopamine (DA) release from DAN-c1 into the lower peduncle (LP) compartment (Figure 7a). When both stimuli coincide, calcium influx from CS activation and Gαs signaling via D1-type dopamine receptors activate the MB-specific adenylyl cyclase, *rutabaga*, which functions as a coincidence detector (Figure 7d). (2) *Rutabaga* converts ATP to cAMP, activating the PKA signaling pathway and modifying synaptic strength between Kenyon cells and mushroom body output neurons (MBONs) (Figure 7d). These changes in synaptic strength underlie learned behavioral responses to future presentations of the same odor. (3) Our results show that D2R is expressed in DAN-c1, and that D2R knockdown impairs aversive learning. Since D2Rs typically inhibit neuronal excitability and reduce cAMP levels[5], we hypothesize that D2R acts as an autoreceptor in DAN-c1 to restrict DA release. When D2R is knocked down, this inhibition is lifted, leading to increased DA release in response to the US (quinine). The resulting excess DA, in combination with CS-induced calcium influx, would elevate cAMP levels in Kenyon cells excessively - disrupting normal learning processes (Figure 7b). This is supported by studies showing that *dunce* mutants, which have elevated cAMP levels, also exhibit aversive learning deficits[6]. (4) The TRPA1 activation results are consistent with our over-excitation model. When DAN-c1 was artificially activated at 34°C in the distilled water group, this mimicked the natural activation by quinine, producing an aversive learning response toward the odor (Figure 2k or new Figure 2i, DW group). Similarly, in the sucrose group, artificial activation mimicked quinine, producing a learning response that reflected both appetitive and aversive conditioning (Figure 2k, SUC group). (5) Over-excitation impairs learning in the quinine group. When DAN-c1 was activated during quinine exposure, both artificial and natural activation combined to produce excessive DA release. This over-excitation likely disrupted the cAMP balance in Kenyon cells, impairing learning and resulting in failure of aversive memory formation (Figure 2k, QUI group). This phenotype closely mirrors the effect of D2R knockdown in DAN-c1. (6) Optogenetic activation of DAN-c1 during aversive training similarly produced elevated DA levels due to both natural and artificial stimulation. This again would result in MBN over-excitation and a corresponding learning deficit. When optogenetic activation occurred during non-training phases (resting or testing), no additional DA was released during training, and aversive learning remained intact (Figure 5b). (7) Notably, when optogenetic activation was applied during training, we observed no aversive learning in the distilled water group and no reduction in the sucrose group (Figure 5c, 5d). We interpret this as evidence that the optogenetic stimulation was strong enough to cause elevated DA release in both groups, impairing learning in a manner similar to D2R knockdown or TRPA1 overactivation. (8) We extended this over-excitation framework to directly activate Kenyon cells (MBNs). Since MBNs are involved in both appetitive and aversive learning, their over-excitation disrupted both types of learning (Figure 6), further supporting our hypothesis. In summary, we propose that DAN-c1 activity is tightly regulated by D2R autoreceptors to ensure appropriate levels of dopamine release during aversive learning. Disruption of this regulation - either through D2R knockdown or artificial overactivation of DAN-c1 - results in excessive DA release, over-excitation of Kenyon cells, and impaired learning. This over-excitation model is consistent with both our experimental results and prior literature.

> *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 S3c 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 originally described by Draper et al.[6]. We attempted to use the same antibody in our experiments; however, we were unable to detect clear signals following staining. This may be due to a lack of specificity for neurons in the Drosophila larval brain or incompatibility with our staining protocol. Unfortunately, we were unable to locate a copy of the Lam (1999) paper for further reference.

