Optogenetic induction of appetitive and aversive taste memories in *Drosophila*

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SUMMARY

Tastes are typically thought to evoke innate appetitive or aversive behaviours, prompting food acceptance or rejection. However, research in *Drosophila melanogaster* indicates that taste responses can be modified through experience-dependent changes in mushroom body circuits. In this study, we develop a novel taste learning paradigm using closed-loop optogenetics. We find that appetitive and aversive taste memories can be formed by pairing gustatory stimuli with optogenetic activation of sensory or dopaminergic neurons associated with reward or punishment. As with olfactory memories, distinct dopaminergic subpopulations drive the parallel formation of short- and long-term appetitive memories. Long-term memories are protein synthesis-dependent and have energetic requirements that are satisfied by a variety of caloric food sources or by direct stimulation of MB-MP1 dopaminergic neurons. Our paradigm affords new opportunities to probe plasticity mechanisms within the taste system and understand the extent to which taste responses are experience dependent.

INTRODUCTION

Food selection is influenced by a complex set of factors including external sensory input, interoceptive circuits signaling internal state, and plasticity driven by past feeding experiences. The gustatory system plays a critical role in evaluating the nutritional qualities of foods, and is generally thought to evoke innate appetitive or aversive behavioural responses. However, the degree to which taste processing can be modified by learning is unclear.

In flies, taste detection is mediated by gustatory receptor neurons (GRNs) located on the proboscis, pharynx, legs, wing margins, and ovipositor [1]. GRNs express a range of chemosensory receptors for detection of sugars, bitters, salts, and other contact chemosensory cues [2–4]. GRNs project to the subesophageal zone (SEZ) of the fly brain where taste information is segregated based on modality, valence, and organ of detection [5].

Although the valence of a specific taste is generally set, the intensity of the response can vary according to a variety of factors, including internal state. For example, starvation increases a fly's sensitivity towards sweet tastes, while blunting responses towards bitters. These starvation-dependent changes occur through direct modulation of GRN activity [6–9]. Moreover, flies lacking essential nutrients such as amino acids and salts exhibit increased nutrient-specific preference towards protein and salt-rich foods [10–12].

In addition to internal state-dependent changes in nutrient drive, fly taste responses can be altered by experience. Most notably, short-term taste-specific suppression of appetitive responses can be achieved through pairing with either bitter taste or noxious heat [13–16]. This plasticity requires an integrative memory association area called the mushroom body (MB), which is known to represent stimuli of different modalities, including taste [13–15]. Thus, while taste responses are carried out by innate circuits, they also exhibit experience-dependent changes driven by the adaptable networks of the MB [14,17,18].

The MB is composed of approximately ~4,000 intrinsic Kenyon cells (KCs), whose dendrites receive inputs from different sensory systems [14,19–21]. KCs form *en passant* synapses with mushroom body output neurons (MBONs), and MBONS send projections to neuropils outside of the MB to modulate behavioural output [20,22]. Connections between KCs and MBONs are depressed during learning, which skews the MB network towards approach or avoidance behaviours [23–25]. Reinforcement signals responsible for synaptic depression are conveyed by heterogenous dopaminergic neuron (DAN) populations when they are activated in coincidence with incoming sensory stimuli [26,27]. DANs are activated in response to rewarding and punishing stimuli within a fly's environment such as sucrose, bitter compounds, or shocks [28,29].

Interestingly, direct activation of DANs can function as an unconditioned stimulus (US) in some *Drosophila* associative learning paradigms [17,30–32]. Optogenetic or thermogenetic activation of the protocerebral anterior medial (PAMs) neurons, the rewarding DAN subpopulation, following or in coincidence with an odor conditioned stimulus (CS) results in the formation of an appetitive memory, while activation of punishing PPL1 DANs leads to the formation of an aversive memory [15,33]. Similarly, recent work on mammalian Pavlovian cue-reward learning shows that phasic optogenetic activation of specific dopaminergic subsets in the absence of a physical reward can lead to the formation of conditioned behaviours in mice [34].

Appetitive short- and long-term memories (STM; LTM) are formed by independent PAM subpopulations, and the nutritional value of the sugar reward and satiation state of the fly contribute to the strength of the association [17,28,33,35]. Whereas STM may be formed under more flexible conditions with a sweet tasting reward, the formation of LTM requires a nutritious sugar. For example, L-glucose, a non-caloric, sweet tasting sugar can be used as a reward for the formation of STM, but only promotes LTM if paired with an energy source such as sorbitol [28,35,36]. Caloric sugars gate memory consolidation by promoting sustained rhythmic activity of MB-MP1 DANs [35,37]. Therefore, nutrient-sensing, internal state, and the gustatory system all play pivotal roles in reward learning [38,39].

Although flies are known to exhibit aversive short-term taste memories, where appetitive taste responses are diminished through punishment [14,15,40,41], the full extent to which taste behaviours are modifiable by learning is unknown. For example, can taste responses be enhanced by appetitive conditioning? Can flies form long-term memories about taste? These are difficult questions to answer using traditional methods for several reasons. First, appetitive association paradigms generally rely on food as the US, which could interfere with the representation of a taste CS and also modify future taste behaviours through changes in satiety state. Second, repeated temporal pairing of a taste CS with a US is difficult to achieve in flies without immobilization, making long-lasting memories difficult to test. To circumvent these issues, we developed an optogenetic learning paradigm that facilitates rapid, repeated CS/US pairing while maintaining similar satiation states between groups. In this paradigm, we couple a taste (the CS) with optogenetic GRN or dopaminergic neuron stimulation (the US) in order to study conditioned taste responses or 'taste memories'. For the purpose of our study, we will define a taste memory as a measurable change in a fly's behavioural response to a previously encountered taste stimulus.

Using our novel paradigm, we show that flies are capable of forming both appetitive and aversive short- and long-term taste memories. As in olfaction, appetitive taste memories are driven by discrete PAM populations, and activation of a single PAM subpopulation is sufficient to induce appetitive LTM. The formation of appetitive LTM requires *de novo* protein synthesis and is contingent on caloric intake. Moreover, sugar, certain amino acids, and lactic acid can

provide the energy required to support LTM formation, and this requirement is also satisfied by thermogenetic activation of MB-MP1 neurons.

RESULTS

Pairing GRN activation with a food source leads to taste memory formation

We previously developed a system called the sip-triggered optogenetic behavioural enclosure (STROBE), in which individual flies are placed in an arena with free access to two food sources [42]. Interactions (mostly sips) with one of those foods triggers nearly instantaneous activation of a red LED, which can be used for optogenetic stimulation of neurons expressing CsChrimson. We reasoned that, if sipping on a tastant (the CS^+) triggers activation of neurons that provide either positive or negative reinforcement, we may observe a change in the number of interactions a fly initiates upon subsequent exposure to the same CS^+ (Figure 1A).

We began by testing the efficacy of the STROBE in inducing aversive and appetitive memories through optogenetic activation of bitter and sweet GRNs, respectively (Figure 1B). Bitter GRN stimulation is known to activate PPL1 DANs, while sweet GRNs activate PAMs [13,15,32,43]. Moreover, bitter or sweet GRN activation with *Gr66a-* or *Gr43a-Gal4* is sufficient for STM induction in taste and olfactory associative learning paradigms [13,33]. Therefore, we tested whether pairing GRN activation with feeding on a single taste modality could create an associative taste memory that altered subsequent behaviour to the taste.

In the aversive taste memory paradigm, interactions with 25 mM sucrose (CS^+) during training triggered LED activation of bitter neurons expressing CsChrimson, which led to CS^+ avoidance relative to plain agar (CS^-) (Figure 1C). During testing, we disable the STROBE lights and measured preference towards 25 mM sucrose (CS^+) relative to agar (CS^-) to see if flies have formed aversive taste memories. Indeed, ten minutes after training, flies that experienced bitter GRN activation during training showed a decreased sugar preference compared to control flies of the same genotype that were not fed the obligate CsChrimson cofactor all-*trans*-retinal, and retinal-fed control genotypes carrying either the Gal4 or UAS alone (Figure 1C). The same effect was produced by activation of $PPK23^{glut}$ 'high salt' GRNs, which also carries a negative valence in salt-satiated flies (Figure S1A, B). Importantly, these effects are not due to heightened satiety in trained flies because training in this paradigm is associated with fewer food interactions than controls.

