Enhanced odour-associated memory performance with a Y-maze assembly in *Drosophila*

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Abstract

The neural basis of behaviour is identified by systematically disrupting the activity of specific neurons and screening for loss in phenotype. High scoring behavioural assays are thus necessary for identifying the neural circuits of novel behaviours. Here, we report the design and use of a Y-maze based classical olfactory learning and memory assay in *Drosophila*. Appetitive memory scores in our Y-maze are considerably greater and longer-lasting than that from a commonly used T-maze design. We show that testing in the Y-maze is key to the improved scores. We also observed considerable 24-hour aversive taste reinforced memory performance with only one training trial using Y-mazes. This allowed us to determine the protein synthesis dependence of long-lasting aversive taste memories for the first time in flies. The Y-maze assembly will make olfactory conditioning more accessible and it will allow the study of novel memory phenotypes in *Drosophila*.

Keywords

Classical Pavlovian conditioning, olfactory conditioning, associative learning, long-term memory, LTM, Y-maze, T-maze, mushroom body, dopaminergic neurons, protein synthesis dependent

Introduction

Behaviour emerges from the activity of neuronal networks in the nervous system. To identify the neural basis of a specific behaviour in flies, it is firstly reliably and robustly replicated in the laboratory. Second, specific brain regions or candidate neuronal subsets are methodically silenced by genetic means to look for change in the behaviour under study. This establishes a strong correlation of specific neural activity to behaviour that can be verified by further genetics, electrophysiology and functional imaging of neural activity (Simpson, 2009; White and Peabody, 2009; Griffith, 2012; Owald, Lin and Waddell, 2015; White, 2016). However, identification of neurons and neural mechanism underlying behaviour becomes difficult when performance scores are low and as a result, a meaningful reduction in behaviour becomes harder to observe. Higher performance scores are thus imperative. Any pronounced improvement over existing behaviour assays could also reveal novel behaviour phenotypes, allowing new lines of enquiry into the function of neural circuits.

In *Drosophila*, scientists have studied the neural mechanisms of learning and memory extensively for almost five decades (Keene and Waddell, 2007; Vosshall, 2007; Harris, 2008; Kahsai and Zars, 2011). The basic design of the Pavlovian associative learning assays, that has been central to this field, has remained unchanged. Groups of flies are sequentially exposed to two sensory stimuli, usually two odours, only one of which overlaps with either punishments like electric shock, heat and aversive taste or rewards like food, water, alcohol and sex (Pitman *et al.*, 2009; Kahsai and Zars, 2011; Kaun *et al.*, 2011; Shohat-Ophir *et al.*, 2012; Das *et al.*, 2014; Galili *et al.*, 2014; Lin *et al.*, 2014; Tabone Christopher and de Belle, 2014; Masek *et al.*, 2015). Subsequently, the degree of avoidance or approach to the associated odour is quantified to derive a memory score via the calculation of a performance index.

In the early days of the field, mutagenesis screens for learning and memory-related genes allowed genetic inroads into memory mechanisms (McGuire, Deshazer and

Davis, 2005; Skoulakis and Grammenoudi, 2006). Thereafter, the marked development of transgenic tools in *Drosophila* for manipulating the activity of specific neural subsets (Yoshihara and Ito, 2012; Owald, Lin and Waddell, 2015; Simpson and Looger, 2018), enabled detailed investigation of the neural circuitry underlying learning, memory-consolidation and recall (Keene and Waddell, 2007; Boto, Stahl and Tomchik, 2020). In the last decade or so, the focus has been on genetic refining of control over increasingly restricted neuronal populations (Pfeiffer *et al.*, 2008, 2010; Jenett *et al.*, 2012), electron microscopy-based circuit reconstruction (Zheng *et al.*, 2018; Pipkin, 2020; Scheffer *et al.*, 2020) and *in vivo* imaging of live fly brains (Simpson and Looger, 2018). This has now led to a richly complex picture of the neuronal connectivity and rationale that underlie learning and memory in flies (Cognigni, Felsenberg and Waddell, 2018; Boto, Stahl and Tomchik, 2020).

In the interim, olfactory conditioning assays have also been through developments of their own. The Benzer laboratory refined one of the earliest associative learning and memory experiments using the countercurrent apparatus to show that flies can learn to avoid odour associated with an electric shock (Quinn, Harris and Benzer, 1974). The same study described the use of a Y-maze to train flies to discriminate between two visible wavelengths of light (Quinn, Harris and Benzer, 1974). The authors used both the apparatus without active odour streams and the experiments yielded low learning scores. Later, although the countercurrent machine was used to condition flies with sugar or shock, learned odour preference was tested in a T-maze (Dudai, 1983; Tempel *et al.*, 1983). Over the ensuing years T-maze design developed to accommodate both training and testing of flies and memory scores improved too (Tully and Quinn, 1985; Schwaerzel *et al.*, 2003; Keene *et al.*, 2006). Authors attributed this improvement to, a) shift from instrumental to classical conditioning where flies are unable to avoid the odours and reward/punishment, b) increased odour concentration and c) reduced ‘distracting’ stimuli like light (Tully and Quinn, 1985). This T-maze design or variants of it are now widely used for assaying learning and memory in flies (Y-C Kim, Lee and Han,

2007; Colomb *et al.*, 2009; Ichinose and Tanimoto, 2016). In contrast, Y-maze based designs have been relatively less utilised for memory assays in *Drosophila.* Current avatars have active odour streams and yield memory scores mostly comparable to the T-mazes (Kaun *et al.*, 2011; Albin *et al.*, 2015; Scaplen *et al.*, 2020).

While effective, T-mazes can be cumbersome to work with and difficult to set up. It needs elaborate vacuum tubing arrangements, airflow, and pressure adjustments for consistent results. Commercially available T-mazes are expensive. Getting one made independently requires the services of a good machine shop. Importantly, both appetitive and aversive food component reinforced memory scores in a T-maze have room for improvement. Any new setup that addresses the above issues will be useful for existent users and those starting classical conditioning experiments in flies.

Here, we describe and benchmark a setup for olfactory conditioning of flies with food-component reinforcement. We trained flies in fly vials fitted with 3D printed odour cups. We tested conditioned odour preference of trained flies in Y-mazes put together from fly vials, 3D printed connectors and standard 1 mL micropipette tips. These assemblies are low cost, easy to assemble, customisable and do not require vacuum generated odour flows. The setups yield significantly higher and more persistent olfactory memory scores when compared to the widely used T-mazes. Our experiments suggest that better scores arise from testing in the Y-mazes and do not depend on the training setup. We also obtained enhanced 24-hour aversive taste memory performance with copper sulphate ($CuSO_4$) reinforcement in our Y-mazes. This allowed us to test, for the first time in flies, the protein synthesis dependence of long-lasting aversive gustatory memories.

Materials and Methods

Flies

All flies were reared on food containing sugar, cornflour, malt extract and yeast at 25℃ and 60 % humidity, with a 12-12 hour light-dark cycle in incubators. We used previously described fly strains for all experiments: Wild type Canton-S (CS), *dumb*^{1} and *dumb*^{2} mutants (Young-Cho Kim, Lee and Han, 2007), *R58E02-GAL4 (Liu et al., 2012)* and *UAS-shi* ^{ts1} (Kitamoto, 2001).

Chemicals and other materials

White oil, a petroleum derived mineral oil, of ISO VG 12 grade (Shield Lubricants), with a specific gravity of ~ 0.8 (23°C) was procured locally. Sucrose (ANJ biomedicals-cat no. 57-50-1), octan-3-ol (SIGMA-Aldrich-cat No. 218405), 4-methyl cyclohexanol (SIGMA-Aldrich- cat no.153095), agar (HiMedia-cat no. GRM026), $CuSO_4.5H_2O$ (SIGMA-Aldrich cat No.- 209198), D-arabinose (MP Biomedicals- cat No.-194023), Whatman filter paper (GE-cat no. 3030-917) , Cycloheximide (MP Biomedicals- cat No.-100183) and Polylactic acid (PLA) black 2.85 mm filament Ultimaker-cat No-1609) were procured from authorised vendors.

Odour preparation

For Y-maze conditioning 0.8 µl of octan- 3-ol (OCT) or 0.9 µl of 4-methyl cyclohexanol (MCH) was diluted in 1.25 mL of white oil. We considered this as ~ 10^{-3} or 1000 fold dilution. For odour standardisation experiments 10^{-3} dilutions were further serially diluted to ~ 10^{-4} or ~ 10^{-6}. For T-maze conditioning, ~ 10^{-3} odour concentrations were used (8-12 µl of OCT and 9 µl MCH in 10 mL white oil).