> *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 re-analyzed the data related to DAN-g1. Interestingly, knockdown of D2R in DAN-g1 larvae trained with quinine (QUI) showed a significant difference in response index (R.I.) compared to the distilled water (DW) control group. However, it also differed significantly from the DAN-g1 genetic control group trained with QUI (two-way ANOVA with Tukey's multiple comparisons, $p = 0.0002$), while it was not significantly different from the UAS-D2R-miR genetic control group ($p = 0.2724$). Furthermore, knockdown of D2R in DAN-g1 did not lead to aversive learning deficits when larvae were trained with a different odorant, propionic acid (ProA; Figure S5a). Similarly, using an RNAi line to knock down D2R in DAN-g1 did not result in learning impairment when larvae were trained with pentyl acetate (PA; Figure S5b). These inconsistencies may stem from differences in stimulus intensity across odorants, as well as the variable efficiency of the knockdown strategies (microRNA vs. RNAi). Based on these results, we propose that D2Rs in DAN-g1 may modulate larval aversive learning in a quantitative manner but do not play as critical a role as those in DAN-c1, where knockdown produces a clear qualitative effect. We have added this paragraph to the Discussion section of the manuscript.

> ***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 Reviewer #1 above.

> ***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 thoughtful suggestions.

Regarding the R76F02AD; R55C10DBD strain, we examined 22 third instar larval brains expressing GFP, Syt-GFP, or Den-mCherry. All brains clearly labeled DAN-c1. In approximately half of the samples, only DAN-c1 was labeled. In the remaining samples, 1 to 5 additional weakly labeled soma were observed, typically without associated neurites. Only 1 or 2 strongly labeled non-DAN-c1 cells were occasionally detected. These additional labeled neurons were rarely dopaminergic. In the ventral nerve cord (VNC), 8 out of 12 samples showed no labeled cells. The remaining 4 samples had 2–4 strongly labeled cells. These results support our conclusion that the R76F02AD; R55C10DBD combination predominantly and specifically labels DAN-c1 in the third instar larval brain. As for the reviewer's question about the expression pattern of R76F02AD; R55C10DBD and D2R in the larval body, we agree that this is a very interesting avenue for further investigation. However, our current study is focused on the central nervous system and larval learning behaviors. We hope to explore this question more fully in future work.

We added the following sentence to the Results section: "Based on analysis of 22 brain samples, we believe this driver strain consistently labels one neuron per hemisphere in the third-instar larval brain (Figure 2a - d, Figure S1c, Table S3)." In addition, we included Table S3 to summarize the DAN-c1 labeling patterns observed across these samples.

> *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 the single-odor larval learning paradigm from Honjo et al., who first developed and validated this method for studying larval olfactory associative learning7,8. To address the reviewer's concern regarding potential non-associative effects from 30-minute exposure to quinine or sucrose, we refer to multiple lines of evidence provided in Honjo's studies: (1) Honjo et al. demonstrated that only larvae receiving paired presentations of odor and unconditioned stimulus (quinine or sucrose) exhibited learned responses. Exposure to either stimulus alone, or temporally dissociated presentations, failed to induce any learning response. (2) When tested with a second, non-trained odorant, larvae only responded to the odorant previously paired with the unconditioned stimulus. This rules out generalized olfactory suppression and confirms odor-specific associative learning. (3) Well-characterized learning mutants (e.g., *rutabaga*, *dunce*) that show deficits in adult reciprocal odor learning also failed to exhibit learned responses in this single-odor paradigm, further supporting its validity. (4) In our study, we used two distinct odorants (pentyl acetate and propionic acid) and two independent D2R knockdown approaches (UAS-miR and UAS-RNAi). We consistently observed that D2R knockdown in DAN-c1 impaired aversive learning. Importantly, naïve olfactory, gustatory, and locomotor assays ruled out general sensory or motor defects. Comparisons with control groups (odor paired with distilled water) also ruled out non-associative effects such as habituation. Taken together, these results strongly support that the single-odor paradigm is a robust and reliable assay for assessing larval olfactory associative learning in *Drosophila*. We have added a section in the Discussion to clarify and defend the use of this paradigm in our study.

