Reward re-exposure driven memory re-evaluation in *Drosophila melanogaster*

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie

vorgelegt der Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Carolin Warnecke

Basel, 2024

Originaldokument gespeichert auf dem Dokumentenserver der Universität Basel
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Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Erstbetreuer:	Dr. Johannes Felsenberg
Zweitbetreuer:	Prof. Dr. Rainer W. Friedrich
Externe Expertin:	Prof. Dr. Amy L. Milton

Basel, den 25. Juni 2024

____

Prof. Dr. Rainer W. Friedrich

Gruppierung I Mitglied des Doktoratskomitees

Prof. Dr. Marcel Mayor

Dekan

--

„Sich verwirrt zu fühlen, ist der Anfang des Wissens.“

“Perplexity is the beginning of knowledge.”

(Khalil Gibran)

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Basel, den 28.05.2024

Acknowledgements

The past five years have been full of new experiences, challenges and development as a scientist and as an individual. However, what impacted the journey of doing the PhD may be the most are the people I was surrounded by and that I got to know. I am truly grateful for all the interactions, support, encouragement, and the friendship I encountered along the way.

First and foremost, I want to thank my mentor Johannes Felsenberg for the opportunity to work on this project. I am especially grateful for the trust you placed in me to join the lab as one of the first and to witness and be part of its evolution. Thank you for all your support, enthusiasm, numerous stimulating scientific discussions, and for always believing in me!

I also want to thank my thesis committee Rainer Friedrich and Amy Milton. Your input and support have been very valuable, always encouraging and appreciated a lot!

A big shout out to the whole Felsenberg lab! It was a pleasure to work with each of you. Every single one of you has become such a valuable colleague and friend, who I wouldn't want to miss. Thanks for being such an inspirational, diverse and supportive group!

Core facilities at FMI are legendary. Special thanks to Peter, Paul and Markus from the workshop, who always manage to come up with solutions that exceed what we had in mind. Thanks to the media kitchen for cooking large amounts of fly food and to FAIM for their support at the microscopes.

I also want to acknowledge the administration, IT, FM, and Piera Cichetti, the guidance counselor at FMI. Knowing that there was always friendly and patient support when needed made many tasks and issues much easier to handle.

FMI is a place where one can meet many outstanding people. Thanks to all for the enriching encounters, conversations, and fun times! A special thanks to those who shared the role of student representative with me – I will always remember it as a great experience and time together!

I had the immense privilege that my PhD project was funded by the Boehringer Ingelheim Fonds. But beyond supporting my project I felt that I was supported as a person. Thanks so much to the whole BIF team! I am grateful and happy to be part of the BIF family.

It might seem a bit unusual, but I also want to thank the team and trainers of the Novartis Fitorama. Especially during the pandemic, but also afterwards, it became a place where I could ground myself, clear my head, release stress, gain new courage and simply have fun.

I cannot imagine how I would have gotten through the last five years without my friends. I want to thank all of you for just being who you are – I am incredibly grateful that our path crossed at different stages of my life. I especially want to say thank you to Theresa Angenlahr, Cora Strobel, Viktoria Anselm, Marie-Theres Och, Lilo Mailänder and Marlena Wisser - you are wonderful - never forget that!

Finally, I want to thank my family! Danke Mama, Papa und Peter! I don’t have words to express how invaluable it is to have you.

Abbreviations

BLA	Basolateral amygdala
CS	Conditioned stimulus
CS-	Conditioned stimulus -
CS+	Condtioned stimulus+
DAN	Dopaminergic neuron
EB	Ethyl butyrate
ER	Endoplasmatic reticulum
GABA	gamma-aminobutyric acid
h	Hours
IgG	Immunglobuline G
IgY	Immunglobuline Y
IPA	Isopentyl acetate
LH	Lateral horn
LHON	Lateral horn output neuron
L-NNA	N_{ω}-Nitro-L-Arginin
LTM	Long-term memory
KC	Kenyon cell
MB	Mushroom body
MBON	Mushroom body output neuron
MCH	4-methylcyclohexanol
min	Minutes
ul	Microliter
ml	Milliliter
mm	Millimeter
NO	Nitric oxide
NOS	Nitric oxide synthase
OCT	3-octanol
PAM	Protocerebral anterior medial
PBS	Phosphate-buffered saline
PBS-T	PBS-Tween20
PFC	Prefrontal cortex
PN	*Projection neuron*
PPL	Protocerebral posterior lateral
PTSD	Posttraumatic stress disorder
RT	Room temperature
shi^{ts}	*Shibire* termosensitive
STM	Short-term memory
Tdc	Tryptophane decarboxylase
TH	Tyrosine hydroxylase
Trh	Tryptophan hydroxylase
TRPA	Transient receptor potential channel subfamily A
US	Unconditioned Stimulus
wt	Wild type

Abstract

In a changing world, re-evaluating learned information allows animals and humans to correct inadequate memories. However, the neuronal principles that govern these memory update processes are little understood. Here we show that manipulating dopamine neurons can bidirectionally change reward-reinforced olfactory memories in *Drosophila melanogaster*. Our data suggest a relationship between pathways involved in memory acquisition and those necessary for changing memory accessibility. Unpaired reward re-exposure that activates dopamine neurons also leads to memory devaluation. However, our data demonstrate that these two phenomena are controlled differently and recruit separate neuronal pathways. In contrast to dopamine driven memory update, sugar-reward-mediated memory devaluation is not only dopamine-independent but also controlled by context. Together our data show that memories are not static and can be updated by multiple pathways. Our findings can provide valuable insights for future investigations in the context of update related strategies to target maladaptive forms of reward memories.

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1 Introduction

1.1 After learning, memories can be maintained, modulated or forgotten.

Every day, animals and humans have to make decisions. What to eat, where to go, whom to interact with, and a lot more. These decisions are mostly guided by previous experience that is stored as memories. However, the world is constantly evolving. Therefore, memories have to be adaptable to allow adequate learned behavior. Understanding the mechanisms that underlie this malleability of memory holds great potential to weaken or alter maladaptive memories.

1.1.1 Associative memories can become maladaptive.

In classical conditioning, a neutral cue, the conditioned stimulus (CS), like a tone, a smell or a location, is paired with an unconditioned stimulus (US), an intrinsically punishing or rewarding stimulus. Consequently, the CS becomes a predictor for the US and elicits a learned response, such as avoidance or approach of the learned cue. The classic example for this form of associative learning stems from Ivan Pavlov's experiments with dogs (1). In his studies Pavlov conditioned dogs to associate the sound of a bell with a food reward. Food is naturally rewarding (US) and elicits a salivation response in dogs. After several repetitions of pairing the tone with the food reward, the dogs learned to associate the sound with feeding. Consequently, they started salivating when they heard the tone in anticipation of the food, even when no food was presented (1).

Acquiring associative memories is crucial for survival and to navigate the world. However, inadequate strong associations can lead to harmful or uncontrollable behavior. They can become maladaptive. In patients suffering from posttraumatic stress disorder (PTSD) cues can lead to the retrieval of the traumatic experience and induce distress and extreme fear (2). Also, in the case of abusive use of drugs, associative memories have been shown to play a crucial role (3, 4). Upon consumption, environmental cues are associated with the rewarding experience (5). In turn, exposure to these learned cues can induce cravings in former drug users (6, 7). Consequently, these cues can enhance or trigger drug seeking even after longer times of abstinence (8). In such cases it is desirable to weaken the predictive power of the associated cue. In the following I will explain ways through which behavioral response after learning of an association can be altered.

1.1.2 Consolidation

Memories are stored as changes in brain function. The physical representation, including the molecular changes, of a memory is referred to as the memory trace. Early after learning memories are labile and therefore vulnerable to disturbance. To persist, memories need to be stabilized, a process called memory consolidation (9, 10). During consolidation, a labile

memory trace is actively stabilized, often in a protein-synthesis-dependent manner (11). In many cases, sleep has been shown to be crucial for consolidation (12). Interference with consolidation, such as through new learning, sleep deprivation, or pharmacological intervention, can lead to amnesia which might or might not be due to the decay of the memory trace (13-15). Therefore, targeted disruption of consolidation in a therapeutical setting can prevent harmful memories from becoming long lived (14, 16). However, rapid action after for example a traumatic experience is needed for this approach. Yet often immediate treatment is not possible. Drug related behaviors are even more complex and such interventions are less feasible (17). Therefore, additional approaches are necessary to target maladaptive memories.