Olfactory associative conditioning using Y-mazes

a. Training setup in fly vials.

5 ml of 0.75 % agar or sucrose (50 mM -to- 2 M) in 0.75 % agar was poured in standard fly rearing vials (inner diameter: 23 mm, outer diameter: 25 mm, height: 90 mm) and

were used for appetitive training. For aversive taste training 80 mM $CuSO_4$ +200 mM arabinose in 0.75 % agar was used. For training with dry sugar, 2.66 M sucrose solution was poured and dried on pieces of filter paper (~8.5 cm*7.3 cm). The sugar papers lined the inner side of the training vials (Fig S2C).

20 µl odour dilutions were spotted on 1 cm * 1 cm Whatman filter paper pieces, which were placed inside custom-designed 3D printed odour cups with slotted base. The cups fit on top of the training vials (Fig 1A, B; Fig S1A, B and supplemental video file 1). The slots in the cup base allow diffusion of the odours into the tube below. We sealed the top of the odour cups with tape to prevent outward odour diffusion. Capped empty vials were used to harbour flies for the 5 minute in between their exposure to the two odours.

b. Differential appetitive and aversive olfactory conditioning (training)

4-8 days old mixed-sex population of flies were used for all experiments. We starved groups of ~ 100 flies in individual vials with 0.75 % agar for ~ 22-24 hours at 25℃. A small strip of filter paper was always provided inside the starvation vials as an additional surface for the flies to climb on. This kept flies from aggregating on the surface of the agar bed and getting their wings stuck wet. Before training we put ~ 20-30 wild-type flies that were not being used for the experiment into each training vial for 20 minutes to imbue the vials with 'fly smell'. This was intended to reduce stress on the experimental flies later. The training tubes were also simultaneously allowed to odorize with the appropriate odour from the odour cups on top.

The conditioned stimuli (**CS**), odours OCT and MCH, were reciprocally paired with only agar (**CS^-** :- CS <u>without</u> unconditioned stimuli) or with sugar reward/aversive taste punishment in agar (**CS^+** :- CS <u>with</u> unconditioned stimuli). To begin, starved flies were transferred to the assigned agar vials to experience the CS^- odour for 5 minutes. The flies were then transferred to empty vials for 5 minutes. Next, they were carried on to the CS^+ odour vial with either sugar-agar reward or aversive substance-agar

punishment for 5 minutes. Any change in association times during training are mentioned where appropriate. Food dyes were added to the CS+ agar to visually monitor feeding during standardization of conditioning protocols. Training vials were inverted during conditioning with aversive substances and during conditioning with shorter association time (2 minutes) to ensure that hungry flies find the agar bed readily due to their negative geotactic tendencies. After training, flies were maintained in 0.75 % agar vials to keep them hungry until testing 24 hours later. For assaying 3, 5 or 7 day memory, flies were starved for ~6 hours after training in 0.75 % agar vials. Subsequently, they were transferred to food vials until 24 hours before planned testing time. They were then shifted back to 0.75 % agar vials for ~ 24 hour starvation until testing.

For blocking specific neuronal populations during training using the *UAS-Shibire*^{ts1} transgene (Kitamoto, 2001), flies in starvation vials were kept at 32°C in a heat chamber specially designed for behavioural experiments for 30 minutes before training (Fig S1E). The training sequence was also carried out at 32°C inside the chamber. After training, flies were shifted back to room temperature (22-23°C) and eventually at 25°C for storage until testing.

c.Y-maze testing setup

The 3D printed parts of the Y-maze were designed on the free online 3D design software TINKERCAD and printed on ULTIMAKER 2 PLUS using PLA filaments. These parts include the central Y-connector with three arms, 3 vial connectors, and a fly loading vial (Fig 1A and S1A). The STL files for all 3D printed parts, including those for training, are available upon request to the corresponding author.

The odour choice vials are the same type as the standard fly-rearing vials used for training. Whatman filter paper pieces (2.5 cm * 2 cm) were spotted with 20 µl of either CS^- or CS^+ odour dilutions and were wedged in at the bottom of the vials. The broader

end of an individual vial connector was attached to each of the two odour vials, with a 1 mL end cut tip fixed into the connector, facing inwards into the vials. The narrow end of a vial connector fitted to individual arms of the central Y connector. The central Y connector has 3 equal length arms that are 120 degrees apart from each other. Snug-fitting at all joints, namely those between the cut tip to vial connector, vial to vial connector, vial connectors to Y connector is ensured by wrapping masking tape onto the appropriate surfaces (Fig S1C). The third arm of the Y connector is connected to the opaque (3D printed) fly loading vial. The odour choice vials are wrapped in black tape to prevent the influence of non-uniform lighting on choice. They can be left uncovered if uniform lighting is ensured to both the choice arms during testing.

d. Conditioned odour preference of flies in Y-mazes (testing)

Before the start of testing, the odour laden filter papers were placed in the appropriate vials and the Y-mazes were assembled as described. They were left to odorize for 20 minutes. Because there are no vacuum-aided odour flows created in the Y-maze, it is important to carry out testing at fixed times after odours are aliquoted and Y-mazes assembled. This is because the strength of the volatile odours tends to change over time and influence choice.

Previously conditioned starved flies were loaded onto the Y-mazes using the 3D printed opaque vial. After loading, the Y-mazes were kept upright in a plastic rack (Figure S1D, E). The experiments should ideally be carried out in stable temperature and uniform light settings. As previously mentioned, we use a custom-designed chamber for testing (Figure S1E). The negative geotactic instinct of flies ensured that they climb up through the Y connector and move towards either one of the odour choice vials. Once individual exploring flies have walked through the cut 1 ml pipette tip into one of the odour vials, they were effectively trapped as they were unable to crawl back into the tip. We generally used a testing time of 30 minutes. As assay scores are determined by trapping the flies, the Y-mazes can be left for a longer period of time for testing. Testing

can also be done with the Y-mazes laid horizontally. In such cases, we suggest that the choice vials are left uncovered to experience uniform light. After testing the Y-mazes were transferred to a freezer for at least 45 minutes to freeze the flies and then their number in each odour vial was counted manually. A memory-driven odour preference score or performance index (PI) was calculated as follows:

Performance Index = (# of flies choosing CS^+ - # of flies choosing CS^-)/ Total # of flies that made choice

To prevent skewed memory scores due to odour bias, half of the fly groups for each condition or genotype is trained with OCT as CS^+ and the other half with MCH as CS^+. Each group of flies, conditioned with either one of the odours is considered as 'n=1'. To reduce variability, scores from any Y-maze where less than 30 flies had made the choice were not included in the final analysis.

Odour balancing in Y-mazes

Memory score is determined by the distribution of a group of conditioned flies between the two odour choice vials (see above). Therefore, establishing odour balance at which naive or unconditioned flies show an equal preference for OCT and MCH, is critical before using the Y-mazes for training. For odour balancing, the Y-maze is prepared with concentrations of OCT and MCH that are presumed to be equally repulsive/attractive to flies naively. ~ 100 naive flies are starved for ~ 22-24 hours on 0.75 % agar and their choice determined as described in the previous section. A preference index of odour choice is calculated as:

Preference Index = (# of flies choosing OCT- # of flies choosing MCH /Total number of flies that made a choice)

Hence a preference index of +1 denotes an absolute OCT preference and -1 denotes an absolute preference for MCH. If the starting odour concentrations yield a skewed distribution, the concentration of the avoided odour can be lowered or the concentration of the preferred odour increased until an average equal preference is achieved.

Important to note here is that odour balances vary with temperature and should be empirically determined as needed.