For the appetitive memory paradigm, we chose 75 mM NaCl as the CS^+, since flies show neither strong attraction nor aversion to this concentration of salt [44]. Interactions with the CS^+ triggered optogenetic activation of sweet neurons, either with *Gr43a-Gal4*, which labels a subset of leg and pharyngeal sweet neurons in addition to fructose-sensitive neurons in the protocerebrum

(Figure 1B) or *Gr64f-Gal4*, which labels most peripheral sweet GRNs (Figure S1A). In both cases, sweet GRN activation produced increased preference for the CS^+ during training and testing 10-minutes later (Figure 1D and Figure S1C). Like the aversive memory paradigm, these effects cannot easily be explained through changes in internal state, since trained flies interacted more with the food during training and therefore should have a lower salt drive during testing. Interestingly, refeeding flies with standard medium directly after training in the appetitive paradigm led to a long-lasting preference for the CS^+, revealed by testing 24-hours later (Figure 1E and Figure S1D). This stands in contrast to the aversive paradigm, where reduced preference for sugar following bitter GRN activation was absent during testing after 24 hours (Figure S1E).

DAN activation is sufficient for the induction of short and long-lasting taste memories

We next asked whether activating DANs during feeding could drive the formation of taste memories. Aversive short-term taste memory depends on multiple PPL1 DANs, including PPL1-α'2 α2 and PPL1-α3[15], while appetitive short-term taste memories have not been previously reported. We first tested whether activating PPL1 DANs coincident with tastant interactions would lead to STM formation in the STROBE. Stimulation of PPL1 neurons reduced sucrose preference during training, and a reduced preference was also observed during short-term memory testing 10 minutes later (Figure 2A). Interestingly, unlike activation of bitter sensory neurons, PPL1 activation also produced a long-lasting aversive memory that was expressed 24 hours after training (Figure 2B).

To test the effect of appetitive DAN activation, we used flies expressing CsChrimson in PAM neurons under control of *R58E02-Gal4*. Intriguingly, although optogenetic activation of PAM neurons signals reward to the MB, it did not affect preference towards light-paired 75 mM NaCl (CS^+) during training (Figure 2C, D). Nonetheless, this pairing resulted in appetitive memory expression during testing 10 minutes and 24 hours after training (Figure 2C, D). Thus, optogenetic activation of PAM neurons in the STROBE was able to write both short- and long-lasting appetitive taste memories in the absence of acute effects on feeding. Importantly, appetitive memories were specific for the CS^+, as flies trained with NaCl as the CS^+ did not exhibit elevated preference for the equally appetitive novel tastant monopotassium glutamate (MPG; Figure S2A)

Consistent with long-term olfactory memories induced by DAN activation, we found long-lasting taste memory consolidation required an energetic food source, and therefore flies were refed for a brief period after training. Flies fed 7 hours post-training, after the memory consolidation time period defined in olfactory memory, did not express taste memories during testing (Figure 2D) [35]. Thus, the contingencies governing the formation and expression of taste memories in *Drosophila* seem to be similar to those previously discovered for olfaction.

The MB is required for the formation of short- and long-lasting taste memories

Prior research indicates that the intrinsic neurons of the MB are required for aversive taste memory formation[15]. To confirm that the MB is required for appetitive taste memory formation, we silenced this neuropil throughout our long-lasting memory assay using tetanus toxin expressed under control of the pan-MB driver *R13F02-LexA*. After pairing Gr43a activation with NaCl feeding, flies with silenced MBs did not exhibit elevated preference for salt during either the short-term or long-lasting taste memory assays (Figure 3A, B). Similarly, PAM activation during feeding led to a sustained increase in preference for the NaCl tastant in control groups for both the STM and long-lasting memory assay, but not in flies with silenced MBs (Figure 3C, D). These findings indicate that MB intrinsic neurons play a pivotal role in the formation of appetitive taste memory (Figure 3F).

To assess whether the molecular underpinnings of 24-hour appetitive taste memory are consistent with classic olfactory LTM – which requires *de novo* protein synthesis during memory consolidation – we fed flies all-*trans*-retinal laced with the protein synthesis inhibitor cycloheximide (CXM) [17]. As expected, flies fed CXM prior to training were unable to form long-term taste memories, in contrast to vehicle controls (Figure 3E). These results confirm that protein synthesis is necessary for long-term taste memory formation (Figure 3F).

Distinct PAM subpopulations induce appetitive short- and long-term taste memories

Distinct, non-overlapping subpopulations of PAM neurons, labeled by *R48B04-Gal4* and *R15A04-Gal4*, mediate the formation of appetitive short- and long-term olfactory memories, respectively [33]. Moreover, it has been hypothesized that two differential reinforcing effects of sugar reward – sweet taste and nutrition, are encoded by these segregated STM and LTM neural populations [33]. We tested both populations in our appetitive STROBE memory assays to determine if the activation of these separate PAM clusters would support the formation of parallel short- and long-term taste memories. *R48B04>CsChrimson* flies formed appetitive short-term but not long-term taste memories, as shown by the higher salt preference of flies expressing active CsChrimson during STM testing but not LTM testing (Figure 4A, B). Conversely, activation of *R15A04-Gal4* neurons produced LTM but not STM (Figure 4C, D). These results indicate that, much like appetitive olfactory memory, short- and long-term taste memories are formed in parallel by discrete PAM sub-populations.

Next, we wondered whether activation of a single PAM cell subtype, PAM-α1, would be sufficient to induce taste memories. PAM-α1 neurons project to an MB compartment innervated by MBON-α1, which in turn feeds back onto PAM-α1 to form a recurrent reward loop necessary for the formation of appetitive olfactory LTM [45,46]. Consistent with its role in olfactory memory, activation of this PAM cell type in the STROBE with drivers *MB043B-Gal4* or *MB299B-Gal4*

was sufficient to drive appetitive long-term, but not short-term, taste memory formation (Figure 4E, F and Figure S3A, B).

Interestingly, activation of another PAM subset labelled by *MB301B-Gal4* produced a higher preference for the salt CS during training, yet no sustained changes in taste preference during short- or long-term memory testing (Figure 4G, H). This demonstrates that the reward signaling associated with PAM cell activation occurs on multiple timescales to produce acute, short-, or long-term changes in behavior. Notably, the trend toward lower salt preference during testing in this experiment may reflect reduced salt drive due to increased salt consumption during training.

Caloric food sources are required for the formation of associative long-term taste memories

Because refeeding shortly after training is required for the consolidation of appetitive long-term taste memories, we next asked what types of nutrients would support this memory formation. As expected, refeeding with L-glucose, a non-caloric sugar, did not lead to the formation of associative long-term taste memories (Figure 5A, B). However, along with sucrose, refeeding with lactic acid, yeast extract, and L-alanine promoted long-term memory, while L-aspartic acid did not. These results indicate that in addition to sucrose, other caloric nutrients can provide sufficient energy for long-term taste memory formation. Moreover, 7-hour delayed refeeding of each nutrient failed to support memory formation (Figure 5B). Thus, similar to olfactory LTM, the formation of appetitive taste LTM is dependent on an energy source being readily available during the memory consolidation window [35,38].

Our findings concerning the formation and expression of appetitive taste LTM bear striking similarities to those of olfactory LTM in terms of MB circuitry, dependence on protein synthesis, and energetic requirements. This led us to wonder if the energy gating performed by MB-MP1 neurons, which signal onto the mushroom body and promote energy flux in MB neurons during LTM, perform a similar function in taste memory [35,47,48]. To test this hypothesis, we activated MB-MP1 neurons directly after training using *UAS-TRPA1* and delayed refeeding to outside the memory consolidation window. Compared to genetic controls, flies in which MB-MP1 neurons were activated post training show significantly elevated memory scores during testing (Figure 6A, B). This confirms that MB-MP1 activation is sufficient to drive memory consolidation during long-term appetitive taste memory formation (Figure 6C).