1.1.3 Memory update

Surroundings undergo perpetual transformation as we transition between diverse environments and circumstances inherently shift over time. Adapting to these changes requires constantly adjusting learned behavior. To be flexible, memories that guide behavior are changeable by new experience. Understanding and controlling underlying processes can help to treat pathologies related to problematic memories.

1.1.3.1 Maintenance or forgetting

Perhaps the simplest form of memory update is forgetting. Generally forgetting is defined as the inability to retrieve a memory. However, this is independent of whether the lack of retrieval is due to the erasure of the memory trace or perturbed access to an existing trace (18). One form of forgetting, intrinsic forgetting, counteracts consolidation and leads to a decay of the memory (19). In contrast, transient forgetting refers to a temporary retrieval deficit, where the memory becomes inaccessible but returns after some time or under the right circumstances (20, 21). Whether a memory trace is maintained or degraded is crucial to prioritize important learned information during decision making. Forgetting allows to reduce unreliable information and to focus on relevant information (22, 23). However, it is a hallmark of problematic memories that they are not spontaneously forgotten (24). Thus, to develop treatments to promote forgetting in a therapeutic setting will be vital to selectively trigger forgetting of harmful memories.

1.1.3.2 Extinction

Extinction is a form of forgetting. Initially learned behavioral responses are suppressed by new learning (25, 26) (Figure 1). Based on previous experience, the brain constantly predicts the future (27). When a novel experience diverges from the expectation this leads to a prediction error, which can trigger an update of the learned information (28). Extinction occurs when, after learning of a “cue–outcome” association, the animal is repeatedly exposed to the cue without reinforcement (1, 29, 30). Consequently, the new information is learned as a parallel “cue–no

outcome" association which opposes the original memory (31) (Figure 1). The integration of the two opposing memories leads to the reduction of the behavioral response. Treatment strategies based on extinction learning, also known as exposure therapy, are prevalent in clinical practice (32-34). However, a notable limitation of this approach is that the initial memory persists and can still influence behavior. For example, which memory guides behavior during retrieval can be context dependent. Thus, in contextual settings very similar to the original learning situation the initial memory is more likely to guide behavior, while in a context that is more similar to the extinction situation learned responses remain reduced – a phenomenon called renewal (26, 30). Further it has been shown that the initial memory can recover over time after extinction, a phenomenon termed spontaneous recovery (1) (Figure 1). Lastly, re-exposure to the unpaired US after extinction can induce the reinstatement of the initial memory (26, 29). In case of drug-related memories cues often acquire reinforcing power themselves, rendering extinction less efficient (35, 36). Thus, though applicable, success of extinction-based therapy is limited (37-39).

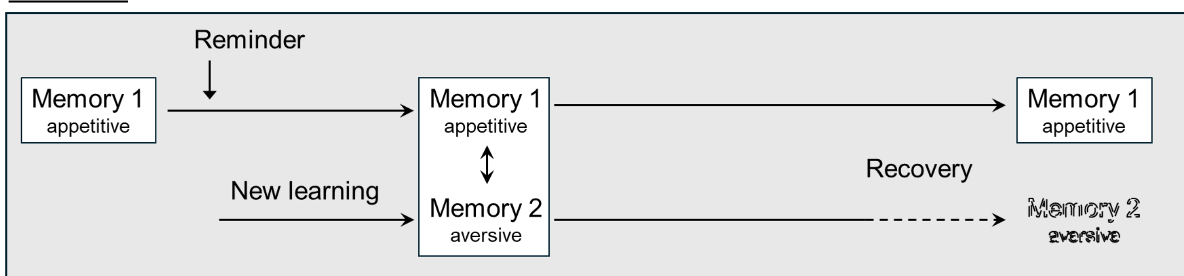
1.1.3.3 Reconsolidation

In contrast to extinction learning, in memory reconsolidation there is the potential that the memories about the CS-US association are updated themselves (40) (Figure 1). Initially, it was assumed that once memories are stabilized, they are unchangeable. However, recent work has shown that stabilized memories can be adjusted. Though initial indications for this process were suggested in the late 1960s by Lewis *et al*., the idea that memory can be updated following a reminder session was invigorated by Nader *et al.* in 2000 (40-42). The work proposed that the retrieval of a memory can induce a second round of consolidation, reconsolidation (40). The idea behind this concept is that the presentation of a reminder cue can switch the consolidated memory into an active state. In this active form, the memory is retrievable but is vulnerable to interference and change. Over time, the memory gets restabilized (Figure 1). Therefore, reconsolidation is discussed as a mechanism to update memories in accordance with changes in the environment (40, 43). Interfering with restabilization leads to the loss of the destabilized memory and therefore holds great potential for application in patients (44-46). This interference can be achieved pharmacologically or with behavioral interventions such as new learning (44, 47-49). However, even after more than 20 years of research many questions in the field of reconsolidation remain open. Especially, the mechanisms behind the destabilization step are not well understood and represent a bottleneck in translation to clinics. Whether retrieval initiates reconsolidation, leaves the memory unchanged or triggers extinction is largely dependent on so called boundary conditions. Boundary conditions include the nature of the memory and circumstances of retrieval i.e., memory type, strength, and age, internal and external context of learning and retrieval as well as the cue used as a reminder (50-52). Extinction and reconsolidation are

presumed to be mutually exclusive, but both are initiated through a prediction error in the retrieval situation (28, 53, 54). When the CS serves as a reminder, longer retrieval sessions, resulting in a larger prediction error, typically induce extinction learning. Conversely, a brief reminder (yielding a smaller prediction error) often triggers destabilization (28, 54). However, what “small” and “large” means is hard to define and differs for individual memories.

Indeed, most studies initiate reconsolidation through exposure to the CS. However, rewarding experiences (US) are typically associated with multiple cues and compared to the laboratory setting there is less of a primary cue present (55). Therefore, identifying the appropriate cue to successfully initiate reconsolidation is not trivial. Even after treatment with one cue, other cues still retain the potential to drive unwanted behavioral patterns (44). On the other hand, the unconditioned stimulus itself, i.e., the effects of the drug, is linked to all cues associated with the reward. Indeed, it has been demonstrated that retrieval through the US as a reminder can initiate reconsolidation (56-58). Interfering with memory restabilization after US-mediated destabilization results in reduced recall of several memories connected to that reward (58). In some cases retrieval sessions including the presence of the US even seem to be necessary to shift stronger memories to a destabilized state (59). Certainly, US-based memory activation poses its own challenges, particularly when it involves administering drugs with high addiction potential. This raises ethical concerns and complicates clinical studies involving humans. One potential approach could involve the use of chemically similar substances such as methylphenidate or methadone. (58, 60).

Extinction:



Reconsolidation:

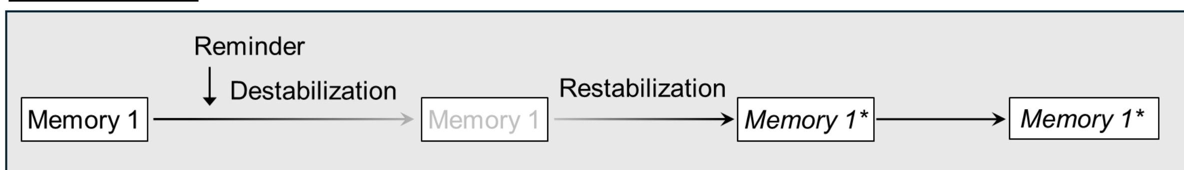


Figure 1: Concept of Extinction and Reconsolidation: Extinction and reconsolidation are memory update mechanisms that can be triggered by a reminder. In extinction behavioral consequences of a memory are suppressed by the formation of a second memory of opposing valence. However, over time the second memory becomes weaker and the initial memory guides behavior again (recovery). Reconsolidation is a sequence of destabilization and restabilization of the memory. In the destabilized state the memory can be changed, and after restabilization persists in the updated form. The memory can be retrieved at any timepoint during this process. White boxes indicate the memory that is guiding behavior in each step.