Olfactory associative conditioning using T-mazes

T-maze conditioning protocols have been extensively described (Pitman *et al.*, 2009; Michael J. Krashes and Waddell, 2011; M. J. Krashes and Waddell, 2011). Following is a brief description.

a. T-maze setup

The T-maze is made of three pieces of acrylic; two side walls screwed to a metal base and a middle piece, with an elevator cavity, sandwiched in between the side walls with clamps (Fig S1F). It has an upper training zone and a lower testing zone that allows binary choice. A single tube connected to an odour source can be fixed at the training zone. The testing zone has two 180° opposing tubes each connected to either one of the odour sources. The elevator in the middle allows the transport of flies from the training to the testing zone. The training and testing zones are connected to a vacuum pump which pulls the training and testing odour streams through the tubes. Constant air pressure was maintained by clamping the T-mazes. Air pressure was frequently checked and adjusted as needed during an experiment using an airflow meter.

b. T-maze training and testing

Flies starved for 24 hours on 0.75 % agar were trained in the upper training tube of a T-maze to pair CS^- odour with 0.75 % agar poured on a Whatman filter paper (4.8 cm * 7.5 cm) or dry filter paper for 2 minutes. CS^- odour source was then disconnected to have only room airflow through the tube for 30 seconds. Following this the flies were transferred to the CS^+ odour tube, lined with a filter paper with a layer of 2 M sucrose (or appropriate sugar/bitter US) in 0.75 % agar, for 2 minutes. Any variation in association times is noted in the appropriate places.

After training, flies were transferred to 0.75 % agar for 24 hours until testing. In some protocols, these are clearly mentioned in relevant places, they were fed briefly ~ 6 hours after training to ensure that enough flies survive for testing. For testing, flies were loaded onto the T-maze in a tube via the upper training zone and lowered to the testing zone, where they are given 2 minutes under uniformly illuminated conditions inside behaviour chambers to choose between the opposing CS^+ and CS^- odour streams. Elevator is raised after 2 minutes, trapping the flies in the individual odour tubes. The flies from these tubes are then transferred to separate vials and subsequently frozen. The number of flies choosing each odour is counted manually. A memory-driven performance index (PI) is calculated the same as with Y-mazes:

Performance Index = (# No. of flies choosing CS^+ - # No. of flies choosing CS^-)/ Total # of flies that made a choice

As with Y-mazes, we trained an equal number of groups with OCT and MCH as the CS^+ odour as much as possible. We considered each group of flies, conditioned with either one of the odours as n=1.

Cycloheximide feeding

As described before, ~ 35 mM of cycloheximide was mixed into 0.75 % agar when flies were being starved before training for 16 hours (Tully *et al.*, 1994; Krashes and Waddell, 2008). Cycloheximide was dissolved in warm water and subsequently mixed into melted agar before it was poured and solidified in the starvation vials.

Data plotting and Statistical analysis

Statistical analysis was performed using the software GraphPad Prism version 8.3.1. All data points are represented as a scatter plot in which each point represents a single 'n' with mean and ± standard deviation (SD). P values for relevant comparison of groups for all data and other relevant statistical details are reported in Supplemental table 1 (ST1).

We plotted our data as estimation plots. These plots include a representation of the 'effect' size, which here is the difference in means of the compared groups (Δ Performance Index- ΔPI). While the data itself were plotted using GraphPad Prism, we used the DABEST ('data analysis with bootstrap-coupled estimation') web application at https://www.estimationstats.com for generating the effect size plots (Ho *et al.*, 2019). The ΔPI or the effect size axis, placed to the right of the main graphs, has its origin at the control group mean. The effect size is aligned with the experimental group mean on this axis (large black dot). The curve represents bootstrap sampling error distribution of ΔPI. The 95 % Confidence Interval (CI) of the effect size is depicted by the two ends of the vertical error bars intersecting the effect size. The software generates the 95 % CI and the error curve by performing 5000 bootstrap (with replacement) resampling of the groups being compared and computing the difference in means of the resampled groups. The software also applies bias-corrected and accelerated (BCa) correction to the resampling bootstrap distributions of the effect size, to account for any skew in it. Further details can be found at https://www.estimationstats.com.

Results

Training in vials and testing in Y-mazes is able to assay robust long-term memory

To simplify the handling and assembly of olfactory conditioning setup in flies, we adapted a Y-maze based design without active (vacuum-assisted) odour streams (Fig 1A, Fig S1A-E). Design, build and details of olfactory conditioning and odour preference testing are described in the Materials and Methods section.

We started by training groups of flies with 2 M sucrose reinforcement and tested for 24-hour memory performance, with 10^{-3}, 10^{-4} and 10^{-6} dilutions of OCT and MCH, for both training and testing (Fig 1B). Naive/untrained flies were found to prefer the two odours equally at the above concentrations (Fig S2A). We observed robust performance with 10^{-3}, drastically lowered but intermediate performance with 10^{-4} and no discernible performance with 10^{-6} odour concentrations (Fig 1B). Based on our results, we decided to train and test flies with 10^{-3} dilutions of OCT and MCH for all subsequent experiments.

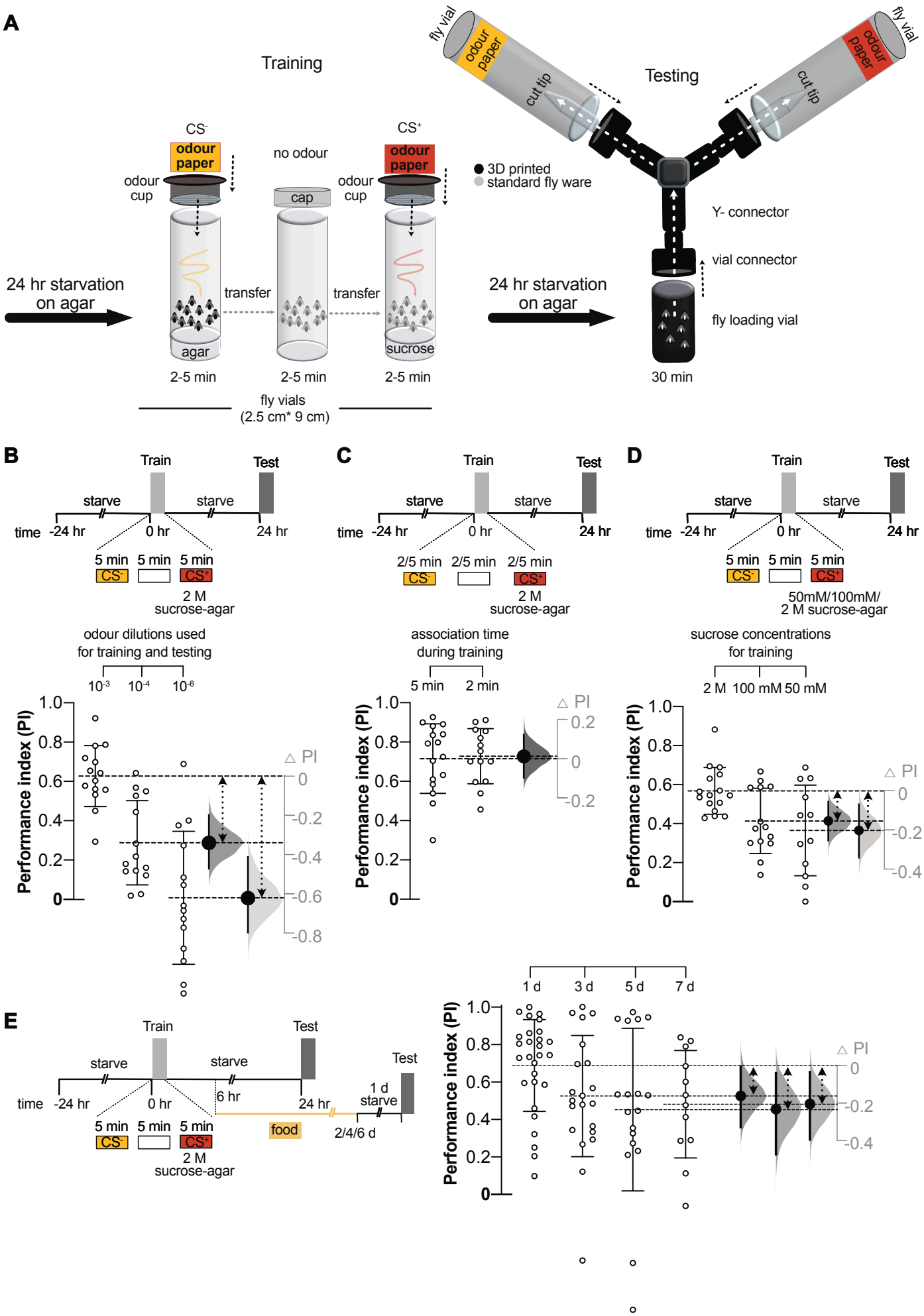
In all above cases flies had 30 minutes to choose between the two odour choice vials as this proved to be a sufficient period to allow most flies to make a choice (Figure S2B). It is important to note here that we observed that a large number of flies are trapped inside the choice vials by ~10-15 minutes (*data not shown)*. But we decided to go with 30 minutes for convenience of use, and to ensure a majority of flies have made a choice. We did not go for a longer choice time to prevent the choice being affected by an altered odour balance.