DISCUSSION

Gustation plays a vital role in determining the suitability of foods for ingestion. Yet, little is known about how experience influences higher-order taste representations and contributes to the continuous refinement of food selection. In fact, in flies, a memory system for the recollection of appetitive taste CS has not been described. In this study, we use the STROBE to establish a

novel learning paradigm and further investigate the formation and expression of taste memories. We demonstrate that flies are capable of forming short- and long-term appetitive and aversive taste memories towards two key nutrients – salt, and sugar. Much like olfactory memory, associative taste memory formation occurs within the MB and follows many of the same circuit and energetic principles.

Appetitive taste memory has been severely understudied in *Drosophila* due to several inherent experimental design issues. First, in other types of associative reward learning – such as olfactory and visual – sucrose is often used as the unconditioned stimulus (US) or reward, which can interact with the representation of a gustatory CS. For example, bitter and sweet tastes interact at both the receptor and circuit levels, and salt responses overlap with most other taste stimuli in the periphery [11,49,50]. Second, changes in internal state due to ingestion of specific nutrients during learning can either influence behavior independent of memory formation, or directly affect memory formation or expression. Most notably, if CS^+ and CS^- groups ingest different amounts of a nutrient during training, it will likely influence the specific drive for the taste of that modality during testing [12,51]. Nevertheless, rats' hedonic response to bitter compounds can be made more positive through pairing with sugar, and human studies suggest that children's taste palates are malleable based on positive experiences with bitter vegetables [52–55]. Thus, despite the constraints of measuring taste memories in a controlled setting, appetitive taste plasticity is very likely an ethologically important process.

To circumvent the complexities associated with appetitive taste memory formation, we decided to directly stimulate DANs as the flies experienced taste inputs through feeding. This also afforded the opportunity to interrogate the roles of specific DAN populations. Taking a hypothesis driven approach, we confirmed that PAM neural subpopulations reinforce taste percepts much like olfactory inputs, and that short- and long-term memories are processed by distinct subpopulations. For example, activating β'2, γ4, and γ5 compartments with *R48B04-Gal4* produces STM in both olfactory and taste paradigms, while activation of α1, β'1, β2, and γ5 with *R15A04-Gal4* produces LTM in both. These results confirm that appetitive STM and LTM are processed in parallel in the MB [33,56]. Given that tastes, like odours, activate the KC calyces, we speculate that optogenetic stimulation of PAM neurons during feeding modulates the strength of KC-MBON synaptic connections [14]. Notably, activation of single PAM cell type produced different forms of memory in the STROBE. For example, stimulating PAM-α1 neurons during feeding drives appetitive taste LTM, while activation of PAM-β'1 was immediately rewarding.

A unique aspect of our long-term taste learning paradigm is that we uncoupled the US from a caloric food source. By doing this we were able to probe the energetic constraints gating LTM formation. For years it has been reported that long-term memory formation in *Drosophila* requires the intake of caloric sugar. Here, we demonstrate that the caloric requirements of long-

term memory formation can be quenched by food sources other than sucrose, such as lactic acid, and protein. Moreover, it seems that at least one amino-acid, L-alanine, is able to provide adequate energy, while L-aspartic acid cannot. We theorize that these foods may provide flies readily accessible energy, as neurons are able to metabolize both lactic acid and L-alanine into pyruvate to fuel the production of ATP via oxidative phosphorylation [57].

Energy gating in the MB is thought to be regulated by the MB-MP1-DANs. MB-MP1 neuron oscillations activate increased mitochondrial energy flux within the KCs, which is both necessary and sufficient to support LTM[48]. To demonstrate sufficiency in our assay, we activated MB-MP1 neurons with TRPA1 directly after fly training, which effectively substitutes for a caloric food source and allows LTM formation (Figure 6C). These results suggest that MB-MP1 neurons integrate energy signals during formation of multiple types of LTM and may be influenced by a variety of caloric foods.

Intriguingly, we observed both positive and negative taste memories following optogenetic GRN stimulation concurrent with taste detection. The activation of bitter GRNs paired with sucrose led to the formation of STM, which agrees with previous research demonstrating that thermogenetic stimulation of bitter GRNs can act as a negative US, and lead to taste learning in short-term paradigms [13]. Strikingly, bitter GRN activation was not sufficient for the formation of LTM in our assay. One possible explanation is that because bitter GRN activation strongly inhibits feeding, the number of pairings between sugar taste and bitter activation is insufficient to induce LTM. By contrast, PPL1 activation is less aversive during training and therefore allows more associations.

Unlike bitter GRNs, activation of sweet GRNs prompted both STM and LTM formation. This indicates that lasting changes in the value of specific tastes can occur in response to temporal association of different taste modalities, and raises the possibility that such plasticity plays an important role in animals' ongoing taste responses. Future experiments using the STROBE paradigm could further probe the molecular and circuit mechanisms underlying such changes and advance our understanding of how taste preferences may be shaped by experience over an animal's lifetime.

MATERIALS AND METHODS

Fly strains

Fly stocks were raised on a standard cornmeal diet at 25°C, 70% relative humidity. For neuronal activation *20XUAS-IVS-CsChrimson.mVenus* (BDCS, stock number: 55135) was used. Dopaminergic PAM expression was targeted using previously described lines: *R58E02-GAL4*[35]*; R58E02-LexA, R48B04-GAL4, R15A04-GAL4, R13F02-LexA,* and *R30E11-LexA* obtained from

Bloomington (BDCS, stock numbers: 52740, 50347, 48671, 52460, 54209); and MB split-GAL4 lines *MB043B-GAL4, MB504B-GAL4, MB299B-GAL4, MB301B-GAL4* from Janelia Farms[23]. GRN expression was driven using *Gr43a-GAL4, Gr64f-GAL4*[58], *Gr66a-GAL4*[59] , and *PPK23*^{glut}-*GAL4* (*PPK23-GAL4, Gr66a-LexA::VP16, LexAop-Gal80*[11]). *LexAop-tnt* was previously described[60]. For temperature activation experiments *LexAop-TrpA1* was used[32].

STROBE experiments

Mated female *Drosophila* were collected 2-3 days post eclosion and transferred into vials containing 1 ml of standard cornmeal medium supplemented with 1 mM all-*trans*-retinal (Sigma #R2500) or an ethanol vehicle control. Flies were maintained on this diet for 2 days in a dark environment. 24 hours prior to experimentation flies were starved at 25°C, 70% relative humidity, on 1% agar supplemented with 1 mM all-*trans*-retinal or ethanol vehicle control.

STROBE training protocol

During the training phase for the short-term memory experiments the STROBE was loaded with 4 μL of tastant (salt: Sigma #S7653 or sucrose: Sigma #S7903) on channel 1 and 4 μL 1% agar on channel 2. The red LED was triggered only when a fly interacted with the tastant in channel 1. The duration of the training period was 40 minutes. For the STM training protocol, flies were then transferred to clean empty vials for 10 minutes while the experimental apparatus was cleaned. The training and testing phases of LTM experiments were performed as described for the STM experiments with the following exception: after the 40-minute training period flies, were transferred individually into vials containing standard cornmeal diet or nutrient of interest (sucrose: Sigma #S7903, L-glucose: Sigma #G5500, lactic acid: Sigma #69785 , yeast extract: Sigma #Y1625, L-alanine: Sigma #05129, L-aspartic acid: Sigma #11230) and allowed to feed for 1 hour. They were then transferred into 1% agar starvation vials and kept at 18°C until the testing component of the experiment. For MB-MB1 activation experiments, after training flies were placed at 29°C, 70% relative humidity for 1 hour on 1% agar starvation vials. They were then transferred to 18°C and refed 8 hours later, outside of the memory consolidation. After 1 hour of feeding they were once again transferred into 1% agar starvation vials and kept at 18°C until the retrieval component of the experiment. The preference index for each individual fly was calculated as: (sips from channel 1 – sips from channel 2)/(sips from channel 1 + sips from channel 2). All experiments were performed with a light intensity of 11.2mW/cm^2 at 25°C, 70% relative humidity.

STROBE testing protocol

During testing, 4 μL of the same tastant (salt: Sigma #S7653, sucrose: Sigma #S7903, MPG: Sigma #G1501) was reloaded into channel one and 4 μL of 1% agar on channel 2. The optogenetic component of the system was deactivated such the red LED would no longer trigger if a fly interacted with the tastant. Flies were reloaded individually into the same arenas. The duration of the testing phase was 1 hour. The preference index for each individual fly was

calculated as: (sips from channel 1 – sips from channel 2)/(sips from channel 1 + sips from channel 2).