1.1.4 Dopamine involvement in learning, forgetting and updating in mammals.

The nervous system constantly receives sensory input from the external environment and integrates this information into activity within neuronal pathways. This activity can induce neuronal plasticity which means changes in connectivity and synaptic strength. Physiologically, this plasticity is substrate for memory trace formation and information storage in memories. Therefore, changing learned information entails adjustments - such as strengthening, reversal or addition - of neuronal connections in the same or other locations, ultimately influencing behavioral outcomes.

The neurotransmitter dopamine plays a crucial role in many memory-related processes. It is involved in signaling reward and punishment, motivation, salience and prediction errors during **learning** (61-70). These processes involve several brain areas such as the amygdala, hippocampus, prefrontal cortex, the striatum, and the ventral tegmental area (VTA). Dopamine release from the VTA neurons is crucial in signaling prediction errors necessary for new learning. Different neuronal populations project from the VTA to different areas in the nucleus accumbens (NAc) shell, signaling either reward or punishment (71, 72). Another study also reports projections to the tail of the striatum to be involved in aversive learning (73).

Dopamine also has been shown to play a dual role in the processes of memory **consolidation, maintenance, and forgetting** (24). After learning, dopamine seems to have a supporting effect on memory consolidation (74-76). However, there are also studies showing the counteracting role of dopamine promoting forgetting. Dopamine signaling to the hippocampus after learning impairs an otherwise long-lasting cocaine conditioned memory (77). On the contrary, blocking dopamine receptors in the hippocampus or the VTA after a weak learning trial enhanced memory performance (77, 78). Further, dopaminergic projections from the VTA to the prefrontal cortex (PFC) are suggested to be involved in retrieval-induced forgetting, in which predominant retrieval of a certain memory leads to forgetting of competing memories (79-81).

As in new learning, dopamine is involved in memory **extinction**. It has been shown that dopamine input from the VTA specifically to the anteromedial part of the NAc signals unexpected omission of the US during early fear extinction trials (82). This signal is triggered by input from the dorsal raphe (DR). Blocking dopamine signaling to the basolateral amygdala (BLA) impairs fear extinction, and specific “extinction neurons” in the BLA increase their response to the CS during fear extinction (83, 84). Further, dopamine-dependent lasting plasticity changes are established in the medial prefrontal cortex (mPFC) during extinction. Dopamine signaling in the infralimbic prefrontal cortex (IL) is crucial during extinction learning

and for retention of extinction memory (84-86). However, the areas of origin of these inputs still need to be discovered.

As in extinction, a prediction error is necessary for initiation of **reconsolidation**. Therefore, it is maybe not surprising that dopamine is involved in the destabilization step of aversive and appetitive memory reconsolidation (87, 88). Perturbing dopamine signaling from the VTA has been shown to prevent appetitive memory destabilization in rats, consistent with VTA output conveying prediction error signals (53, 89, 90). Similarly, dopamine input to the BLA is crucial for appetitive memory destabilization (91). Furthermore, dopamine signaling in the hippocampus has been implicated in gating destabilization with respect to internal state of the animal (92).

Overall, dopamine plays a diverse role in memory processing. However, due to the intricate nature of the mammalian nervous system, elucidating the precise functions of dopamine, its receptors, and associated pathways poses a significant challenge. Yet, a deeper understanding of the underlying neuronal mechanisms, particularly those involving dopamine signaling, is crucial for effectively modulating memory reconsolidation and other update mechanisms in a controlled manner. Moreover, the availability of measurable parameters indicating whether a memory has been destabilized or not would significantly improve our ability to intervene in the restabilization process with precision. Given that the principles of extinction and reconsolidation seem to be similar across species, the use of model organisms such as the fruit fly *Drosophila melanogaster* becomes advantageous. Its numerically simpler, genetically accessible brain system provides an excellent opportunity to uncover mechanistic insights that can subsequently guide investigations in more complex brain systems.

1.2 *Drosophila* as a model organism to study learning and memory

1.2.1 *Drosophila* general

The fruit fly *Drosophila melanogaster* is widely used as a model organism in biomedical research. While sharing around 61% to 73% of disease related genes with humans (93, 94), they are easy to maintain, quick in reproductivity and genetically accessible. In 2000, the whole fly genome was sequenced allowing for more targeted investigations of genes of interest (95). Moreover, binary expression systems, such as the UAS/GAL4 system, have enabled precise targeting of gene expression at the cellular level (96). This includes the expression of thermo- or optogenetic tools to manipulate neuronal activity. Light-sensitive ion channels, like channelrhodopsin CsChrimson or the thermosensitive drosophila transient receptor potential channel subfamily A member 1 (dTRPA1), enable targeted cell activation (97, 98). Conversely, a conformational change of the dynamin mutant *shibire*^{ts} (*shi*^{ts}) leads to inhibition of small vesicle release at high temperatures (99). The fly brain consists of around 200 000 neurons

with a more or less stereotypical wiring pattern and therefore is considerably less complex and more accessible for study than the mammalian counterpart (100). Despite this relative simplicity, flies exhibit complex behaviors conserved over species rendering them a suitable model to understand underlying circuit motives of such. Research of the last decades has led to many achievements in understanding perception, sleep, navigation, motor activity, social behaviors, and learning and memory on the fly. In 2020, the electron microscopy based connectome of a female fly brain was made available being a powerful tool to track individual neurons and their connectivity, expanding the potential of *Drosophila* as a model organism for system neuroscience (101, 102).

1.2.2 Olfactory learning/ MB network

Olfactory learning has been a major paradigm to study learning and memory in fruit flies. Flies can learn to associate odors with either reward or punishment (103, 104) (Figure 3). The antennal olfactory receptor neurons sense odors and sensory neurons that express the same receptor type project to one out of 43 glomeruli in the antennal lobes where they connect to projection neurons (105). There are two kinds of projection neurons signaling to central brain areas of the fly: uniglomerular excitatory projection neurons and multiglomerular inhibitory projection neurons. Both types are connected to the lateral horn, one of two higher brain areas involved in olfactory processing (106-108). Additionally, uniglomerular projection neurons connect to the calyx of the mushroom body (MB), the memory center of insects (107-109) (Figure 2). The MB comprises ~2000 cholinergic neurons per hemisphere, known as Kenyon cells (KC), which receive their inputs within the so-called calyx and project into the MB lobes (110). These Kenyon cells encode odor identities as sparse activity patterns (111). Apart from olfactory inputs, KCs also receive visual, tactile hygro- and thermosensory information (102, 112, 113). Anatomically and functionally KCs can be divided into three major types: γ-, α′/β′-, and α/β- KCs with their axons forming the respective lobes (114, 115) (Figure 2). Concerning memory storage, they seem to serve different functions. While short-term memory (STM) is thought to be stored in γ KCs, long-term memory (LTM) rather involves α/β KCs (116, 117). The α'/β' lobe is important during memory acquisition and consolidation (117-119). Downstream of KCs, postsynaptic mushroom body output neurons (MBONs) mediate behavioral approach or avoidance in response to a cue (120-122). Thereby, dendritic zones of MBONs innervate discrete compartments in the MB lobes that fulfill different functions (122, 123). These compartments are named with the letter of the respective lobe followed by the position in the lobe with the highest number labeling the last compartment at the tip of a lobe (Figure 2). In general, MBONs getting input in the vertical lobe are mainly approach coding MBONs, whereas those receiving input in the horizontal lobes mostly mediate avoidance. During learning KC to MBON signaling is modulated by the aminergic system (124-127) (Figure 2). While plasticity has classically been thought to occur presynaptically, i.e., in the KC,

there is also evidence for post synaptic plasticity in the MBONs (128-130). Therefore, memories are stored in the MB at the conjunction of KC to MBONs.