We next examined the effect of CS^+ association time on memory performance. Decreasing odour with 2 M sucrose association time down to 2 minutes from 5 minutes did not affect performance (Fig 1C). However, flies trained for 5 minutes with sucrose showed lower mortality upon subsequent starvation (data not shown). For most experiments, we subsequently preferred to use the 5 minute association time due to the

lower mortality rate and also because the 5-5-5 minute (CS^--no odour-CS^+) training regimen conveniently facilitated the conditioning of multiple groups of flies simultaneously.

We also investigated the effect of training with different concentrations of sucrose on 24-hour memory scores. We observed considerably reduced memory scores when flies were trained with 100 mM or 50 mM sucrose compared to with 2 M sucrose training (Fig 1D). Since training with 2 M sucrose facilitated the highest 24-hour memory performance, we used this concentration of sucrose for further experiments. We also trained flies with 2.66 M saturated sucrose solution poured and dried on paper, which lined the training vials. Here, the scores were robust too, but reduced compared to training with 2M Sucrose in agar for the same association time of 2 minutes (Fig S2C, Fig 1C). Next, we tested how long memory lasted in our setup. For this, we tested 2 M sucrose (in agar) reinforced memory performance 1, 3, 5 and 7 days after training. We found that despite expected decay of memory score over time, 7-day memory performance was robust (Fig 1E).

Figure 1


A
Training
CS-
odour paper
odour cup
no odour
cap
CS+
odour paper
odour cup
24 hr starvation on agar
transfer
transfer
agar
sucrose
2-5 min
2-5 min
2-5 min
fly vials (2.5 cm* 9 cm)
Testing
fly vial
odour paper
cut tip
3D printed
standard fly ware
Y- connector
vial connector
fly loading vial
24 hr starvation on agar
30 min
B
Train
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time -24 hr
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CS- CS+
2 M sucrose-agar
odour dilutions used for training and testing
10-3 10-4 10-6
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time -24 hr
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2/5 min 2/5 min 2/5 min
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association time during training
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5 min 5 min 5 min
CS- CS+
50mM/100mM/ 2 M sucrose-agar
sucrose concentrations for training
2 M 100 mM 50 mM
Performance index (PI)
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Test
starve
starve
Test
time -24 hr
0 hr
6 hr
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1 d starve
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CS- CS+
2 M sucrose-agar
food
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Performance index (PI)
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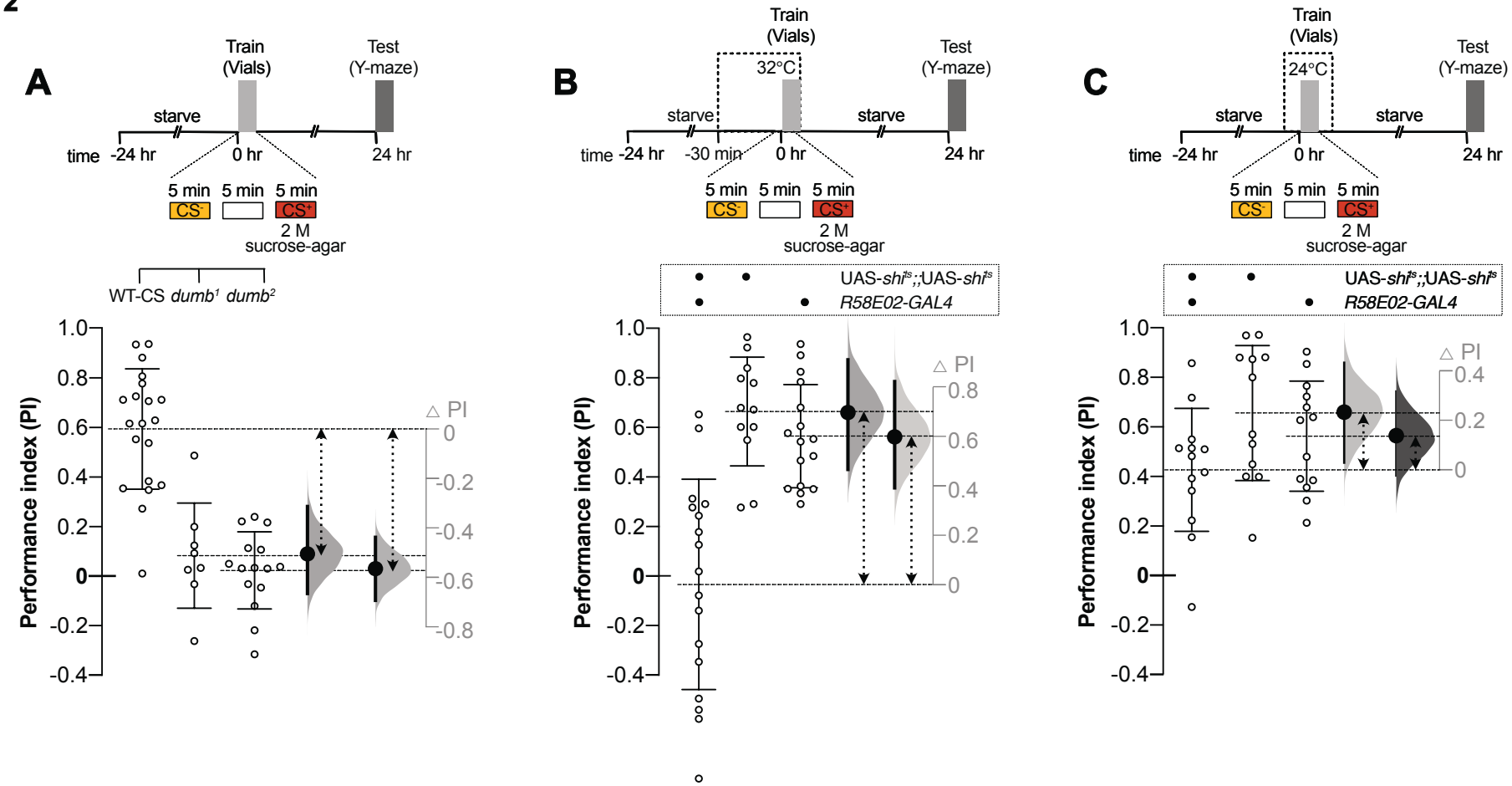
Figure 1: Robust and long-lasting memory performance using Y-mazes: (A) Protocol for classical odour conditioning of flies in vials and subsequent testing of conditioned odour preference in Y-mazes without vacuum-assisted active airflows. The setups are built with a combination of readily available fly plasticware and custom-designed 3D printed parts. **(B)** Using ~ 10^{-3} fold dilution of OCT and MCH for both training and testing yielded the maximum score, compared to ~ 10^{-4} or ~ 10^{-6} dilutions , *n=14; CS*$^{+}$ *OCT=7, MCH=7* for all groups. **(C)** Varying odour and 2 M sucrose association time from 5 minutes (*n=16; CS*$^{+}$*OCT=8, MCH=8*) to 2 minutes (*n=14; CS*$^{+}$ *OCT=7, MCH=7*) during training, did not change 24-hour memory performance. **(D)** Lowering sucrose concentration used for training from 2 M (*n=16; CS*$^{+}$ *OCT=8, MCH=8*) to 100 mM (*n=14; CS*$^{+}$ *OCT=7, MCH=7*) and 50 mM (*n=12; CS*$^{+}$*OCT=6, MCH=6*) greatly reduced 24-hour memory performance. **(E)** 2 M sucrose reinforced memory performance with Y-mazes at 1 day (*n=28; CS*$^{+}$*OCT=14, MCH=14*), 3 day (*n=21; CS*$^{+}$*OCT=11, MCH=10*), 5day (*n=18; CS*$^{+}$*OCT=10, MCH=8*) and 7 day (*n=12; CS*$^{+}$*OCT=6, MCH=6*) after conditioning. Substantial 2 M sucrose reinforced memory performance is seen even at the 7-day timepoint. To the right of each graph, effect size or the difference in mean performance index (ΔPI) plots are shown. The curve represents the bootstrap sampling error distribution of ΔPI. The observed effect size between experimental and a control group is depicted as a large black dot and the 95 % CI is indicated by the ends of the vertical error bars. Double-headed dashed arrows between the group means indicate the groups being compared. *Also, see supplementary Figure S1 and S2*.