> *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.*

We gave 5 min during the testing stage to allow the larvae to wander on 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 approaches -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.*

Shibire^{ts1} 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 recycling[7]. 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 *Shibire*^{ts1} insensitive ways of dopamine release exist. However, blocking dopamine release from DAN-c1 with *Shibire*^{ts1} 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*[9], 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 learning[10,11]. 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 described this in the Materials and Methods part, "All control strains used in learning assays were homozygous (except DAN-c1×WT), while all experimental groups (D2R knockdown and thermogenetics) used were heterozygous by crossing the corresponding control strains".

We also re-organized the Figure S4e and S5c along with the control groups to make it 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 appreciate the reviewer's suggestion. We read through this literature, which also addresses the question we mentioned in the Discussion section, about the discrepancy between the cAMP elevation in the mushroom body neurons and the reduced MBN-MBON synaptic plasticity after olfactory associative learning in *Drosophila*. The author gave an explanation to the existing D1R-cAMP elevation-MBN-MBON LTD axis, which is really helpful to our understanding about the learning mechanism. However, unfortunately, we do not think this offers a possible explanation for our D2R-related mechanisms. We added this literature into our citation.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Throughout the behavioral experiments, a defect in aversive learning is defined as a relative increase in the response index (RI) after olfactory training with quinine (red) and a defect in appetitive learning as a relative decrease in RI after training with sucrose (blue). Training with distilled water (yellow) is intended to be a control for comparisons within genotypes/treatment groups but causes interpretation issues if it is also affected by experimental manipulations.*
>
> *The authors typically make comparisons between quinine, water, and sucrose within each group, but this often forces readers to infer the key comparisons of interest. For example, the key comparison in Figure 2h is the statistically significant difference between the red groups, which differ only in the temperature used during training. Many other figure panels in the paper would also benefit from more direct statistical comparisons, particularly Figure 2k.*
>
> *While I recognize the value of the water control, I strongly recommend that the authors make statistical comparisons directly between genotypes/treatment groups where possible and to interpret results with more caution when the water RI score differs substantially between groups. Also, since the authors are conducting two-way ANOVAs before Dunnett's multiple comparisons tests, they ideally should report the p-value for the main effect of each factor, plus the interaction p-value between the two factors before making multiple comparisons.*

We appreciate the reviewer's suggestion. In response, we re-analyzed all learning assay data in Figures 2 and 4 using two-way ANOVA followed by Tukey's multiple comparisons test. Unlike our previous analysis, which only compared each experimental group to its corresponding DW control, we now compared all groups against one another. First, we found that most R.I. values from different temperature conditions (Figure 2) or genotypes (Figure 4) trained with DW were not significantly different, with the exception of the data in Figure 2i (formerly Figure 2k; discussed further below). The R.I. from DAN-c1 × D2R-miR larvae trained with QUI was significantly different from both genotype control groups (DAN-c1 × WT and UAS-D2R-miR), while no significant difference was observed between the two controls trained with QUI. Thus, this more comprehensive statistical approach supports the conclusions we previously reported. Second, as the reviewer noted, the new analysis allows for a more direct interpretation of our findings. For example, in the thermogenetic experiments using the Shibire^{ts1} strain, the R.I. of DAN-c1 × UAS-Shibire^{ts1} larvae trained with QUI at 34°C was not significantly different from the DW group at 34°C, but was significantly different from the QUI group at 22°C. Both findings support our conclusion that blocking dopamine release from DAN-c1 impairs larval aversive learning (Figure 2f).

In the dTRPA1 activation experiments, the R.I. of DAN-c1 × UAS-dTRPA1 larvae trained with DW at 34°C was significantly lower than that of the DW group at 22°C and the QUI group at 34°C, but not significantly different from the QUI group at 22°C (Figure 2i). These results

indicate that activating DAN-c1 during training is sufficient to drive aversive learning even in the absence of QUI. Interestingly, when DAN-c1 × UAS-dTRPA1 larvae were trained with QUI at 34°C, their R.I. was significantly higher than that of the DW group at 34°C and significantly different from the QUI group at 22°C, but not significantly different from the DW group at 22°C (Figure 2i). We interpret this as evidence that simultaneous activation of DAN-c1 by both QUI and dTRPA1 leads to over-excitation, which in turn impairs aversive learning.