1.2.3 Dopamine neurons in learning, updating and forgetting of memory in *Drosophila*.

As in mammals, dopamine in *Drosophila melanogaster* is well known for its role in learning and memory consolidation as well as memory maintenance, forgetting and memory update (31, 69, 126, 131, 132).

In flies, memories are established in the lobes of the MB at the synapses between KCs and postsynaptic MBONs (129). During **learning,** reward or punishment is conveyed by dopaminergic neurons (DANs) from the protocerebral anterior medial (PAM) or protocerebral posterior lateral (PPL1) cluster that innervate the MB lobes (124, 133-135). The system is built in a way that appetitive coding DANs innervate compartments where avoidance coding MBONs receive their input and punishment coding DANs innervate those where approach coding MBONs receive input. The coincident activity of KCs and DANs activates the cAMP-PKA signaling pathway through adenylyl cyclase rutabaga (136, 137). Subsequently, this leads to depression of KC-MBON synapses resulting in reduced avoidance or approach, respectively (Figure 2). For example, activity in reward coding PAM-γ5 and PAM-β’2 during learning leads to depression in avoidance-promoting MBON-β′2mp and MBON-γ5β′2a favoring approach behaviors, while aversive learning leads to depression in approach-promoting MBON-γ1pedc mediated by aversive reinforcing PPL1- γ1pedc (120, 121). On the level of DANs there is further heterogeneity within each cluster: Different subsets of DANs have the potential to reinforce either only a short-term or long-term memory. When either of these MBONs is blocked during sugar learning, flies lack either the expression of the long-term or the short-term memory respectively (138, 139). Furthermore, different types of rewards are processed in different compartments: while PAM-β’1 DANs have been shown to be crucial for long-term water memory, γ5 and α1 PAM DANs are crucial for long term memory of a sugar reward (118, 138, 140). The reward of absence of shock in reversal learning has been shown to be reinforced by PAM-β'2a DANs (141). Recently it has been shown that if multiple DANs project to one compartment, they can be even further divided into subcategories. For instance, γ5 PAMs can be categorized into five groups based on their inputs, with some neurons providing the teaching signal during sugar learning and different PAM- γ5 DANs are involved in memory re-evaluation (142).

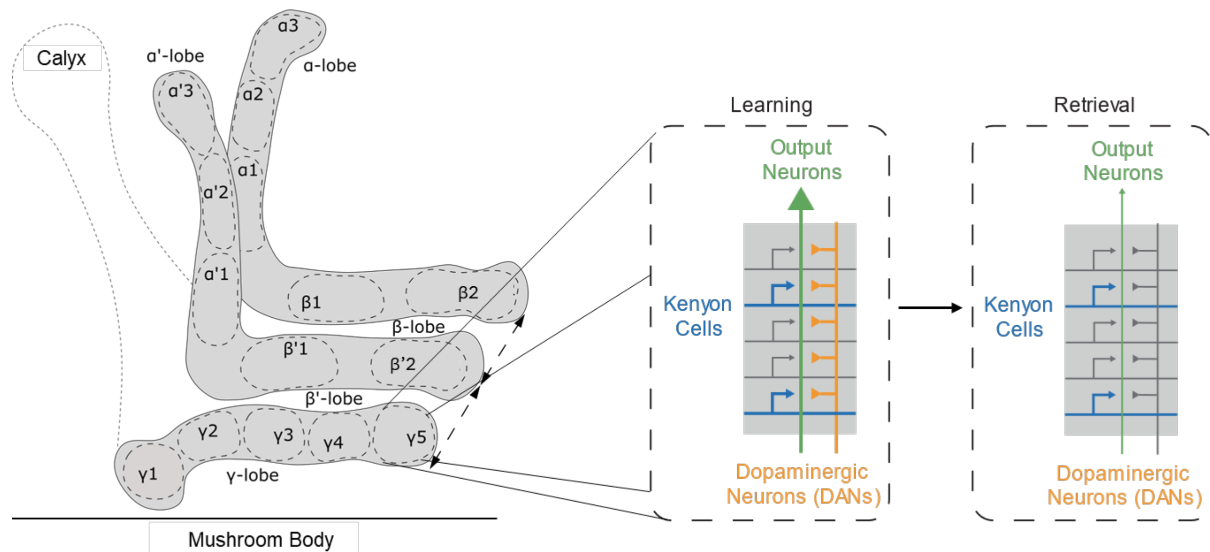


Figure 2: Schematic of the mushroom body. Simplified, the mushroom body consists of the calyx (dendritic field of KCs) and three lobes that are formed by the axons of different classes of KCs. The lobes are divided into different compartments. In each of these compartments distinct output neurons receive input from KCs. DANs innervate the same compartment signaling naïve valence. During learning, output from these DANs modulates the KC to MBON synapse (Learning). Subsequent odor exposure after learning evokes a reduced response in the output neuron (Retrieval).

Additionally to this feed forward signaling, the three-parted KC-DAN-MBON system features further complexity: Individual Kenyon cells give inhibitory axo-axonal input onto neighboring Kenyon cells, contributing to sparsity of odor patterns and therefore preventing unspecific learning (143). Further, connections between DANs and KCs are reciprocal. Alongside the input DANs give to KCs, cholinergic axo-axonal synapses from KCs to DANs influence ongoing activity within DANs and also seem to amplify the reinforcement signal of DANs at connection sites with active KCs (144). Moreover, MBON axons project back to DAN dendrites, forming feedback loops onto the MB system (31, 102, 118, 123, 131, 145, 146). These feedback connections can on the one hand provide a mechanism by which previous experience influences dopamine responses to repeated exposure to a cue (31, 131). On the other hand, they can extend the duration of a signal, as proposed for the PAM-α1 MBON-α1 loop during **consolidation** (118). Besides their prominent role in conveying the reinforcement signal, DANs also provide information about the fly's internal and behavioral state to the memory system and integrate this information into the learning and retrieval process. Many DANs show ongoing activity which can be modulated by nutritional status of the animal as well as by sleep, movement, arousal and mating (19, 147-150). This activity of DANs influences consolidation, memory maintenance/ retrievability and forgetting (19, 150-153). For example, activity in PPL1-γ1pedc neurons is high in fed and low in starved flies. Low activity allows retrieval of appetitive STMs in a state-dependent manner (151). Further, oscillations in PPL1-γ1pedc and PPL1-γ2α'1 after learning are only present in fed flies and required for aversive and appetitive LTM consolidation (150, 153). Interestingly, arousal dependent activity in the same neurons leads to active forgetting of aversive memories after learning ((132, 154, 155) reviewed in (156)). Accordingly, artificial activation of PPL1 DANs after learning diminishes memory

performance (132). Same applies when an unpaired shock is delivered after aversive learning. On the contrary, inhibiting PPL1 DANs after one trial of aversive training delays the decay of memory (132). Consolidated memories can be transiently forgotten after exposure to an aversive stimulus. However, the memory trace is maintained and forgetting is mediated by PPL1 DANs different to those involved in learning and active forgetting (20). Corresponding to aversive memories, activating PAM DANs after appetitive learning, leads to loss of memory performance in the testing situation (155). This raises the question: How can the same neurons exert two opposing processes? The answer to this question seems to be two-fold. Firstly, dopamine can activate different dopamine receptors at the postsynapse. In *Drosophila* there are four dopamine receptor types known: the D1 like (referring to the mammalian counterpart) receptors Dop1R1 and Dop1R2 (DAMB), the D2 like receptor Dop2R and the receptor DopEcR that additionally binds ecdysone and 20E (157). New learning involves Dop1R1 dopamine receptors at the KC postsynapse. Dop1R1 is coupled to Gs protein and activates adynyl cyclase rutabaga resulting in increased cAMP levels (125). On the contrary active forgetting, transient forgetting and backward conditioning (when the CS is presented after the US and therefore predicts the end to a meaningful event) have been shown to depend on Dop1R2 (DAMB) receptor signaling via Gq, IP3 and on the small G protein Rac1 (20, 132, 158, 159). Interestingly Rac1 has also been shown to regulate forgetting processes in mammals (160-163). Both receptors have a different sensitivity to dopamine (158). Therefore, it has been proposed that high dopamine levels during learning predominantly activate Dop1R1, whereas low ongoing activity afterwards engages more on Dop1R2 signaling leading to forgetting (127). However, it has been shown that during learning dopamine signals are highest during simultaneous activity in DANs and KCs, with no difference in the release pattern whether KC activity precedes or follows DAN activation (126). Still, Dop1R2 dependent Ca^{2+} release from the endoplasmatic reticulum (ER) depends on relative timing of CS and US. Handler *et al.* suggest that calcium-sensitive IP_3 receptors at the ER sense the relative timing between DAN and KC activity (126). In the absence of IP_3 elevated calcium levels suppress receptor activation. Only after IP_3 has bound an activating Ca^{2+} binding site becomes accessible (164). Therefore, higher calcium levels resulting from KC activity must not precede but follow DAMB activation to activate IP_3 receptors and subsequent Ca^{2+} release from the ER.