Immunofluorescence microscopy

Brain staining protocols were performed as previously described[49]. Briefly, brains were fixed for 1 hour in 4% paraformaldehyde and dissected in PBS + 0.1% TritonX. After dissection brains were blocked in 5% NGS diluted with PBST for 1 hour. Brains were probed overnight at 4°C using the following primary antibody dilutions: rabbit anti-GFP (1:1000, Invitrogen #A11122), and mouse anti-brp (1:50, DSHB #nc82). After a 1hour wash period secondary antibodies goat anti-rabbit Alexa-488 (1:200, Invitrogen #A11008) and goat anti-mouse Alexa-568 (1:200, Invitrogen #A11030) were applied and incubated for 1 hour at room temperature to detect primary antibody binding. Slowfade gold was used as an antifade mounting medium.

Slides were imaged under a 25x water immersion objective using a Leica SP5 II Confocal microscope. All images were taken sequentially with a z-stack step size at 1 μm, a line average of 2, speed of 200 Hz, and a resolution of 1024 x 1024 pixels. Image J was used to compile slices into a maximum intensity projection[11].

Statistical analysis

All statistical analyses were executed using GraphPad Prism 6 software. Sample size and statistical tests performed are provided in the Figure legends. For Dunnett's post hoc analyses, the lowest statistically significant value is shown on top of the experimental group as compared to various controls. Replicates are biological replicates, using different individual flies from 2 or more crosses. Sample sizes were based on previous experiments in which effect size was determined. Data was excluded on the basis of STROBE technical malfunctions for individual flies and criteria for data exclusion are as follows: i) if the light system was not working during training for individual arenas ii) if during training or testing a fly did not meet a standard minimum # of interactions for that genotype iii) if during training or testing the STROBE recorded an abnormally large # of interactions for that genotype iiii) technical malfunctions due to high channel capacitance baseline activity v) if a fly was dead in an arena.

DATA AVAILABILITY

All data are available in the manuscript and Supplementary Information, or are available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

M.J, P.Y.M, and M.D.G conceived the project. M.J and M.D.G wrote the manuscript. M.J ran all experiments. P.Y.M and P.J offered experimental advice. M.D.G supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information includes 3 figures.

REFERENCES

1. Stocker, R. F. The organization of the chemosensory system in Drosophila melanogaster: a rewiew. *Cell Tissue Res*. **275**, 3–26 (1994).
2. Hiroi, M., Meunier, N., Marion-Poll, F. & Tanimura, T. Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste inDrosophila. *J. Neurobiol*. **61**, 333–342 (2004).
3. Thorne, N., Chromey, C., Bray, S. & Amrein, H. Taste Perception and Coding in Drosophila. *Curr. Biol*. **14**, 1065–1079 (2004).
4. Zhang, Y. *et al*. Coding of Sweet, Bitter, and Umami Tastes: Different Receptor Cells Sharing Similar Signaling Pathways. 9 (2003).
5. Marella, S. *et al*. Imaging Taste Responses in the Fly Brain Reveals a Functional Map of Taste Category and Behavior. *Neuron* **49**, 285–295 (2006).
6. Inagaki, H. K. *et al*. Visualizing Neuromodulation In Vivo: TANGO-Mapping of Dopamine Signaling Reveals Appetite Control of Sugar Sensing. *Cell* **148**, 583–595 (2012).
7. Inagaki, H. K., Panse, K. M. & Anderson, D. J. Independent, Reciprocal Neuromodulatory Control of Sweet and Bitter Taste Sensitivity during Starvation in Drosophila. *Neuron* **84**, 806–820 (2014).
8. LeDue, E. E. *et al*. Starvation-Induced Depotentiation of Bitter Taste in Drosophila. *Curr. Biol*. **26**, 2854–2861 (2016).
9. Marella, S., Mann, K. & Scott, K. Dopaminergic Modulation of Sucrose Acceptance Behavior in Drosophila. *Neuron* **73**, 941–950 (2012).
10. Corrales-Carvajal, V. M., Faisal, A. A. & Ribeiro, C. Internal states drive nutrient homeostasis by modulating exploration-exploitation trade-off. *eLife* **5**, e19920 (2016).

11. Jaeger, A. H. *et al*. A complex peripheral code for salt taste in Drosophila. *eLife* **7**, e37167 (2018).

12. Steck, K. *et al*. Internal amino acid state modulates yeast taste neurons to support protein homeostasis in Drosophila. 29 (2018).

13. Keene, A. C. & Masek, P. Optogenetic induction of aversive taste memory. *Neuroscience* **222**, 173–180 (2012).

14. Kirkhart, C. & Scott, K. Gustatory Learning and Processing in the Drosophila Mushroom Bodies. *J. Neurosci*. **35**, 5950–5958 (2015).

15. Masek, P., Worden, K., Aso, Y., Rubin, G. M. & Keene, A. C. A Dopamine-Modulated Neural Circuit Regulating Aversive Taste Memory in Drosophila. *Curr. Biol*. **25**, 1535–1541 (2015).

16. Tauber, J. M. *et al*. A subset of sweet-sensing neurons identified by IR56d are necessary and sufficient for fatty acid taste. *PLOS Genet*. **13**, e1007059 (2017).

17. Colomb, J., Kaiser, L., Chabaud, M.-A. & Preat, T. Parametric and genetic analysis of Drosophila appetitive long-term memory and sugar motivation. *Genes Brain Behav*. **8**, 407–415 (2009).

18. Krashes, M. J. *et al*. A Neural Circuit Mechanism Integrating Motivational State with Memory Expression in Drosophila. *Cell* **139**, 416–427 (2009).

19. Schwaerzel, M. *et al*. Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in *Drosophila*. *J. Neurosci*. **23**, 10495–10502 (2003).

20. Tanaka, N. K., Tanimoto, H. & Ito, K. Neuronal assemblies of the Drosophila mushroom body. *J. Comp. Neurol*. **508**, 711–755 (2008).

21. Vogt, K. *et al*. Shared mushroom body circuits underlie visual and olfactory memories in Drosophila. *eLife* **3**, e02395 (2014).

22. Crittenden, J. R., Skoulakis, E. M. C., Han, K.-A., Kalderon, D. & Davis, R. L. Tripartite Mushroom Body Architecture Revealed by Antigenic Markers. *E R N N G* 14 (1998).

23. Aso, Y. *et al*. Mushroom body output neurons encode valence and guide memory-based action selection in Drosophila. *eLife* **3**, e04580 (2014).

24. Cohn, R., Morantte, I. & Ruta, V. Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in Drosophila. *Cell* **163**, 1742–1755 (2015).

25. Perisse, E. *et al*. Different Kenyon Cell Populations Drive Learned Approach and Avoidance in Drosophila. *Neuron* **79**, 945–956 (2013).

26. Gervasi, N., Tchénio, P. & Preat, T. PKA Dynamics in a Drosophila Learning Center: Coincidence Detection by Rutabaga Adenylyl Cyclase and Spatial Regulation by Dunce Phosphodiesterase. *Neuron* **65**, 516–529 (2010).

27. Tomchik, S. M. & Davis, R. L. Dynamics of Learning-Related cAMP Signaling and Stimulus Integration in the Drosophila Olfactory Pathway. *Neuron* **64**, 510–521 (2009).

28. Burke, C. J. & Waddell, S. Remembering Nutrient Quality of Sugar in Drosophila. *Curr. Biol*. **21**, 746–750 (2011).

29. Mao, Z. & Davis, R. L. Eight different types of dopaminergic neurons innervate the Drosophila mushroom body neuropil: anatomical and physiological heterogeneity. *Front. Neural Circuits* **3**, (2009).

30. Aso, Y. *et al*. Three Dopamine Pathways Induce Aversive Odor Memories with Different Stability. *PLoS Genet*. **8**, e1002768 (2012).

31. Claridge-Chang, A. *et al*. Writing Memories with Light-Addressable Reinforcement Circuitry. *Cell* **139**, 405–415 (2009).

32. Liu, C. *et al*. A subset of dopamine neurons signals reward for odour memory in Drosophila. *Nature* **488**, 512–516 (2012).