Memory formation in our setup is dopamine signalling and PAM cluster activity dependent

Olfactory appetitive memory formation in a T-maze requires dopamine release from the rewarding dopaminergic neurons of the protocerebral anterior medial (PAM) cluster and the dopaminergic receptor DopR1 in the mushroom body neurons (Young-Cho Kim, Lee and Han, 2007; Burke *et al.*, 2012; Liu *et al.*, 2012). To test whether appetitive memories formed in our setup were also dopamine signalling dependent, we first tested 2 M sucrose reinforced 24-hour memory performance in two strains that are null mutants for the dopamine receptor DopR1, namely $Dumb^{1}$ and $Dumb^{2}$, along with wild type CS control flies (Young-Cho Kim, Lee and Han, 2007). Olfactory memory performance was almost completely abolished in the $Dumb^{1}$ and $Dumb^{2}$ mutants compared to the robust performance in the control (Fig 2A).

Next, we wanted to test whether the rewarding PAM dopaminergic neurons were required for appetitive memory formation during training in our setup . For this, we expressed the dominant temperature-sensitive *UAS-Shibire*^{ts1} transgene (Kitamoto, 2001) in almost all PAM neurons using the *R58E02-GAL4* driver line (Liu *et al.*, 2012; Huetteroth *et al.*, 2015; Yamagata *et al.*, 2015). This allowed us to transiently silence the rewarding PAM dopaminergic neurons at the restrictive temperature of 32°C during training with 2 M sucrose reinforcement. Upon testing memory performance at 24-hour, we saw that while robust memory performance is seen in the parental controls, the experimental flies, expressing *UAS-Shibire*^{ts1} in *R58E02-GAL4* labelled neurons, exhibited no apparent memory performance (Fig 2B). Importantly, such memory defects were not observed in the same genotypes when the experiment was performed at the permissive temperature of 24℃ (Fig. 2C). Together, the above results ascertained that learning in our assay setup required dopamine release from the PAM neurons and also required the function of the DopR1 dopamine receptor expressed in the mushroom body neurons.

Figure 2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(Vials)
Test
(Y-maze)
starve
time -24 hr
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24 hr
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5 min
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CS+
2 M
sucrose-agar
WT-CS dumb1 dumb2
Performance index (PI)
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C
24°C

Figure 2: Appetitive memory formation is dopamine signalling dependent: (A) 24-hour Memory formation is DopR1 dopamine receptor-dependent. Compared to wild type CS flies *(n=20; CS$^+$ OCT=10, MCH=10)*, appetitive memory performance is abolished in *dumb^1* (*n=8; CS$^+$ OCT=5, MCH=3*) and *dumb^2* (*n=15; CS$^+$ OCT=7, MCH=8*) mutants. **(B)** Activity of the reward dopaminergic neuron in the PAM cluster is required for appetitive memory formation during training in vials. Flies were trained with 2 M sucrose reinforcement at 32°C to block PAM dopaminergic neurons during training. *R58E02-GAL4* drove *UAS-Shibire^{ts1}* transgene in reward PAM dopaminergic neurons *(n=17; CS$^+$ OCT=9, MCH=8*). Memory performance was severely impaired in these flies compared to the two parental lines, *UAS-Shibire^{ts1}(n=12; CS$^+$ OCT=6, CS$^+$ MCH=6)* and *R58E02-GAL4 (n=16;CS$^+$ OCT=8, CS$^+$ MCH=8)*. **(C)** Room temperature control experiment for (B) shows that the experimental genotype, *R58E02-GAL4/UAS-Shibire^{ts1} (n=13;CS$^+$ OCT=7; CS$^+$ MCH=6)* performs as well as the parental controls *UAS-Shibire^{ts1}(n=12;CS$^+$ OCT=7, CS$^+$ MCH=5)* and *R58E02-GAL4 (n=13;CS$^+$ OCT=7, CS$^+$ MCH=6)*. Effect size or the difference in mean performance index (ΔPI) plots are shown for all. They show the observed effect size of the experimental group compared to a control (large black dots), 95 % CI (end of vertical error bars) and the bootstrap resampling error (curve) of effect size. Double-headed dashed arrows mark the groups being compared.

Testing in the Y-mazes is key to improved scores over the T-maze

While our average Y-maze performance indices for 24-hour memory are consistently higher (~0.6) than reported using the T-maze, we wanted to do a side by side comparison with a frequently used T-maze paradigm (Tully *et al.*, 1994; Michael J. Krashes and Waddell, 2011; M. J. Krashes and Waddell, 2011). For this, we trained groups of flies with 1 M sucrose reinforcement in vials or in T-maze tubes for the same CS^+ association time of 2 minute. From both training conditions, half of the groups were tested in Y-mazes and the other half in the T-mazes for 24-hour memory. Hence, we had 4 groups in each experiment; a) vial-trained and Y-maze tested (VY), b) vial-trained and T-maze tested (VT), c) T-maze tube trained and Y-maze tested (TY) and d) T-maze tube trained and T-maze tested (TT) (Fig 3A, schema).

We observed that regardless of the training condition, in vials or in T-maze tubes, the groups tested in the Y-mazes showed consistently greater mean performance than the groups tested in the T-mazes (VY > VT and TY>TT). This strongly suggested that testing in the Y-maze assembly was critical for the higher scores obtained. Also, groups tested in the same apparatus had similar scores regardless of the training conditions (VY≈TY and VT≈TT), again highlighting the importance of testing paradigms in the magnitude of memory performance (Fig 3A).

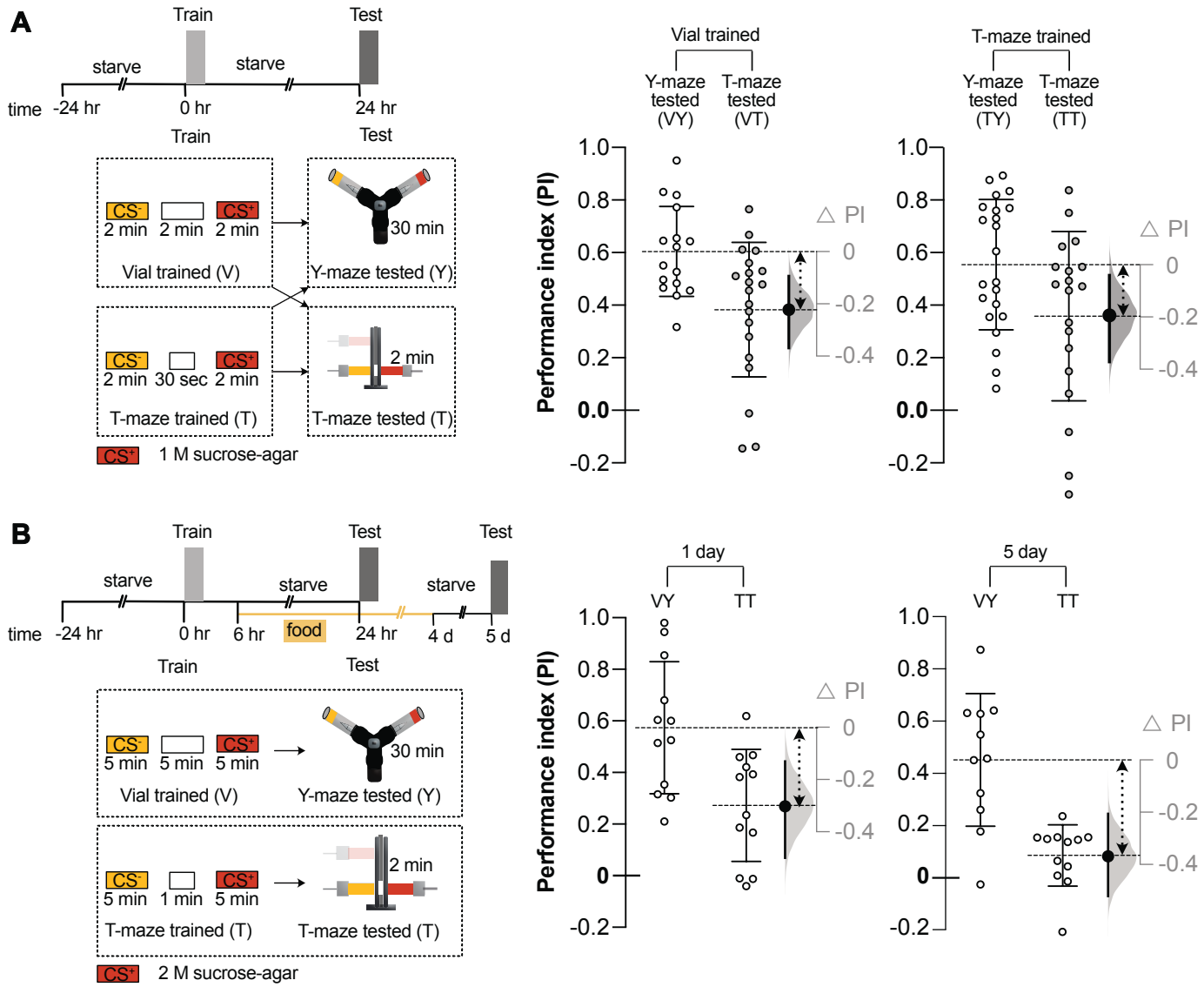
Next, we directly compared the longevity of memory performance using Y-mazes and T-mazes. Fly groups were trained in vials and T-maze tubes with 2 M sucrose reinforcement for 5 minute CS^+ association time. The memory performance of both these groups were tested 1 day and 5 days after training with the vial trained flies tested in Y-mazes (VY) and the T-maze tube trained flies tested in T-mazes (TT).