A second explanation might be the release of co-transmitters from DANs. Gamma aminobutyric acid (GABA) and glutamate are transmitter reported to be released from DANs and involved in forgetting of appetitive or aversive memory, respectively (155). Alongside these classical neurotransmitters, DANs can also signal via other transmitter systems like neuropeptides, gaseous molecules or electric synapses (165, 166). Nitric oxide (NO) is a gaseous transmitter that is produced from arginine by the nitric oxidase (NOS) and can passively diffuse through the membrane to reach the postsynaptic receptors. During learning

NO release from certain DANs leads to the formation of a memory of opposing valence that emerges with slower dynamics than the memory implemented by dopamine therefore promoting forgetting (166).

However, dopamine not only plays a role in the two extremes of memory maintenance and forgetting. Both extinction and reconsolidation in the fruit fly are dependent on dopamine signaling (31, 131, 167). Since **extinction** entails the formation of a new memory trace of opposing valence, it is intuitive that this involves DANs of the corresponding opposite cluster compared to the learning situation. Meaning that the extinction of a reward memory requires neurons from the PPL1 cluster whereas extinction after aversive learning recruits DANs from the PAM cluster (31, 131). Interestingly, the PAM-DANs that mediate extinction of aversive memory and sugar reward learning are anatomically similar and innervate the same compartment. However, recent work has shown that these neurons are functional heterogeneous with distinct neurons encoding sugar reward or the reinforcement in the case of the omission of expected punishment during extinction (142).
Two studies investigate reconsolidation in *Drosophila*. Both use the conditioned stimulus as a reminder cue (131, 168). In **reconsolidation** PPL1 DANs have been shown to be involved in the restabilization step of reconsolidation of a reward memory (131). However, whether exposure to the US can induce reconsolidation and whether DANs are involved in destabilization of the memory remains to be explored.

Thus, despite significant progress in understanding the roles of DANs in memory revaluation and update, a lot remains to be elucidated. This includes investigating whether and which DANs are involved in memory destabilization and whether and how forgetting and memory destabilization differ from each other.

1.2.4 Other players giving input to the MB

Beyond the main circuits, there are additional inputs into the learning network.

There are around 80 **serotonergic neurons** in the *Drosophila* brain and the MB expresses serotonergic receptors (169-171). Further, serotonergic signaling seems to be crucial in aversive olfactory conditioning (172). For long-lasting memories the serotonergic neuron SPN has been shown to gate LTM formation by increasing PPL1-γ1pedc oscillations after spaced aversive training (173). Another significant serotonergic input to the MB comes from the **DPM** (dorsal paired medial) neuron, which arborizes all over the MB. This neuron has been implicated in consolidation and sleep promotion during the latter (174-177).

Like the DPM neuron, the GABAergic **APL** (anterior paired lateral) neuron spans the entire MB. When activated by active KCs it ensures sparse odor representation by inhibition of non-

activated KCs (111, 178). Additionally, APL can release **octopamine,** the analog of vertebrate norepinephrine in insects. Octopamine influences a wide range of behaviors, including sleep, feeding, locomotion, reproduction and learning (133, 179-185). Aside from the APL, four classes of octopaminergic neurons are innervating the mushroom body: OA-VUMa2, OA-VPM3, OA-VPM4, and OA-VPM5. OA-VUMa2 and OA-VPM5 innervate only the calyx; OA-VPM3 innervates only the γ-lobe, while OA-VPM4 gives input to both the calyx and the y-lobe (186). Octopamine receptors in the KCs are important for aversive and appetitive conditioning (133, 187-189). Further PAM DANs receive octopaminergic input, which is crucial for formation of a short-lasting sweet taste memory (124).

Besides the mushroom body, olfactory information is computed in the **lateral horn**. Similar to the MB, the lateral horn gets additional input from other sensory modalities (190). Unlike the MB, the input to the lateral horn is stereotyped and it is primarily associated with innate olfactory processes (191). However, we have contributed to a study showing that neuronal pathways impinging on the lateral horn are modulated by associative learning (192). Further, several lateral horn output neurons (LHONs) not only provide direct input to KCs but also connect to PAM-and PPL1 DAN dendrites, suggesting that information from the lateral horn influences dopamine signaling to the mushroom body (101, 190). Thus, the lateral horn can modulate the MB-network based on its computations regarding the innate valence of a stimulus, depending on the context and state of the animal (190). On the other hand, the LH itself receives input from the MB. MBONs not only project to dendrites of LHONs, potentially forming feedback loops, but MBON and LHON signals also converge downstream through axo-axonal connections (190, 193). Therefore, the combined signaling from the MB and LH can integrate innate and learned information, leading to an appropriate behavioral response.

Additionally to neurons, **glia** cells have long been known to be a substantial part of the nervous system. However, their direct involvement in the learning process is a rather novel insight. Only recently it has been reported that vesicle release from ensheathing glia is important for aversive learning and even further that artificial activation of ensheathing glia can serve as a reinforcement signal during learning (194). Therefore, in the future glia should be considered as a substantial part of learning networks in the mushroom body.

All in all, olfactory learning paradigms in *Drosophila* offer great opportunities to investigate the various players involved in memory processes. Decades of research have already yielded valuable insights across many topics that future research can build on.

1.3 Aim of this thesis

Inadequate strong associative memories can underlie maladaptive memory disease like posttraumatic stress disorder or substance abuse. Therefore, targeting these memories to prevent harmful behavior is fundamental in treatment. While most strategies focus on CS-initiated memory update, this approach has the caveat that only weakening the specific CS-US connection may not fully prevent unwanted behavior, as other related cues can still drive it. Using the US as a reminder could overcome this problem, since the US is part of all memories associating a cue with the respective outcome. However, the principles by which exposure to the US can lead to the updating of a memory are poorly understood.

This thesis aimed to gain insight into US-mediated memory update processes using *Drosophila melanogaster* as a model organism. Therefore, we established a paradigm in which sugar-trained flies are re-exposed to the unpaired reward after training. This re-exposure leads to devaluation of the learned CS-US association, independent of the consolidation state of a memory. Applying this paradigm, we aimed to investigate the following questions:

1) Is memory devaluation reward-specific, and does it affect multiple CS-US associations?
2) What are the circuits underlying sugar memory devaluation?
3) Are there mechanistic differences to active forgetting, in which ongoing activity of reinforcing DANs leads to fading of unconsolidated memories over time?

Our results show that exposure to the unpaired US can lead to devaluation of multiple associations. This devaluation happens in a context dependent manner. Further, there is a certain specificity regarding the reward that can trigger memory update. Despite our efforts, our results do not reveal the circuit motives underlying sugar memory devaluation. However, our data demonstrates that vesicle release from most of the main MB network candidates is not involved in US-mediated devaluation, which is as surprising as it is fascinating. Finally, though we observe dopamine-dependent forgetting in an artificial setting, sugar memory devaluation seems to be a separate mechanism which follows distinct rules.