33. Yamagata, N. *et al*. Distinct dopamine neurons mediate reward signals for short- and long-term memories. *Proc. Natl. Acad. Sci.* **112**, 578–583 (2015).

34. Saunders, B. T., Richard, J. M., Margolis, E. B. & Janak, P. H. Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nat. Neurosci.* **21**, 1072–1083 (2018).

35. Musso, P.-Y., Tchenio, P. & Preat, T. Delayed Dopamine Signaling of Energy Level Builds Appetitive Long-Term Memory in Drosophila. *Cell Rep.* **10**, 1023–1031 (2015).

36. Musso, P.-Y., Lampin-Saint-Amaux, A., Tchenio, P. & Preat, T. Ingestion of artificial sweeteners leads to caloric frustration memory in Drosophila. *Nat. Commun.* **8**, 1803 (2017).

37. Pavlowsky, A., Schor, J., Plaçais, P.-Y. & Preat, T. A GABAergic Feedback Shapes Dopaminergic Input on the Drosophila Mushroom Body to Promote Appetitive Long-Term Memory. *Curr. Biol.* **28**, 1783-1793.e4 (2018).

38. Fujita, M. & Tanimura, T. Drosophila Evaluates and Learns the Nutritional Value of Sugars. *Curr. Biol.* **21**, 751–755 (2011).

39. Huetteroth, W. *et al*. Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in Drosophila. *Curr. Biol.* **25**, 751–758 (2015).

40. Kim, H., Kirkhart, C. & Scott, K. Long-range projection neurons in the taste circuit of Drosophila. *eLife* **6**, e23386 (2017).

41. Masek, P. & Scott, K. Limited taste discrimination in Drosophila. *Proc. Natl. Acad. Sci.* **107**, 14833–14838 (2010).

42. Musso, P.-Y. *et al*. Closed-loop optogenetic activation of peripheral or central neurons modulates feeding in freely moving Drosophila. *eLife* **8**, e45636 (2019).

43. Miyamoto, T., Slone, J., Song, X. & Amrein, H. A Fructose Receptor Functions as a Nutrient Sensor in the Drosophila Brain. *Cell* **151**, 1113–1125 (2012).

44. Zhang, Y. V., Ni, J. & Montell, C. The Molecular Basis for Attractive Salt-Taste Coding in Drosophila. *Science* **340**, 1334–1338 (2013).

45. Aso, Y. & Rubin, G. M. Dopaminergic neurons write and update memories with cell-type-specific rules. *eLife* **5**, e16135 (2016).

46. Ichinose, T. *et al*. Reward signal in a recurrent circuit drives appetitive long-term memory formation. *eLife* **4**, e10719 (2015).

47. Plaçais, P.-Y. *et al*. Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in Drosophila. *Nat. Neurosci.* **15**, 592–599 (2012).

48. Plaçais, P.-Y. *et al*. Upregulated energy metabolism in the Drosophila mushroom body is the trigger for long-term memory. *Nat. Commun.* **8**, 15510 (2017).

49. Chu, B., Chui, V., Mann, K. & Gordon, M. D. Presynaptic Gain Control Drives Sweet and Bitter Taste Integration in Drosophila. *Curr. Biol.* **24**, 1978–1984 (2014).

50. Jeong, Y. T. *et al*. An Odorant-Binding Protein Required for Suppression of Sweet Taste by Bitter Chemicals. *Neuron* **79**, 725–737 (2013).

51. Lin, S. *et al*. Neural correlates of water reward in thirsty Drosophila. *Nat. Neurosci.* **17**, 1536–1542 (2014).

52. Breslin, P. A. S., Davidson, T. L. & Grill, H. J. Conditioned reversal of reactions to normally avoided tastes. *Physiol. Behav.* **47**, 535–538 (1990).

53. Figueroa, J., Gasalla, P., Müller, M. & Dwyer, D. Socially conditioned flavor preferences with fluids: Transfer with solid foods, palatability, and testing constraints. *Physiol. Behav.* **223**, 112976 (2020).

54. Forestell, C. A. & LoLordo, V. M. Can Orally Consumed Calories Condition Preferences for Relatively Unacceptable Tastes? *Learn. Motiv.* **31**, 153–179 (2000).

55. Wadhera, D., Capaldi Phillips, E. D. & Wilkie, L. M. Teaching children to like and eat vegetables. *Appetite* **93**, 75–84 (2015).

56. Trannoy, S., Redt-Clouet, C., Dura, J.-M. & Preat, T. Parallel Processing of Appetitive Short- and Long-Term Memories In Drosophila. *Curr. Biol.* **21**, 1647–1653 (2011).

57. de Tredern, E. *et al.* Glial glucose fuels the neuronal pentose phosphate pathway for long-term memory. *Cell Rep.* **36**, 109620 (2021).

58. Dahanukar, A., Lei, Y.-T., Kwon, J. Y. & Carlson, J. R. Two Gr Genes Underlie Sugar Reception in Drosophila. *Neuron* **56**, 503–516 (2007).

59. Wang, Z., Singhvi, A., Kong, P. & Scott, K. Taste Representations in the Drosophila Brain. *Cell* **117**, 981–991 (2004).

60. Liu, S., Liu, Q., Tabuchi, M. & Wu, M. N. Sleep Drive Is Encoded by Neural Plastic Changes in a Dedicated Circuit. *Cell* **165**, 1347–1360 (2016).

FIGURES

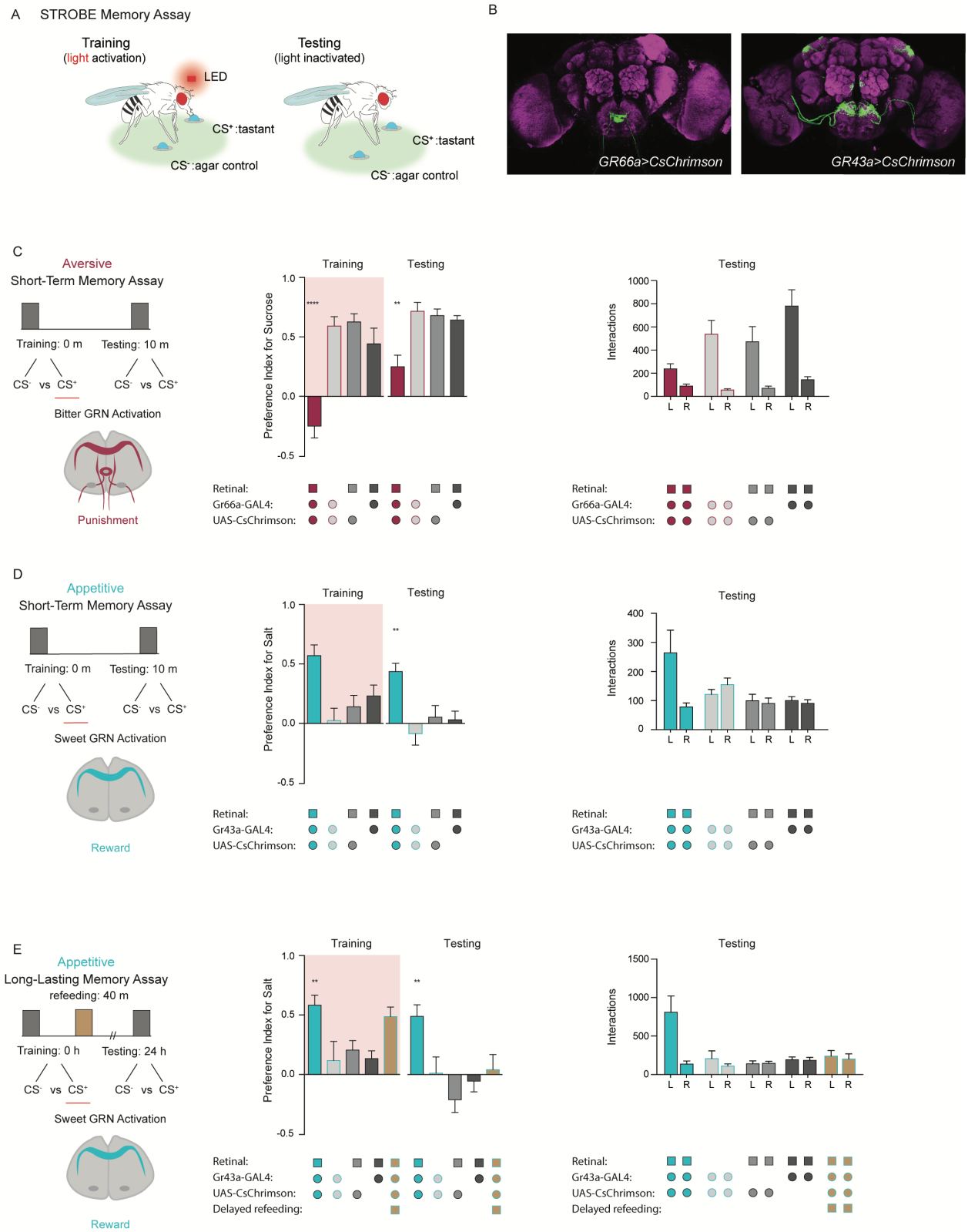
A
STROBE Memory Assay
Training
(light activation)
LED
CS+ :tastant
CS- :agar control
Testing
(light inactivated)
CS+ :tastant
CS- :agar control
B
GR66a>CsChrimson
GR43a>CsChrimson
C
Aversive
Short-Term Memory Assay
Training: 0 m
Testing: 10 m
CS- vs CS+
CS- vs CS+
Bitter GRN Activation
Punishment
Training
Testing
Preference Index for Sucrose
Retinal:
Gr66a-GAL4:
UAS-CsChrimson:
Testing
Interactions
L R L R L R L R
D
Appetitive
Short-Term Memory Assay
Training: 0 m
Testing: 10 m
CS- vs CS+
CS- vs CS+
Sweet GRN Activation
Reward
Training
Testing
Preference Index for Salt
Retinal:
Gr43a-GAL4:
UAS-CsChrimson:
Testing
Interactions
L R L R L R L R
E
Appetitive
Long-Lasting Memory Assay
refeeding: 40 m
Training: 0 h
Testing: 24 h
CS- vs CS+
CS- vs CS+
Sweet GRN Activation
Reward
Training
Testing
Preference Index for Salt
Retinal:
Gr43a-GAL4:
UAS-CsChrimson:
Delayed refeeding:
Testing
Interactions
L R L R L R L R L R
Retinal:
Gr43a-GAL4:
UAS-CsChrimson:
Delayed refeeding:

Figure 1: GRNs produce punishment and reward signals capable of facilitating taste memory formation. (A) Diagram outlining STROBE memory paradigm. Training: starved flies freely interact with a LED-activating tastant for 40 minutes. *CsChrimson* induces bitter or sweet neuron stimulation upon LED-activation, pairing feeding with punishment or reward. Testing: associative memory is measured by assessing preference for the tastant compared to agar for a 1-hour time period. (B) Expression of *Gr66a-Gal4* and *Gr43a-Gal4* in the fly brain. (C) Schematic of aversive STM timeline and a diagram of activated bitter neurons (left). Preference indices (middle) and tastant interaction numbers (right) for *Gr66a>CsChrimson* flies compared to genetic controls during training and 10 minutes later upon testing (n=16-30). (D, E) Schematic depicting appetitive short- and long-lasting memory assay timelines, and representative model of sweet GRNs in the SEZ (left). Preference index (middle) and interactions numbers (right) for *Gr43a>CsChrimson* flies fed all-*trans*-retinal compared to controls in the short-term (n= 12-23) (D), and long-lasting (n= 14-30) (E) memory assays (right). Preference index is mean ± SEM, One-way ANOVA, Dunnett's post hoc test, with : $^{**}p < 0.01$, $^{****}p < 0.0001$.

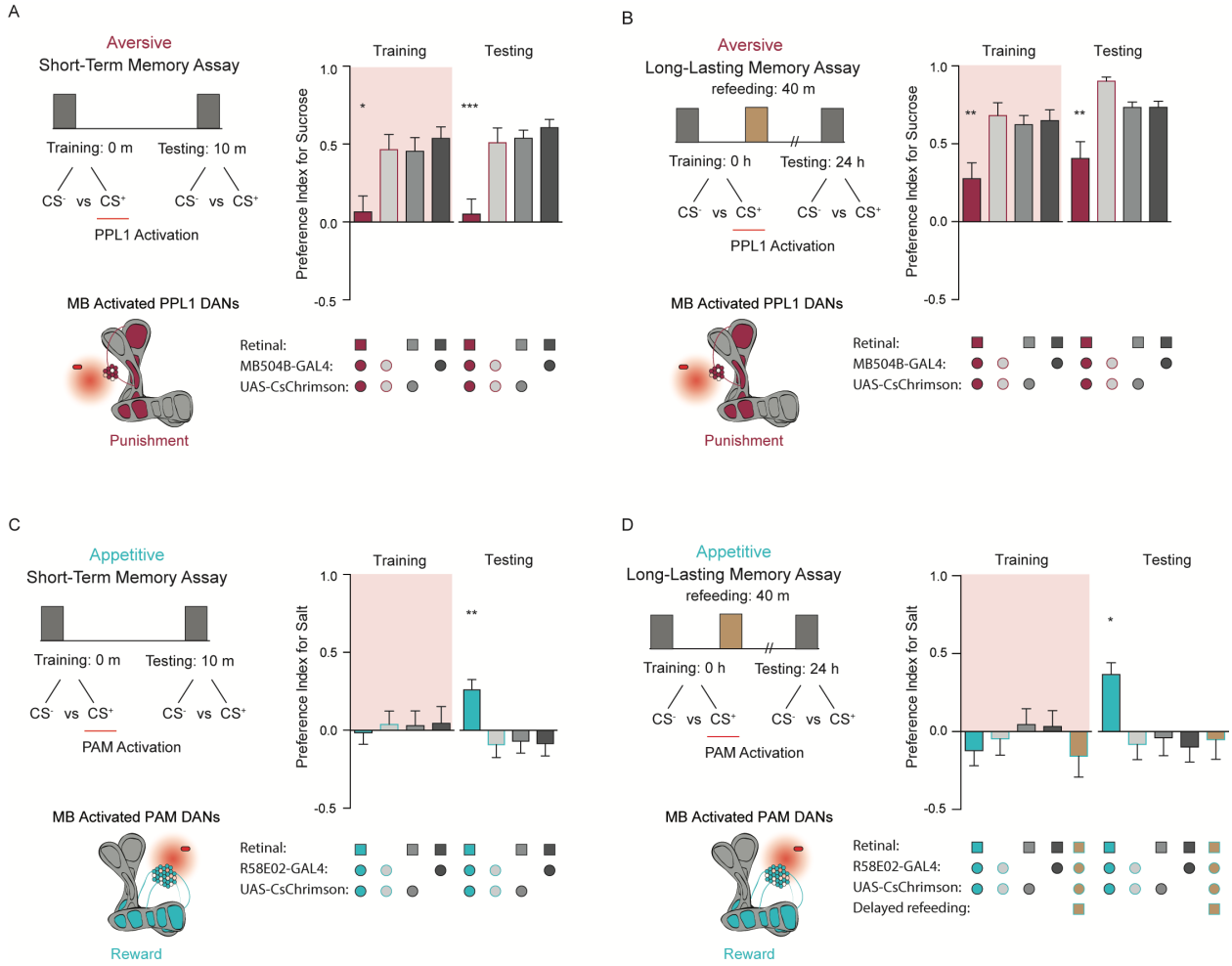

Figure 2: PPL1 and PAM neural activation is sufficient for the induction of short and long-lasting taste memories. (A, B) Paradigm timeline schematic and MB model indicating PPL1 compartments activated by optogenetics (left). Preference indices for *MB504B>CsChrimson* flies and genetic controls in the short-term (n=19-31) (A), and long-lasting (n=20-33) (B) memory assays (right). (C, D) Assay timeline, and MB model with activated PAM compartments highlighted (left). Preference index for *R58E02>CsChrimson* flies comparted to controls in the short-term (n=25-38) (C), and long-lasting (n=17-35) (D) memory assays (right). Preference indices are mean ± SEM, One-way ANOVA, Dunnett's post hoc test: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