We found that, as before (Fig.1D), robust 1-day and 5-day memory performance could be seen in VY groups. At both time points, TT groups showed considerably reduced

memory scores compared to the VY groups. However, the reduction was more pronounced on the 5th day, with a markedly weakened TT group mean score (Fig. 3B).

This suggested that workable memory scores were hard to derive with the T-mazes beyond 5 days, whereas robust 7-day memory performance was clearly attainable with Y-mazes (Fig 1D). Therefore, clearly, the Y-maze assemblies yielded superior scores over a longer time too.

Figure 3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Vial trained (V)
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Y-maze tested (Y)
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5 min
1 min
5 min
T-maze trained (T)
2 min
T-maze tested (T)
CS+
2 M sucrose-agar
1 day
5 day
VY
TT
Performance index (PI)
△ PI

Figure 3: Olfactory memory assay using Y-mazes markedly improves scores over T-maze based assaying. (A) Direct comparison of training and testing in Y-mazes and T-mazes. Results suggest that irrespective of training condition, in vials or in T-maze tubes, testing in Y-mazes yields superior scores to that of the T-mazes. The Vial trained and T-maze tested group (VT; *n=20; CS*$^{+}$ *OCT=11, MCH=9*) has considerably reduced performance from the Vial trained and Y-maze tested group (VY; *n=16; CS*$^{+}$ *OCT=8, MCH=8*). Similarly, the T-maze tube trained and T-maze tested group (TT; *n=19; CS*$^{+}$ *OCT=9, MCH=10*) also has reduced performance from the T-maze tube trained and Y-maze tested group (TY; *n=22; CS*$^{+}$ *OCT=11, MCH=11)*. **(B)** 1 day and 5 day memory performance comparison between Y-mazes and T-mazes. Significant 1 day scores are seen in both the Vial trained and Y-maze tested group (VY) and the T-maze tube trained and T-maze tested group (TT). However, VY group (1 day; *n=12; CS*$^{+}$ *OCT=6, MCH=6*) has substantially higher memory performance than the TT group (1 day; *n=12; CS*$^{+}$ *OCT=6, MCH=6*). Similar trend is seen on day 5 for the two groups. However, while VY *(*5 day; *n=11; CS*$^{+}$ *OCT=5, MCH=6*) performance is still robust, TT group (5 day; *n=12; CS*$^{+}$ *OCT=6, MCH=6)* performance is close to zero. Effect size or the difference in mean performance index (ΔPI) plots are shown for all. They show the observed effect size of the experimental group compared to a control (large black dots), 95 % CI (end of vertical error bars) and the bootstrap resampling error (curve) of effect size. Double-headed dashed arrows mark the groups being compared.

24-hour sweet and aversive taste memories are protein synthesis-dependent

Previously, it had been difficult to see any bitter or aversive taste reinforced memory scores 24 hours post-training in flies (Das *et al.*, 2014). Based on above results, it is possible that T-maze conditioning protocols that do not normally yield scores at a certain time point, may do so with Y-maze testing. We therefore verified whether assaying aversive taste learning in Y-mazes could enable the study of long-term taste aversion.

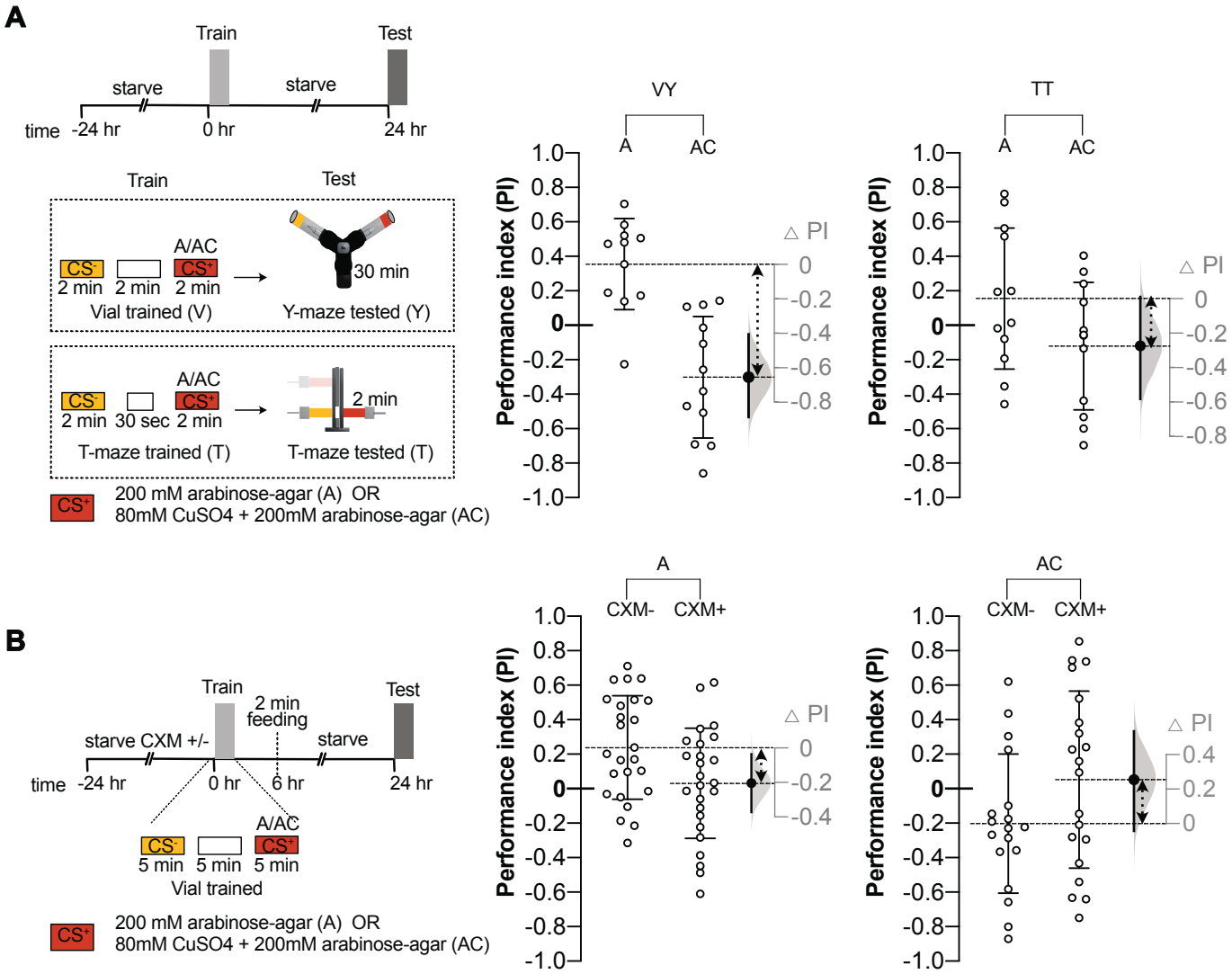
Copper sulphate ($CuSO_4$), a compound listed as a pesticide, is toxic to flies (Živanov-Čurlis *et al.*, 2006; Alaraby, Hernández and Marcos, 2017; Balinski and Woodruff, 2017; Halmenschelager and da Rocha, 2019), and our unpublished data suggests that flies avoid it. Therefore, we decided to condition ~ 24 hour starved flies both in T-maze tubes and in training vials with 80 mM $CuSO_4$ along with 200 mM arabinose, a sweet but not metabolisable sugar, as a carrier to promote ingestion of $CuSO_4$. Parallelly, flies were also conditioned with 200 mM arabinose only. 24 hours after training, T-maze trained flies were tested in T-mazes (TT) and vial-trained flies were tested in Y-mazes (VY).