Together, our results for the first time report the reward-initiated update of reward memory in *Drosophila*. While open questions remain, insights achieved during the course of this thesis can serve as a basis for further research on mechanisms underlying US mediated memory update.

2 Material

2.1 Antibodies

anti GFP (chicken)	Abcam (ab13970)	1:2000
Anti bruchpilot (mouse)	DSHB (nc82)	1:50
Goat anti-Chicken IgY (H+L) Alexa Fluor™ 488	Invitrogen (A-11039)	1:300
Goat anti-Mouse IgG (H+L) Alexa Fluor™ 633	Invitrogen (A-21052)	1:300

2.2 Chemicals

4-methylcyclohexanol (98%)	Sigma-Aldrich	Cat#218405
3-octanol (99%)	Sigma-Aldrich	Cat#153095
Active dry yeas	Sigma-Aldrich	Cat#789093
All trans-Retinal	Sigma-Aldrich	Cat#116-31-4
Arabinose	Carl Roth AG	Cat# 3051.2
Ethanol absolut	Sigma-Aldrich	Cat#1.00983.1000
Ethyl butyrate	Sigma-Aldrich	Cat#E15701
Goat serum	Thermo Fisher Scientific	Cat# 16210064
Isopentyl acetate (also known as isoamyl alcohol)	Sigma-Aldrich	Cat#112674
L-NNA (Nω-Nitro-L-Arginin)	Sigma-Aldrich	Cat#2149-70-4
Mineral oil	Sigma-Aldrich	Cat#M5904
Sucrose	Carl Roth AG/ Sigma-Aldrich	Cat# 4661.1/ Cat#S7903
Triton X	Carl Roth AG	Cat# 3051.2
Vectashield mounting medium	Vectorlabs	Cat#VC-H-1000-L010
Agar	Fisher Scientific	Cat#10346693
Soy flour	VWR	Cat#0296002405
Active dry yeast	Milian	Cat#789093
Cornmeal	E. Zwicky AG	Cat#1054942
Molasses	Milian	Cat#789214
Nipagin	Sigma-Aldrich	Cat#H5501

2.3 Equipment and consumables

Ultimate flypad	Milian
Klimazellen	K.Schweizer
T-mazes and T-maze tubes	In house design
Red LEDs 634 nm, Osram Opto Semiconductors	Distrelec
Optogenetic situmlators	In house design
Dehydrator	WMF
Forceps	FST
Whatman paper	Sigma-Aldrich

Fly bottles	Milian
Fly vials	Milian

2.4 Fly food and buffers

2.4.1 Fly food

Water	10 000 ml (~82.7%)
Agar	75 g (~0.6%)
Soy flour	100 g (~0.8%)
Yeast	185 g (~1.5%)
Cornmeal	900 g (~7.4%)
Sugar beet syrup	400 g (~0.3%)
Molasses	400g (~0.3%)
nipagin	25 g (~0.02%)

2.4.2 PBS (pH 7.3)

NaCl	8.00 g/l
KCl	0.20 g/l
$Na_2HPO_4 \cdot 2H_2O$	1.44 g/l
KH_2PO_4	0.20 g/l

2.5 Organisms/ strains

D. melanogaster: R58E02-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_41347
D. melanogaster: R58E02-lexA	Bloomington *Drosophila* Stock Center	RRID: BDSC_52740
D. melanogaster: 0273-GAL4	Waddel lab Oxford	
D. melanogaster: R15A04-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_48671
D. melanogaster: R15A04-lexA	Bloomington *Drosophila* Stock Center	RRID: BDSC_52483
D. melanogaster: R48B04-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_50347
D. melanogaster: 0279-GAL4	Waddel lab Oxford	
D. melanogaster: MB194B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68269
D. melanogaster: MB043C-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68363
D. melanogaster: R56H09-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_39166
D. melanogaster: R87D06-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_40487
D. melanogaster: MB315C-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68316
D. melanogaster: MB025B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68299
D. melanogaster: NP5272-GAL4	Kyoto Stock Center (DGRC)	113659
D. melanogaster: MB056B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68276

D. melanogaster: MB032B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68302
D. melanogaster: MB441B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68251
D. melanogaster: MB312B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68314
D. melanogaster: MB058B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68278
D. melanogaster: MB296B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68308
D. melanogaster: MB099C-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68290
D. melanogaster: MB320C-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68253
D. melanogaster: MB630B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68334
D. melanogaster: MB438B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68326
D. melanogaster: MB504B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68329
D. melanogaster: MB301B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68311
D. melanogaster: MB032B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68302
D. melanogaster: TH-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_51982
D. melanogaster: MB299B-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68310
D. melanogaster: R13F02-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_48571
D. melanogaster: Tcd2-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_9313
D. melanogaster: Trhn-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_38388
D. melanogaster: 5015(DPM)-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_2721
D. melanogaster: LH989-GAL4/ SS04956-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_86697
D. melanogaster: Gr64f-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_57669
D. melanogaster: repo-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_7415
D. melanogaster: empty-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_68384
D. melanogaster: empty split-GAL4	Bloomington *Drosophila* Stock Center	RRID: BDSC_79603
D. melanogaster: UAS-*shi*^{ts}	Waddel lab Oxford	
D. melanogaster: UAS-CsChrimson	Waddel lab Oxford	
D. melanogaster: UAS-dTRPA1	Bloomington *Drosophila* Stock Center	RRID: BDSC_26263
D. melanogaster: UAS-dTRPA1, lexAOP-CsChrimson; R58E02-GAL4		This study
D. melanogaster: UAS-dTRPA1, lexAOP-CsChrimson; R15A04-GAL4		This study

D. melanogaster: UAS-dTRPA1, lexAOP-CsChrimson; 0273-GAL4		This study
D. melanogaster: UAS-dTRPA1, lexAOP-CsChrimson; R48B04-GAL4		This study
D. melanogaster: UAS-CsChrimson; UAS- shi^{ts}		This study
D. melanogaster: UAS-GFP; 247-LexA, LexAOP-RFP	Waddel lab Oxford	

2.6 Software

GraphPad Prism version 8.3.0 to 10.2.2	GraphPad Software, La Jolla, CA
Fiji	NIH; Schindelin et al., 2012
Adobe Illustrator CC	Adobe Systems, San Jose, CA

3 Methods

3.1 Fly keeping

Drosophila melanogaster strains were reared and kept on standard cornmeal-agar food either at 60% humidity and 22°C or 25°C in a 12:12 h light-dark cycle. As wildtype strain Canton S flies were used. All other lines are listed in the resource table. Flies expressing *CsChrimson* were kept and handled in the dark or under dim light conditions and flipped to food containing 125 mM all-trans retinal 3 days prior to the experiment. Males from GAL4 or lexA driver lines were crossed to virgins from the UAS or lexAOP lines.

3.2 Behavioral experiments

For behavioral experiments mixed-sex populations of 2-8-day-old flies were used. Approximately 80-100 flies were starved for 16-20 h in a 25 ml vial containing 3 ml of 1% agar as water source and a 15 × 30 mm filter paper. As odors 4-methylcyclohexanol (MCH), 3-octanol (OCT), ethyl butyrate (EB) and isopentyl acetate (IPA) diluted approximately 10^{-3} in mineral oil were used. Standard experiments were performed at 23°C. For thermogenetic activation of neurons by dTRPA1 or inhibition by *shibire*^{ts} temperature was raised to 32°C. In experiments using *shibire*^{ts} flies were exposed to the restrictive temperature 30 min before the respective experimental phase.

<u>Odor avoidance assay:</u>

For odor avoidance experiments flies were given the choice between 3-octanol and solvent-immersed air in the dark for 2 min. Flies were placed into the restrictive temperature of 32°C for *shi*^{ts} inhibition 30 min before and throughout testing. Afterwards flies were collected from either side of the T-maze and counted accordingly. Avoidance index was calculated as the difference between number of flies approaching the 3-octanol and the solvent odor, divided by the total number of flies. One N consists of two sets of flies alternating the side of the T-maze where the odor was presented.