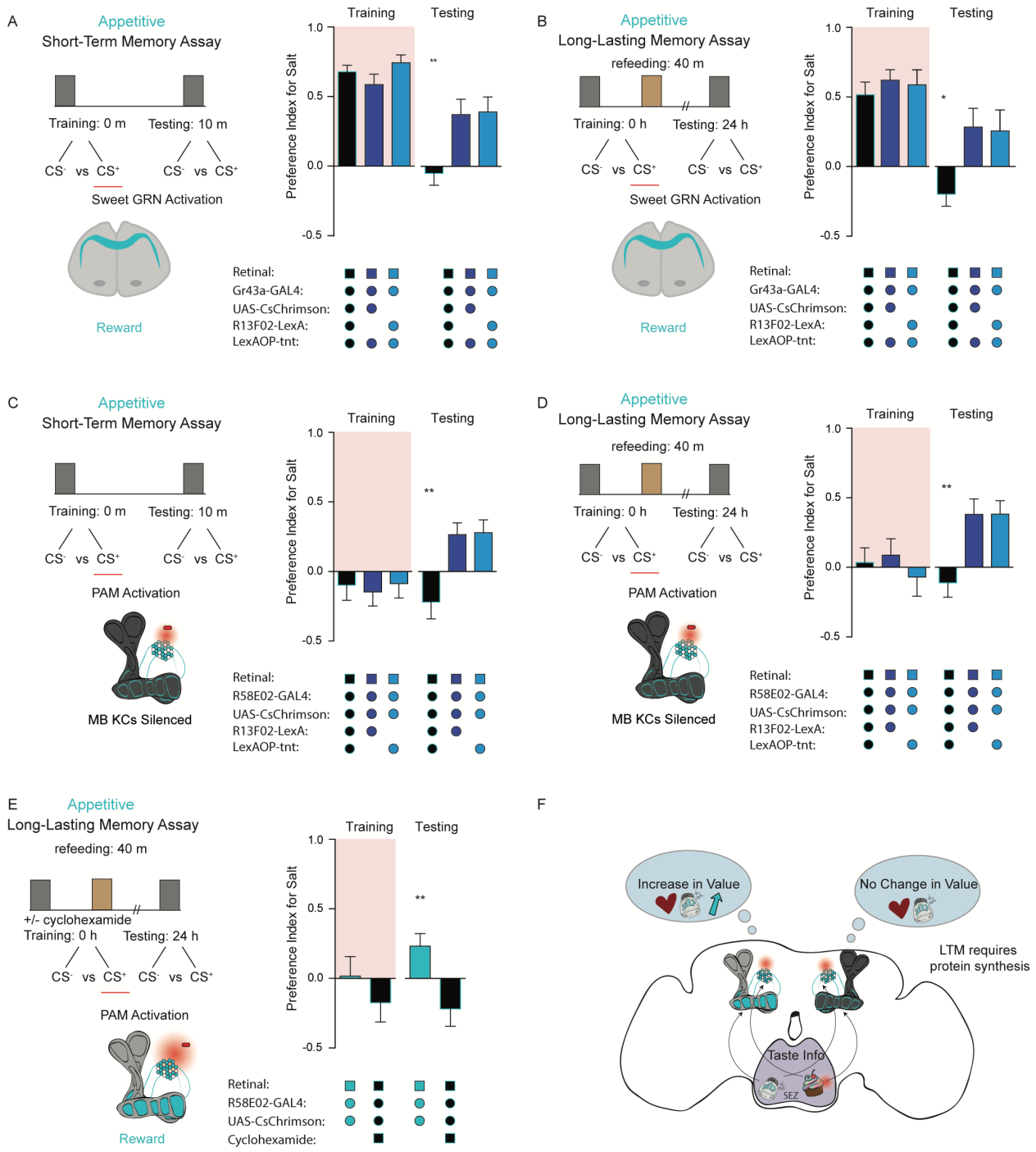

Figure 3: The MB is required for the formation of short- and long-term taste memories
(A, B) Preference indices for *Gr43a>CsChrimson* flies in the short-term (n=16-34) (A) and long-lasting (n=13-27) (B) memory assays when the MB is silenced, compared to controls. (C, D) Preference indices for *R58E02>CsChrimson* flies when the MB is silenced in the short-term (n=24-28)(C) and long-lasting (n=17-23) (D) memory paradigms, compared to controls. (E)

Preference indices in the long-lasting memory assay for *R58E02>CsChrimson* flies fed protein synthesis inhibitor cycloheximide compared to vehicle-fed controls (n=17-22). (F) Model of appetitive taste memory formation via GRN/PAM activation. Preference indices are mean ± SEM, *t*-test/One-way ANOVA, Dunnett's post hoc test: $*p < 0.05$, $**p < 0.01$.

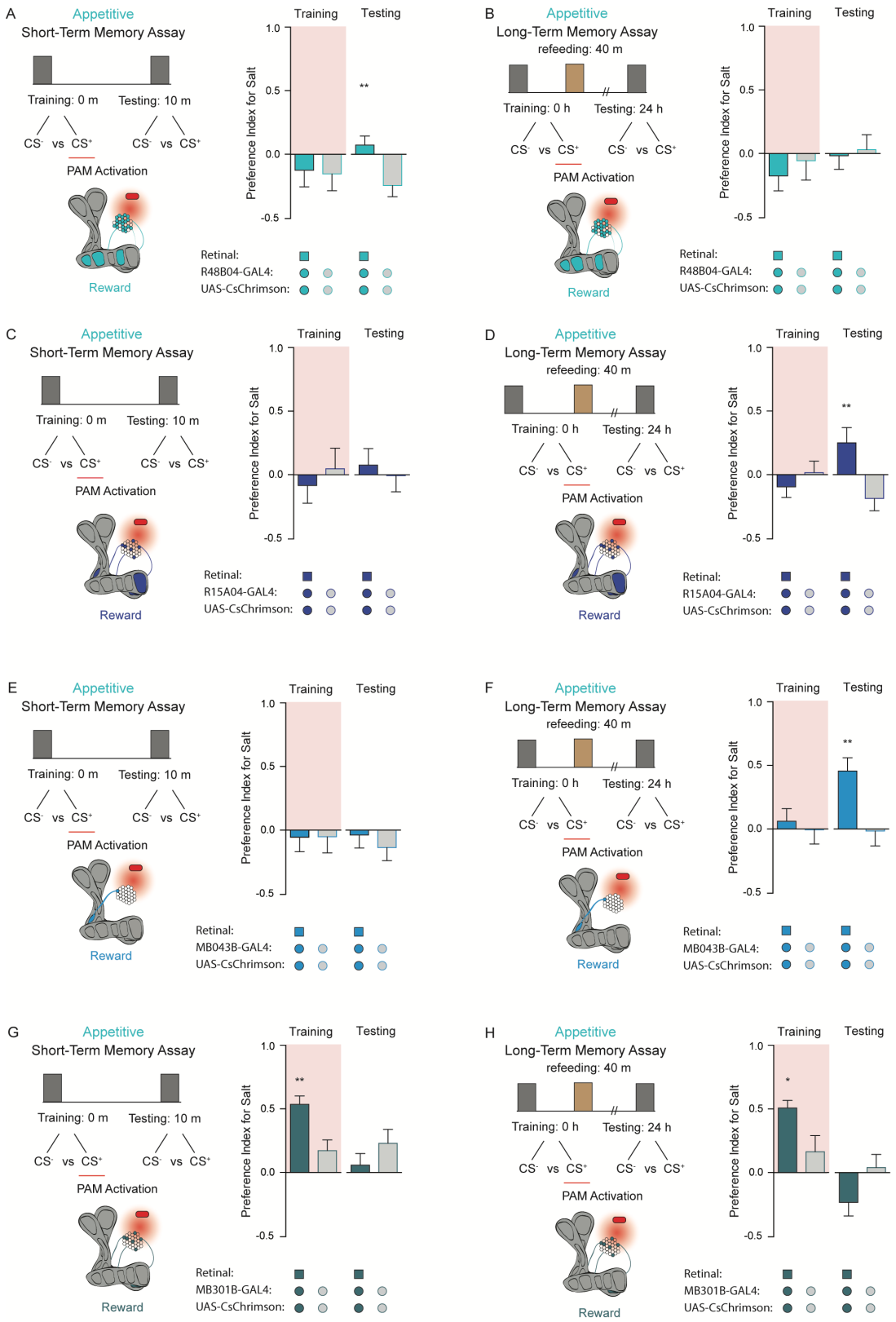

Figure 4: Discrete non-overlapping PAM subpopulations induce appetitive short- and long-term taste memories. (A, B) PAM subpopulation R48B04 innervates highlighted MB