We have revised the figures (Figures 2, 4, 5, and 6) and updated the corresponding Results sections to reflect this new statistical analysis. Additionally, we now report the p-values for interaction, row factor, and column factor - either in Table S4 (for Figure 2) or in the figure captions for Figures 4, 5, 6, S4, S5, and S7.

> *(2) The authors' motivation to find tools that label DANs other than DAN-c1 was unclear until much later in the paper when I saw the screening experiments in Figures S4 and S5. The authors could provide a clearer justification for why they focus on DAN-c1 in Figure 2 rather than another DAN for which they found a specific driver in Figure 1. The motivation for looking at individual pPAM neurons was also unclear.*

We sincerely appreciate the reviewer's thoughtful suggestion. Our study was initially motivated by the goal of characterizing the expression pattern of D2R in the larval brain. From there, we aimed to identify DAN drivers that label specific pairs of dopaminergic neurons, enabling us to assess the functional role of D2R in distinct DAN subtypes through targeted knockdown experiments. This approach ultimately led us to focus on DAN-c1, as it was the only neuronal population for which D2R knockdown resulted in a learning deficit. We then returned to examine the functional significance of DAN-c1 in aversive learning. While we recognize that a more comprehensive narrative might be desirable, the current structure of our manuscript reflects the most logical progression of our work based on our research priorities and experimental outcomes. We did explore alternative manuscript structures - such as beginning with the D2R expression pattern - but found that the current format best conveys our findings and rtionale.

Regarding our motivation to study individual PAM neurons: we aimed to identify whether D2R plays a role in a specific pair of pPAM neurons involved in larval appetitive learning. However, we were unable to find a driver that exclusively labels DAN-j1, which we believe to be the key neuron in this context (see Figure 1). As a result, our investigation into appetitive learning did not progress beyond the observation of D2R expression in pPAM neurons (Figure 3d), and we did not proceed with learning assays in this context. While we acknowledge the limitations of our study, we believe that our focus on DAN-c1 is well-justified based on both our findings and the tools currently available. We respectfully note that a major restructuring of the manuscript would not necessarily clarify the rationale for focusing on DAN-c1, and therefore we have maintained the current organization.

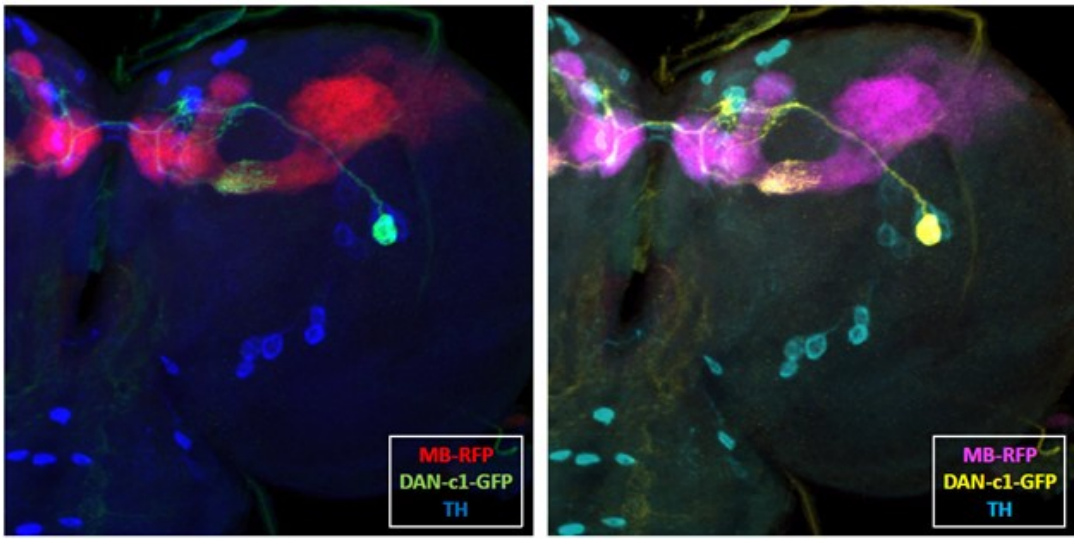
> *(3) The authors should also double-check and update the expression patterns of the drivers in Table 1 using references such as the FlyLight online resource. For example, MB438B labels PPL1-α'2α2, PPL1-α3, PPL1-γ1pedc according to FlyLight, not just PPL1-γ1pedc as initially reported by Aso and Hattori et al. (2014).*