<u>Sugar conditioning:</u>

Appetitive training was performed as described before (104). Flies were exposed to the unpaired odor for two minutes without reward, followed by 30 s of clean air. Subsequently flies encountered the paired odor for 2 min together with dry sucrose.

<u>Electroshock conditioning:</u>

Aversive olfactory conditioning using electro shocks was performed as described previously (103, 120) Flies were exposed to the CS+ for 1 min while delivering electricshocks (by twelve 90 V electric shocks at 5-second intervals). Afterwards they received clean air for 45 s followed by 1 min of CS- without punishment.

Artificial training/ activation:

To activate *dTRPA1* expressing neurons flies were flipped into preheated (32°C) vials. As an exception, flies expressing *dTRPA*1 together with *CsChrimson* were not flipped but placed in heat in their original vials. For optogenetic activation using CsChrimson, flies were exposed to red light (λ=634 nm, 500 Hz, 19.5 lm/W).

When the training in the T-maze was done artificially flies received the paired odor in the heat (pre-heated T-maze tubes) or during red light exposure, instead of being exposed to dry sugar or shock.

Sugar re-exposure:

Sugar re-exposure was performed for 5 min either in 25 ml vials containing 3 ml of 1% agar or in T-maze tubes. To present the sugar, vials/ tubes were lined with a filter paper covered in dry sucrose.

Test:

For the test flies were given the option between two odors in the T-maze. Testing was performed in the dark for 2 min. Afterwards flies were collected from either side of the T-maze and counted accordingly. Performance index was calculated as the difference between number of flies approaching the paired odor and the unpaired odor, divided by the total number of flies. One N consists of two sets of flies trained reciprocally in terms of odor identity for CS- and CS+.

Pharmacology:

NOS activity was inhibited by feeding flies L-NNA. L-NNA was either given in food containing 100 mM L-NNA or on filter papers soaked with saturated L-NNA solution in tab water.

3.3 Immunohistochemistry

Fly brains were fixed for 30 min in 4% formaldehyde after dissection. Blocking was done overnight at 4°C with 5 % goat serum in PBS containing 0.3% Triton X (PBS-T). Afterwards brains were incubated with the primary antibodies (chicken anti-GFP, Abcam (ab13970) (diluted 1:2000); mouse anti-bruchpilot, DSHB (nc82) (diluted 1:50) for at least 5 h at RT, sufficiently rinsed in PBS-T and afterwards incubated with the secondary antibodies (diluted 1:300) (Goat anti-Chicken IgY (H+L) Alexa Fluor™ 488; Goat anti-Mouse IgG (H+L) Alexa Fluor™ 633) over night at 4°C. For mounting, Vectashield mounting medium was used. Imaging was done with a Zeiss Axio Imager M2 equipped with a spinning disk confocal scanning unit (Yokogawa CSU W1 with Dual T2, Pinhole size: 50 µm) using a 20x air objective. Images were analyzed with Fiji (Schindelin et al. 2012).

3.4 Data analysis

Data was analyzed with GraphPad Prism version 8.3.0 to 10.2.2. Groups were analyzed for Gaussian distribution using Shapiro-Wilk normality test (Significance level alpha = 0.05). For normally distributed data one-sample t-test was applied to check for significant positive or negative performance indices (Significance level alpha = 0.05). Data sets containing two groups with normal distribution were analyzed for significant differences using an unpaired t-test ($P<0.05$ for significance). When data points were not normally distributed a Mann-Whitney U test was performed ($P<0.05$ for significance). One-way ANOVA was applied on data sets containing more than two groups. For correction of multiple comparisons, a Dunnett’s test was performed.

4 Results

4.1 Reward re-exposure diminishes expression of olfactory reward memory.

How learned information guides future behavior depends on the reliability of the related association. Though it has been shown that the re-exposure to the conditioned stimulus alone can reduce learned responses, the consequences of re-exposure to the unconditioned stimulus are less well understood. Here we test how US re-exposure after learning impacts memory retrieval of appetitive memories in Drosophila melanogaster. Flies form long lasting sugar memories after a single trial of differential conditioning where an unpaired odor is presented alone (unpaired odor, conditioned stimulus-, CS-) followed by the pairing of a second odor (paired odor, conditioned stimulus+, CS+) paired with sugar reward (unconditioned stimulus, US). When tested immediately or a day later flies approach the paired odor in a forced choice test against the unpaired odor (Figure 3, Supplemental figure 1A). To explore the effects of reward re-exposure we re-exposed flies to dried sugar for 5 min in starvation vials 3 h after training. In a parallel control group, flies were handled equally but not exposed to dried sugar. Indeed, when tested at 3 h after the sugar re-exposure, appetitive memory was diminished compared to a handling control (Figure 4A). This decrease in expression of reward memory is persistent and does not recover after 24 h (Figure 4B). Interestingly, exposing flies to dried sugar 3 h or 6 h before training did not change 6 h memory performance compared to a handling control (Figure 4C, Supplemental figure 1B). Given that memory strength is assessed as relative choice between the paired and unpaired odor after the sugar re-exposure, a diminished score can be explained by changes in the value of the unpaired odor. To test for such generalization, we repeated the re-exposure experiment (re-exposure at 3 h), but in the test we provided a choice between the paired odor versus a novel odor. Compared to the handling control, the learned approach to the paired odor is reduced, demonstrating that the deficit in memory retrieval is due to a change in preference to the paired odor (Figure 4D). Thus, these results show that experiencing the sugar reward without the trained odor after the training devalues the sugar-odor association memory and reduces the expression of the memory.

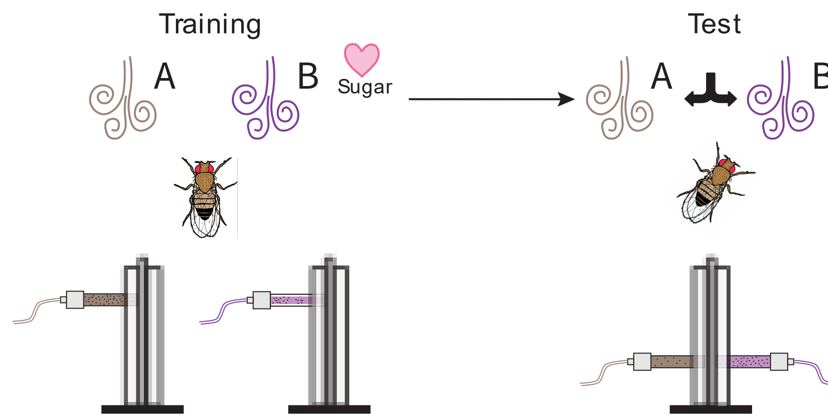


Figure 3: Appetitive olfactory learning scheme. During training (left side) groups of starved flies are exposed to one unpaired odor (A) followed by an odor paired with a sugar reward (B) in the upper part of the T-maze. During the test (right side) flies are exposed to a forced choice between both odors in the two arms of the lower part of the T-maze. After successful training the majority of flies will choose odor B in the test phase.

4.2 Reward re-exposure devalues reward memory independent of memory consolidation.

Memories can be distinguished into different phases. For example, a short-term memory phase that drives learned behavior in the first minutes to hours after learning and long-term memory that allows retrieval even after days. Previous experiments have shown that in flies long-lasting sugar memories need to be stabilized. This consolidation phase requires coordinated neuronal activity as well as molecular processes including protein synthesis in defined neurons within the first hours after learning (104). Before being consolidated memories are vulnerable and can be lost by disruptions, such as new learning, sleep deprivation, or pharmacological interventions (11, 15, 195, 196). Thus, we tested whether sugar re-exposure can interfere with memory when given outside of the consolidation window. Prior studies have shown that memories are stabilized within 90 min or 2 h after training but it is commonly agreed that memories are fully consolidated 24 h after training.(104, 131). Therefore, we re-exposed flies to sugar 24 h after training, when memory was clearly stabilized and tested 3 h later (Figure 4B). Compared to the handling control, learned approach is significantly reduced. These experiments suggest that olfactory reward memories are diminished independent of the timepoint of the re-exposure.