compartments (left), and preference indices of *R48B04>CsChrimson* flies for 75mM NaCl is tested in the short-term (n=21-28) (A), and long-term (n=15-17) (B) memory assays with or without retinal. (C, D). PAM subpopulation R15A04 innervates non-overlapping MB sub-regions compared to R48B04. Preference indices for *R15A04>CsChrimson* flies fed all-*trans*-retinal in the short-term (n=11-15) (C) and long-term (n=20-27) (D) taste memory assays with or without retinal. (E, F) PAM-α1 innervates a single compartment in the MB. Preference indices of *MB043B>CsChrimson* flies in the short-term (n=11-14) (E) and long-term (n=19-22) (F) memory assays with or without retinal. (G, H) PAM-β2β′2a synapses on the highlighted MB compartment. Preference indices for *MB301B>CsChrimson* flies during the short-term (n=20-27) (G) and long-term (n=10-15) (H) memory assays with or without retinal. Preference indices are mean ± SEM, *t*-test: $^{**}p < 0.01$.

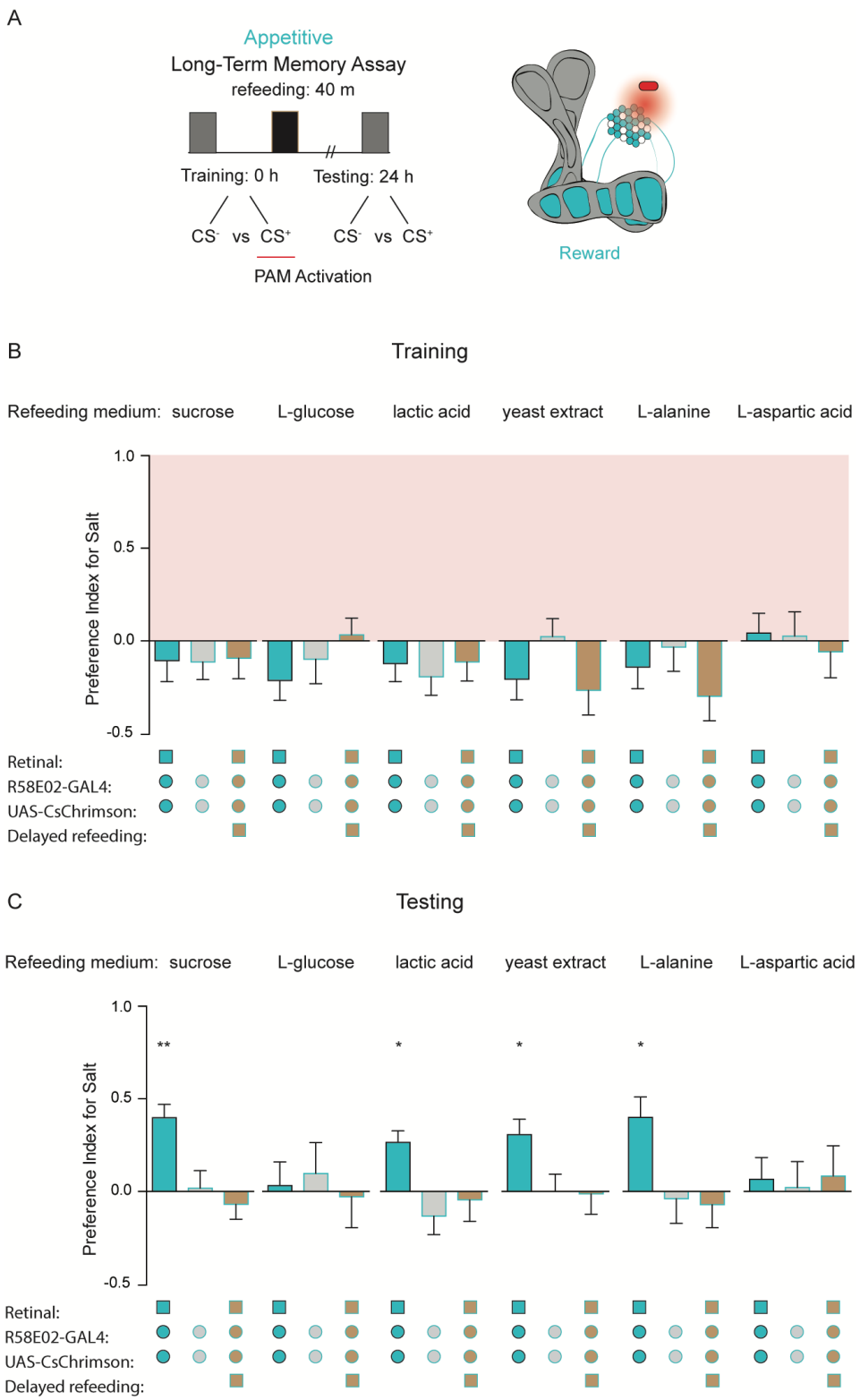

Figure 5: Caloric food sources are required for the formation of associative long-term taste memories. (A) Graphic of the timeline followed for the long-term taste memory paradigm, and the MB compartments innervated by PAM driver *R58E02-Gal4*. (B, C) Preference indices for

R58E02>CsChrimson and control flies during training (B), and testing (C), after being refed with a caloric or non-caloric medium (n=13-28). Preference indices are mean ± SEM, One-way ANOVA, Dunnett's post hoc test: $*p < 0.05$, $**p < 0.01$.

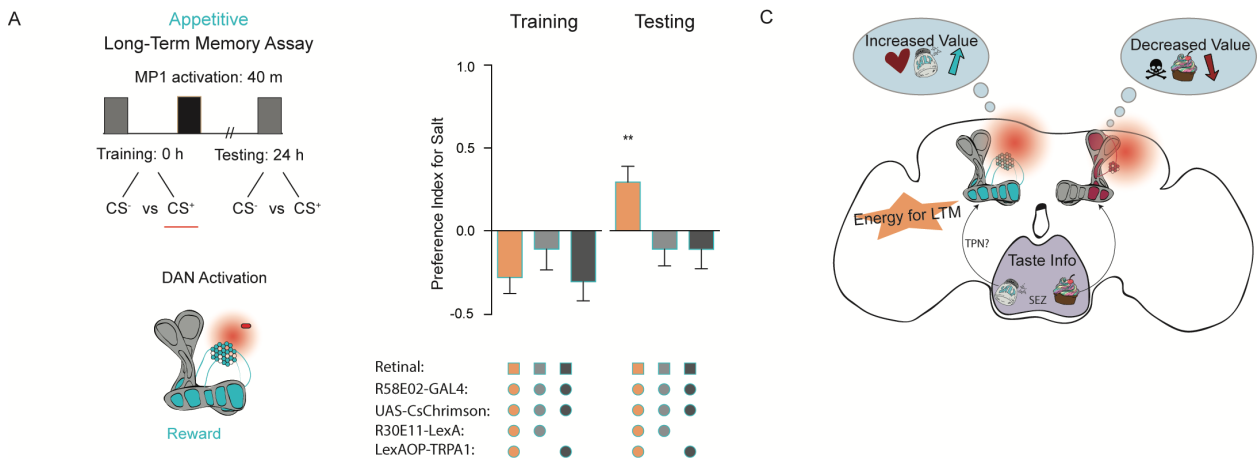

Figure 6: MB-MP1 neuron activation post-training replaces energy signal required for the formation of LTM. (A) Graphic of timeline followed for the LTM taste assay. Preference indices during training and testing for *R58E02>CsChrimson* flies fed all-*trans*-retinal, with MB-MP1 neurons thermogenetically activated post training using *R30E11>TRPA1*, compared to controls without MB-MP1 activation (n=18-29). (C) Schematic depicting pathway of activation. Preference indices are mean ± SEM, One-way ANOVA, Dunnett's post hoc test: $^{**}p < 0.01$.