We appreciate the reviewer's suggestion. We have double-checked and updated the driver expression patterns in Table 1, using FlyLight data as a reference.

> *(4) Interpreting overlaid green-and-red fluorescence confocal images would be difficult for any colorblind readers; I suggest that the authors consider using a more friendly color set.*

We thank the reviewer for the suggestion. In our study, we need three distinct colors to represent different channels. We also tested an alternative color scheme using and cyan , magenta, and yellow (CMY) instead of the standard red, green, and blue (RGB). As a comparison (see below), we used a R76F02AD;R55C10DBD (DAN-c1) GFP-labeled brain as an example. In our evaluation, the RGB combination provided clearer visualization and appeared more natural, while the CMY scheme looked somewhat artificial. Therefore, we decided to retain the original RGB color scheme and did not modify the colors in the figures.

Author response image 1.



> *(5) For Figure 4d, counting each DAN as an individual N would violate the assumption of independence made by the unpaired t test, since multiple DANs are found in each brain and therefore are not independent. Instead, it would be better to count each individual N as the average intensity of the four DANs measured in each brain.*

We revised the analysis of microRNA efficiency by averaging the fluorescence intensity of DANs within each brain, treating each brain as a single sample. Based on this approach, we re-plotted Figure 4d.

> *(6) Finally, the authors ought to make it clearer throughout the paper that they have implicated a pair of DAN-c1 neurons in aversive learning, not just a single DAN as currently stated in the title.*

We thank the reviewer for the suggestion about the phrase we are using under this scenario. We have changed all “single neuron” to “a pair of neurons”.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *(1) The results section presents: "Activation of DAN-c1 with dTRPA1 at 34°C during training induced repulsion to PA in the distilled 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."*
> *An alternative interpretation is that 30 min of TrpA activation depletes synaptic vesicle pool, or inactivates neurons because of prolonged depolarization, or DAN shows firing rate adaptation (e.g. see Pulver et al. 2009; doi:10.1152/jn.00071.2009). In such a case DA release would be reduced and not increased. Therefore, the interpretation that DAN-*

> *c1 activation is both necessary and sufficient in larval aversive learning is difficult to be sustained.*
>
> *In this regard it is important to know how the sensory motor abilities are during a thermos-induction at 34°C during 30 min.*

We thank the reviewer for the thoughtful suggestion. Regarding the concern about potential dopamine depletion or neuronal inactivation, we believe a comparison with the Shibire^{ts1} experiments helps clarify the interpretation. Activation of Shibire^{ts1} during training with distilled water did not result in aversive learning (Figure 2f), which is a distinct phenotype from that observed with dTRPA1 activation (Figure 2i). This suggests that the phenotypes seen with dTRPA1 activation are not due to reduced dopamine release. Additionally, as the reviewer suggested, we have revised our conclusion to state that "DAN-c1 is important for larval aversive learning," rather than claiming it is both necessary and sufficient.