We saw that training and testing in the T-maze yielded a low positive score with 200 mM arabinose and a low aversive score with 80mM $CuSO_4$ + 200 mM arabinose (Fig 4A). Previous work suggests that when mixed appetitive and aversive stimuli are used for training, the memory score is the sum of the parallel and opposing memories from the two components (Das *et al.*, 2014). Therefore, the difference between the means of the two groups (arabinose and $CuSO_4$+ arabinose) is a measure of the aversiveness of $CuSO_4$. This difference is modest in the case of the TT groups and makes it difficult to further investigate (Fig 4A). In contrast, with the VY groups, both the approach scores with 200 mM arabinose and the avoidance scores with 80mM $CuSO_4$ + 200 mM arabinose are greater and the difference between their means substantial (Fig 4A). We

concluded that Y-maze testing can enhance aversive memory scores too, making it possible to study the underlying neural mechanisms.

Because we could see a robust $CuSO_4$ reinforced 24-hour aversive memory, we decided to investigate whether such memory is protein synthesis-dependent using Y-mazes. For this, experimental flies were kept on 35 mM of cycloheximide, a protein synthesis inhibitor, mixed in 0.75 % agar during the ~ 17 hour starvation period before training. Control flies were not exposed to cycloheximide. As before, flies were conditioned with 200 mM arabinose or 80 mM $CuSO_4$ + 200mM arabinose and tested for 24-hour memory. They were briefly fed, for 2 minutes on 2 M sucrose, 6 hours after training to keep enough flies alive in the wake of cycloheximide exposure. We observed that both 200 mM arabinose memory and 80 mM $CuSO_4$ + 200mM arabinose reinforced memories were reduced in the CXM fed (CXM+) group compared to the control (CXM-) group. We concluded that formation of both arabinose reinforced appetitive and $CuSO_4$ reinforced aversive memories were sensitive to CXM treatment and thus likely to be protein synthesis dependent (Fig 4B).

Figure 4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2 min 30 sec 2 min
2 min
T-maze trained (T)
T-maze tested (T)
CS+
200 mM arabinose-agar (A) OR
80mM CuSO4 + 200mM arabinose-agar (AC)
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Performance index (PI)
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TT
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AC
Performance index (PI)
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A/AC
CS-
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5 min 5 min 5 min
Vial trained
CS+
200 mM arabinose-agar (A) OR
80mM CuSO4 + 200mM arabinose-agar (AC)
A
CXM-
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Performance index (PI)
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AC
CXM-
CXM+
Performance index (PI)
△ PI

Figure 4: Testing in Y-mazes enables studying of novel memory phenotypes: testing protein synthesis dependence of sweet and aversive taste reinforced memories. (A) Enhanced $CuSO_4$ reinforced aversive memory is seen in the Y-maze tested groups (VY) compared to the T-maze tested group (TT). In the Y-maze tested groups, the difference in memory performance between 200 mM arabinose training (VY-A; *n=12; CS*$^+$ *OCT=6, MCH=6*) and the 80 mM $CuSO_4$ + 200 mM arabinose training (VY-AC; *n=12; CS*$^+$ *OCT=6, MCH=6*) was substantial. In contrast when the same experiment was done with T-mazes, memory performance difference between 200 mM arabinose training (TT-A; *n=11; CS*$^+$ *OCT=5, MCH=6*) and 80 mM $CuSO_4$ + 200 mM arabinose training (TT-AC; *n=12; CS*$^+$ *OCT=6, MCH*=6) was modest. **(B)** To ascertain protein synthesis dependence of robust aversive taste memories, flies were trained in vials and tested for 24-hour memory performance in Y-mazes. 200 mM arabinose reinforced appetitive memory performance is reduced upon cycloheximide treatment (A-CXM+; *n= 25; CS*$^+$ *OCT=12, MCH*= 13) compared to the control (A-CXM-; *n=25; CS*$^+$ *OCT=12, MCH=13*). Further, 80 mM $CuSO_4$ + 200mM arabinose reinforced aversive memory performance upon cycloheximide treatment (AC-CXM+; *n=18; CS*$^+$ *OCT=9, MCH*=9) was also reduced compared to the control (AC-CXM-; *n=20; CS*$^+$ *OCT=10, MCH=10*). To increase survival, all flies were briefly fed for 2 minute on 2 M sucrose, 6 hours after training. To the right of each graph, effect size or the difference in mean performance index (ΔPI) plots are shown. These plots depict the bootstrap sampling error distribution curve of ΔPI. The actual mean difference of the groups is depicted as a large black dot and the 95 % CI is indicated by the ends of the vertical error bars. Double-headed dashed arrows between the group means indicate the groups being compared.

Discussion

Y-maze designs for olfactory behaviours

Effective study of neural circuitry requires robust behavioural assays. It was the development of reliable learning and memory assays that enabled the neurogenetic study of fly learning and memory (Quinn, Harris and Benzer, 1974; Tully *et al.*, 1994; Pitman *et al.*, 2009; Tabone Christopher and de Belle, 2014). The olfactory conditioning and testing assembly we have described here has the potential to uncover novel memory phenotypes by virtue of the superior scores it generates. The Y-mazes are easy to build and costs a fraction of other conditioning setups currently in use. The lack of vacuum-assisted odour streams does away with the need of tubing, clamps, vacuum pumps and airflow monitoring, making it much simpler to handle.

Y-maze setups allowing free movement are common in rodent studies to measure spatial working memory and exploratory behaviour (Kraeuter, Guest and Sarnyai, 2019). Miniature single fly Y-mazes have been used in flies for the same purpose (Buchanan, Kain and de Bivort, 2015; Lewis *et al.*, 2017; Cleal *et al.*, 2020). Y-mazes have also been used for naive and learned odour preferences in insects other than flies (Dupuy *et al.*, 2006; Carcaud *et al.*, 2009; Josens, Eschbach and Giurfa, 2009). One of the first conditioning studies with *Drosophila* included Y-maze based visual conditioning experiments. Flies were trained in one arm of the Y-maze to avoid a specific wavelength of light paired with aversive quinine taste. However, scores in this visual discrimination assay were quite low (Quinn, Harris and Benzer, 1974). Y-mazes have also been used recently for testing learned odour preference of flies previously conditioned with sugar or alcohol (Kaun *et al.*, 2011; Albin *et al.*, 2015; Nunez, Azanchi and Kaun, 2018). In these designs active and competing odour flows were generated in the arms of the Y-mazes for flies to choose between the conditioned odour stimuli. T-mazes and more often Y-mazes have been used as ‘olfactometers’ to measure olfactory choice, acuity and habituation in flies (Rodrigues and Siddiqi, 1978; Chakraborty, Goswami and

Siddiqi, 2009; Das *et al.*, 2011; Simoes, Ott and Niven, 2011; Simonnet, Berthelot-Grosjean and Grosjean, 2014).

Y-mazes against T-mazes

We have compared our Y-maze assembly directly to the T-maze design refined in (Tully and Quinn, 1985). Our appetitive and aversive gustatory cue reinforced memory scores from the Y-mazes were markedly greater than from the T-mazes (Fig 4). We also report durable appetitive memory scores up to 7 days in the Y-mazes. In contrast, T-maze PI scores were lower than 0.1 by day 5 in our hands (Fig 3). This suggests that memory performance will most likely be non-existent by day 7 in the T-maze. This trend is in agreement with earlier studies where training was done with dried sugar (as opposed to sugar in agar here) and tested in conventional T-mazes (Krashes and Waddell, 2008; Colomb *et al.*, 2009). Going by the 7-day score with Y-mazes, flies are likely to exhibit memory performance even at later time points. Previously, such long-lasting appetitive scores from testing in T-mazes, have only been reported upon artificial induction of appetitive memory in flies (Huetteroth *et al.*, 2015).

Our experiments suggest that testing in the Y-mazes is key to the improved scores. We speculate that this improvement over T-mazes is due to some or all of the following possibilities; a) an increased path length from the end of the cut pipette tips to the Y-junction and hence an extended odour gradient, b) increased time for choice, coupled with factors outlined in (a), might allow for a more reflective than reflexive choice, c) The trapping mechanism that prevent change in preference once choice is made d) reduced crowding at the choice point, preventing a ‘stampede’ like effect (Quinn, Harris and Benzer, 1974) and e) lack of airflow in the setup could help increase correct choice as flies do not have to struggle against fast odour streams.