> *(2) The GRASP system can label the contact of a cell in close proximity like synaptic contacts, but also other situations like no synaptic contact. It would be useful to use a more specific synaptic labelling tool, like the trans-synaptic tracing system (Talay et al., 2017 https://doi.org/10.1016/j.neuron.2017.10.011), which provides a better label of synaptic contact.*

We really appreciate the reviewer's suggestion. First, we acknowledge that there are four general methods to reveal synaptic connections between neurons: immunohistochemistry (IHC), neuron labeling, viral tracing, GRASP, and electron microscopy (EM). Among these, IHC is not sufficiently convincing, viral tracing is challenging and rarely used in Drosophila, and EM, while the most accurate, is prohibitively expensive for our current goals. For these reasons, we chose the GRASP system to demonstrate the synaptic connections from dopaminergic neurons to the mushroom body. Second, we utilized an activity-dependent version of the GRASP system, linking split-GFP1-10 with synaptic proteins (e.g., synaptobrevin)[12] rather than with cell surface proteins like CD4 or CD8. This version significantly reduces false positive signals compared to the previous version, which was tagged with cell surface proteins. While we admit that this method does not provide as solid evidence of synaptic connections as EM, it is the most efficient method available to us for showing the synaptic connections from dopaminergic neurons to the mushroom body. Finally, we thank the reviewer for suggesting the literature on trans-synaptic tracing methods. Unfortunately, this method is not suitable for our goal, as it labels the entire postsynaptic neuron. In our study, we use GRASP to identify the specific dopaminergic neurons based on the synaptic locations and compartments within the mushroom body lobe. We require a labeling system at the subcellular level because, as noted, DAN-c1 forms synapses specifically in the lower peduncle (LP) of the mushroom body lobe, which is part of the axonal bundles from mushroom body neurons. Using the trans-synaptic tracing method would label the entire mushroom body, making it impossible to distinguish DAN-c1 from other DL1 dopaminergic neurons.

> *(3) Previously, Honjo et al (2009) used a petri dish of 8.5 cm and a filter paper for reinforcement of 5.5 cm. In this study the petri dish was 10 cm and the size of the filter paper was not informed. That is important information because it will determine the probability of conditioning.*

A piece of filter paper (0.25cm^2 square) was used to hold odorants in this study. We have added this information to the Materials and Methods.

> *(4) Statistic analysis of Behavioral performance of Fig 2H-I was made by ANOVA followed by Dunnett multiple comparisons test. Which was the control group? In each graph 2*

> *independent Dunnett tests were performed against the DW control group?*

We have re-analyzed the data using a two-way ANOVA followed by Tukey's multiple comparison test, as suggested by Reviewer #1. In Figure 2f-j (previously Figure 2h-l), the DW groups serve as the control groups. In our new analysis, we compared data across all groups using Tukey's multiple comparison test, with particular focus on comparisons to the corresponding DW control groups.

> *(5) The sample size in staining experiments of figures 1-4 were not informed.*

We have added Table S2 in the supplementary materials to provide the N numbers for brain samples used in the figures.

> *(6) Color code in Fig 5 is missing, I assumed that is the same as in figure 4e*

We added color code in the figure legend of Figure 5.

> *(7) Line 506 "0.1% QH solutions" should be 0.1% QUI solutions*

Changed.

> *(8) There is no information on the availability of data*

We added Data Availability Statement: Data will be made available on request.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Axes of behavioural experiments should better show the full span of possible values (-1;1) to allow a fair assessment.*

We have adjusted the axes in all learning assay graphs to a range from -1 to 1 for consistency and clarity.

> *(2) Ns should better be given within the figures.*

We have added Table S2 in the supplementary materials to provide the N numbers for brain samples used in the figures. Additionally, Tables S4 to S6 include the N numbers for the learning assays. While we initially considered including the N numbers within the figure captions, we found it challenging to present this information clearly and efficiently. Therefore, we decided to summarize the N numbers in the tables instead.

> *(3) Dot- or box-plots would be better for visualizing the data than means and SEMs.*

We agree with the reviewer's suggestion. In the behavioral assay graphs, both dot plots and mean ± SEM have been included for better visualization of the data.

> *(4) The paper reads as if Dop2R would reduce neuronal activity, rather than "just" cAMP levels. Such a misunderstanding should be avoided.*

We appreciate the reviewer's comment. Under most conditions, dopamine binding to D2Rs activates the Gαi/o pathway, which inhibits adenylyl cyclase (AC) and reduces cAMP levels. This reduction in cAMP ultimately leads to decreased neuronal activity. In other words, D2R activation typically has an inhibitory effect on neurons. Additionally, D2R can exert inhibitory effects through other signaling pathways, such as the inhibition of voltage-gated

associative learning, we continue to emphasize the importance of the D2R-mediated AC-cAMP-PKA signaling pathway. However, we do not rule out the potential involvement of additional signaling pathways, such as inhibition of voltage-gated calcium channels via Gβγ subunits[5]. As noted in the Introduction, dopamine receptors are also involved in other signaling cascades, including PKC, MAPK, and CaMKII pathways. In the context of our study, based on current understanding of molecular signaling in Drosophila olfactory, we still think D2R mediated AC-cAMP-PKA signaling pathway would be the most important one. However, we cannot rule out the involvement of other signaling pathways.

> *(5) It would be better if citations were more clearly separated into ones that refer to adult flies versus work on larvae.*

We separated the citations related to adult flies from those working on larvae.

> *(6) Line 81-83. DopECR is not found in mammals, is it?*

You are correct. DopECR is not found in mammals. This non-canonical receptor shares structural homology with vertebrate β-adrenergic-like receptors. It can be activated rapidly by dopamine as well as insect ecdysteroids[13,14].

> *(7) Line 99: Better "a" learning center (some forms of learning work without mushroom bodies).*

We have revised the text from "the learning center" to "a learning center," as suggested by the reviewer.

> *(8) Supplemental figures should be numbered according to the sequence in which they are mentioned in the text.*

We have rearranged the sequence of supplemental figures to match the order in which they are referenced in the text.

> *(9) It is striking that dTRPA1-driving DANc1 is punishing in the water condition but that this effect does not summate with quinine punishment (but rather seems to impair it). Maybe you can back this up by ChR- or Chrimson-driving DANc1? Or by silencing DANc1 by GtACR1?*

We appreciate the reviewer's suggestion. Indeed, we observed similar but not identical results when we used ChR2 to activate DAN-c1 during the training stage (Figure 5b and c). We found that activating DAN-c1 with quinine (QUI) impaired aversive learning (Figure 5b), consistent with our findings using dTRPA1 activation of DAN-c1 when trained in QUI at 34°C (Figure 2i). We propose that the over-excitation of DAN-c1, whether induced by QUI or artificial manipulation (optogenetics and thermogenetics), impairs aversive learning, which aligns with our findings for D2R knockdown (Figure 4e). However, there are some differences between dTRPA1 and ChR2 activation. While dTRPA1 activation induced aversive learning when trained with distilled water (DW) at 34°C (Figure 2i), ChR2 did not induce aversive learning under the same conditions (Figure 5c). We believe this difference is due to the varying activation levels between the two manipulations. Our optogenetic stimulus may have been stronger than the thermogenetic one, potentially leading to over-excitation in the DW group, preventing aversive learning. In the QUI group, the more severe over-excitation impaired aversive learning, producing a phenotype similar to that observed with other over-excitation methods (e.g., thermogenetics or D2R knockdown), where the phenotype reached a maximum level. We have also addressed these points in the Discussion section.

> *(10) Unless I got the experimental procedure wrong, isn't it surprising that Figure S7b does not uncover a punishing effect of driving TH-Gals neurons?*

This optogenetic experiment with ChR2 expression in TH-GAL4 neurons was a pioneering attempt to activate DAN-c1 using ChR2. As explained in response to question (9), the failure to observe a punishing effect in the DW group when TH-GAL4 neurons were activated during training may be due to our optogenetic stimulus being too strong. This likely resulted in over-excitation of DAN-c1 (among the neurons labeled by TH-GAL4), impairing aversive learning and preventing the appearance of typical aversive behaviors.

> *(11) It seems that Figure1f is repeated, in a mirrored manner, in Figure 2e.*

We have removed Figure 2e, as it was deemed redundant and not necessary for this section.

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