We also demonstrate that, like in T-mazes, memory formation in our setup depends on dopamine signaling to the mushroom body neurons. We did not directly test whether mushroom body neuronal activity is required for memory retrieval in the Y-mazes.

Typically, when using T-mazes, scores from two fly groups reciprocally trained with the two odours and tested in the same T-maze, are averaged to compute a single data point. This is done to negate any intrinsic odour bias in the specific T-maze machine used. However, as no single Y-maze was reused for testing, instead of averaging reciprocal odour-trained groups, all Y-maze tested groups were represented as individual data points. Further, to maintain parity we also adapted a similar representation for T-maze data in all experiments here. Importantly, not averaging the memory scores from pairs of reciprocally trained groups, the mean of the data population is not altered. In fact, averaging decreases the population standard deviation. We do maintain an equal number of groups conditioned with each odour to balance out odour biases in experiments.

The Y-mazes are versatile

Testing in Y-mazes can be potentially coupled to other training protocols. For example, electric shock conditioned flies can be tested in Y-mazes to improve test scores. Y-mazes are compatible with common genetic manipulation techniques used in flies. We demonstrate its use with thermogenetic silencing of neurons. It is easy to carry out both training and testing at 32°C by simply placing the training vials and the Y-mazes inside a chamber of appropriate temperature (Fig S1F). Care should be taken to balance odour concentrations at the testing temperature to ensure equal distribution of naive flies. With slight modifications, the Y-mazes should be optogenetic compatible too. As is, training is done in transparent vials. The testing setup can be made optogenetics-ready by using transparent Y-connectors. The Y-mazes described here do not require a vacuum source to work, making them readily portable and can be easily used for science outreach outside the laboratory.

A perceived drawback of our assay is that it is fully manual. Training, testing and counting flies are not automated. But the setup is simple enough to be amenable to mechanization. Another drawback could be the number of flies that are used (~50-100 for n=1). While the strength of group-based memory assays is in the numbers, one can easily shrink the dimensions of the connectors to fit with smaller tubes and perform the assay with ~ 25-50 flies. Assaying memory with even smaller group sizes may increase the variability of the data as choice of individual flies will have a greater influence on the score.

Revealing novel memory phenotypes with the Y-mazes

When flies are odour-conditioned for a single round with heat or bitter tasting compounds, the memories formed decay within a few hours (Quinn, Harris and Benzer, 1974; Tully and Quinn, 1985; Das *et al.*, 2014; Galili *et al.*, 2014; Masek *et al.*, 2015). Single round of shock reinforced memories also decays within a few hours or show relatively weak 24-hour memory performance, mainly depending on the voltage, protocol and setup used (Quinn, Harris and Benzer, 1974; Tully and Quinn, 1985; Tully *et al.*, 1994). Robust 24-hour aversive memory reinforced by shock stimulus requires multiple rounds of massed or spaced training (Tully *et al.*, 1994). However, no such effect is reported for training with heat, bitter taste or other aversive stimuli. Our improved 24-hour appetitive memory scores with the Y-mazes encouraged us to test whether robust taste reinforced aversive memories can be assayed. In keeping with our appetitive memory scores, here we report the presence of a robust 24-hour aversive taste memory reinforced by $CuSO_4$. This then prompted us to test whether this long-lasting aversive memory was protein synthesis-dependent.

Protein-synthesis dependence, often tested by the use of pharmacological blockers of protein synthesis, has been considered a hallmark of ‘long-term memory’ (LTM) in flies and other species (Davis and Squire, 1984; Hernandez and Abel, 2008). Memory

formed by single session shock training is not sensitive to the feeding of the protein synthesis inhibitor cycloheximide, unless flies are starved before training and fed immediately afterwards (Tully *et al.*, 1994; Hirano *et al.*, 2013; Plaçais and Preat, 2013). Formation of protein synthesis dependent shock-reinforced aversive memories are also seen upon multiple session spaced training, but not upon massed training (Tully *et al.*, 1994).

A recent study in flies highlighted the importance of the order of CS^+ odour-shock association during spaced training. They showed that when CS^+ precedes CS^-, both a CS^+ linked aversive memory and a CS^- linked appetitive 'safety' memory is formed. Thus the 24-hour memory performance, showing aversion to the CS^+ odour, is ultimately a summation of both these memories (Jacob and Waddell, 2020). Interestingly, only the CS^- linked 24-hour appetitive safety memory, not the CS^+ linked aversive memory, is protein synthesis dependent. Thus adding clarity to why spaced trained flies show reduced aversive memory performance upon cycloheximide exposure (Tully *et al.*, 1994). In contrast, when CS^- precedes CS^+ during spaced shock training, no parallel safety memory is formed (Jacob and Waddell, 2020).

Incidentally, protein synthesis independent, shock reinforced aversive memory, for 14 days and possibly more, have been reported in flies. This memory, called context dependent LTM (cLTM), is retrieved only when the context of copper wires that deliver shock during training is presented during testing inside the T-maze choice tubes. However, while the formation of cLTM is dopamine dependent, its retrieval is mediated through the lateral horn neurons (Zhao *et al.*, 2019).

In our experiments, we found that the aversive memory formed by a single 5 minute session of 80mM $CuSO_4$ (with 200 mM arabinose carrier) training, is severely impaired when flies were exposed to the protein-synthesis inhibitor cycloheximide. This was also the case for memory reinforced by 200 mM arabinose alone. Since neither aversive nor

sweet taste reinforced 24-hour memories have been observed robustly before, their protein synthesis dependence had not been tested. Previous studies suggest that starvation biases the brain away from protein synthesis dependent LTM formation. If flies are food replete or have been fed proximally enough to when they are conditioned with shock-they form LTM (Hirano *et al.*, 2013; Plaçais and Preat, 2013). Similarly, feeding after 3 hours of conditioning with sweet only sugar aids in the formation of LTM. However, no LTM is formed when flies are fed after 6 hours of conditioning with sweet only sugar (Musso, Tchenio and Preat, 2015). In our case flies were starved before training, kept starved after training, but they were briefly fed for 2 minutes with 2 M sucrose, 6 hours after training. We cannot rule out that this brief feeding might induce LTM formation without thorough investigation. Nonetheless, our results imply that either a) gustatory reinforcement forms LTM independently of energy status or b) LTM formation can be induced with a brief dose of feeding even 6 hours after training. Our ongoing studies are pursuing these and other related questions regarding the nature of long lasting aversive memories using the Y-mazes.

In conclusion, we have highlighted the potential benefits of a newly designed Y-maze assembly, the most important of which is improved memory scores. Potential users can also easily modify parts of the assembly to suit their needs. We anticipate that the Y-maze assembly described here will be widely adopted by researchers investigating mechanisms of learning and memory in flies.

Acknowledgements

We would like to thank all our colleagues in the brain and feeding behaviour laboratory, NCCS, for their support, help with experiments and for providing feedback on the manuscript. We also thank Suewei Lin, Vincent Croset, Emamanuel Perisse, Johannes Felsenberg, Wolf Huetteroth and Scott Waddell for their critical reading of the manuscript and providing valuable feedback and insights. We thank Paola Cognini- our vial training paradigm has been inspired by her similar design. We thank Rajkumar Pawar for lab ordering, making fly food, stock maintenance and Abhay Jadhav for washing fly bottles and vials. RM is funded by a PhD fellowship from NCCS. MRT is funded by a Department of Biotechnology (DBT) doctoral scholarship. FMI and MS were funded from a Ramanujan Fellowship to GD. GD is a Ramanujan Fellow (SB/S2/RJN-048/2017) awarded by the Science and Engineering Research Board (SERB), part of the Department of Science & Technology (DST), Government of India. The lab is also funded by a Core Research Grant (CRG/2019/005587) from SERB and by generous intramural funding support from NCCS.

Contributions

G.D. and R.M. conceived the design of this particular Y-maze. Initial trials and design refinement was done by R.M., M.S. and G.D. All experiments described here were performed by R.M., F.M.I and M.T. G.D. directed the research and wrote the manuscript with inputs from R.M.

Competing interests

The authors declare no competing interests.

Data availability

All raw data, including STL files for the Y-maze parts are available upon request to the corresponding author.

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