The expression of reward memories at different timepoints after training depends on specific subpopulations of KCs (116, 117). Short- and mid-term memories dominate minutes to hours after learning, while long-term memories influence behavior days later. These memory types appear to be established in parallel by distinct dopamine pathways (138, 139). Previous work has shown that activating DANs artificially can diminish subsequent memory expression (20, 127, 132, 156). To confirm these findings, we expressed the *dTrpA1*-encoded transient receptor potential (dTRPA1) channel in most DANs from the PAM cluster (R58E02-GAL4). Raising the ambient temperature above 25°C activates the temperature dependent dTRPA1

and therefore depolarizes neurons that express this channel (98). In our experiments, activating dopamine neurons for 30 min at 3 h after sugar training diminished learned approach behavior in the test (Figure 4H). Importantly, *dTRPA1* expressing flies show the same sugar learning scores at 6 h as controls (Supplemental figure 1C). Further, exposing wild type flies to heat at 3 h after training leaves reward memory expression unaltered (Supplemental figure 1D).

Artificial training with thermo- or optogenetic activation of specific reward DANs substituting for the sugar presentation during training leads to appetitive memory formation. Depending on which subsets of dopamine neurons are activated the dynamics of memory formation are different (138). We confirm these findings by showing that activating short-term memory DANs (STM-DANs, R48B04-GAL4, labeling DANs projecting to β’2, γ1, γ2, γ4, γ5) during training leads to memories that decay within the first hours, whereas artificial training with long-term DANs (LTM-DANs, R15A04-GAL4, labeling DANs projecting to β2, α1, β’1, γ5 and the pedunculus) exclusively form a long-term memory (Supplemental figure 1E &F). Given that these separable dopamine pathways are related to STM or LTM we tested whether their activation after sugar learning can affect specific memory phases. Flies expressing *dTRPA1* in STM-DANs (R48B04-GAL4), LTM-DANs (R15A04-GAL4) or in both (R58E02-GAL4) were sugar trained and tested 27 h later. In independent experiments, flies were shifted to >25°C for 30 min either 15 min or 24 h after training. Interestingly, we find that compared to a handling control, activating LTM-DANs (R15A04-GAL4) or LTM-DANs together with STM-DANs (R58E02-GAL4) abolishes retrieval of 27 h memory (Figure 4E &F). In contrast, activating STM-DANs (R48B04-GAL4) at these timepoints leaves the memory unaltered (Figure 4G). However, when flies were trained and tested at 1 h after learning and STM-DANs are activated 15 min after the training, learned approach is diminished (Figure 4G). Interestingly, activating only LTM-DANs (R15A04-GAL4) shows a similar effect (Figure 4F). These results suggest that the long-term component of sugar memory is only sensitive to interference from LTM-DANs but not STM DANs, whereas for 1 h memory we do not observe such specificity. However, artificial training with STM and LTM DANs can induce a partial 6 h memory (Figure 2G, Supplemental figure 1E &F). In 6 h memory experiments, activation of STM- or LTM-DANs alone at 3 h leaves the reward memory unaltered (Figure 4I). However, activation of both STM- and LTM-DANs together at 3 h diminishes 6 h memory retrieval (Figure 4H). To test whether more refined subsets of dopamine neurons can diminish 6h memory we screened candidate neurons for their capacity to interfere with 6 h memory However, neither 3 h activation of subsets of PAM DANs nor the activation of punishment coding DANs from the PPL1 cluster were potent to reduce 6 h memory (Supplemental figure 1H, for labeled DAN subsets see supplemental table 1). Increased memory retrieval after activation of neurons labeled by MB025B-GAL4, MB301B-GAL4 and MB056B-GAL4 are likely caused by the genetic

background of the flies, since 6 h memory controls show elevated memories in these flies (Supplemental figure 1I & J).

Together our data suggest that sugar re-exposure after training can devalue sugar related memories independent of memory phases. However, memories in different time phases seem to be vulnerable to the activation of distinct DANs, which drive behavior through independent circuit motifs.

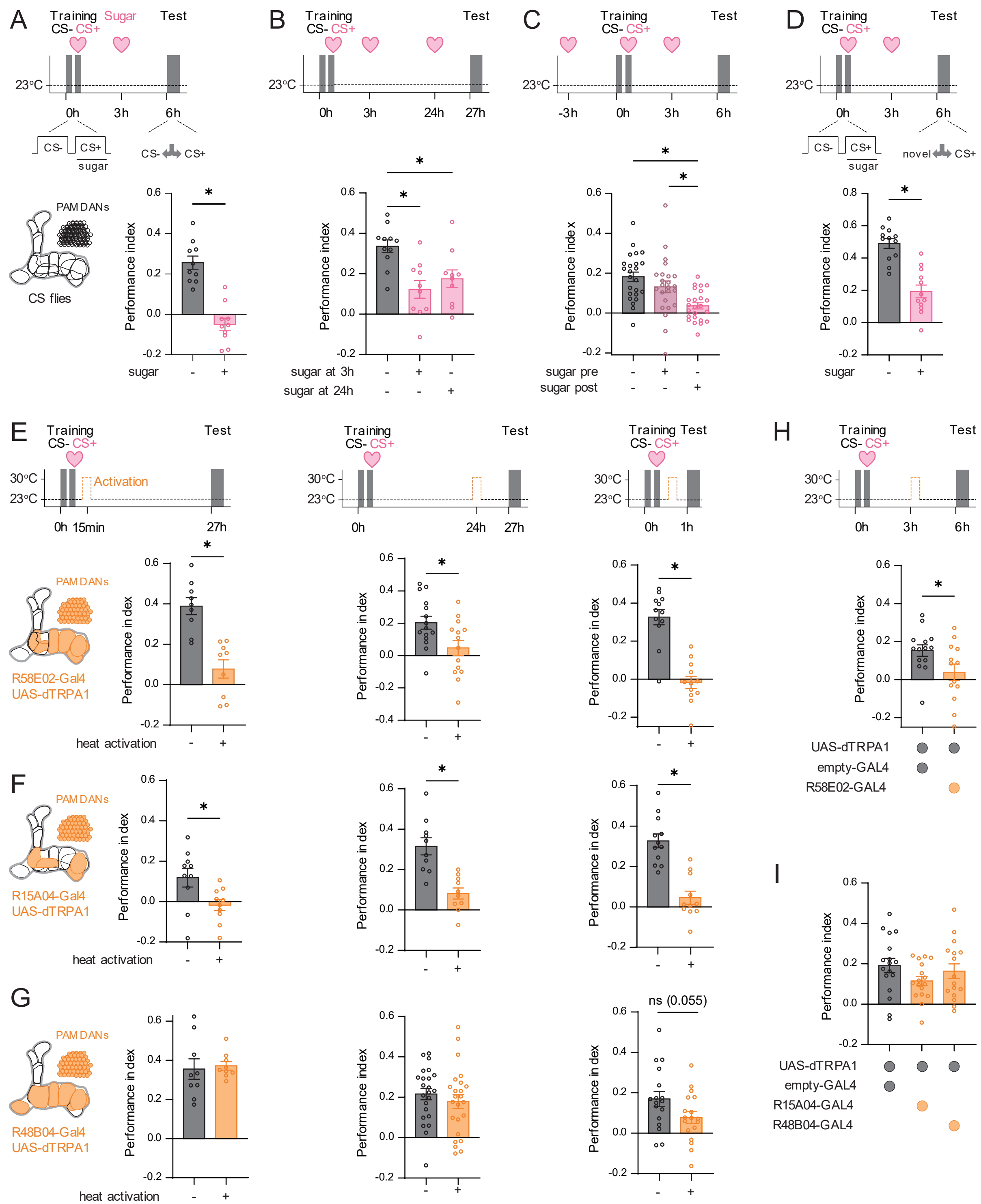


Figure 4: Reward re-exposure diminishes expression of olfactory reward memory. Re-exposing flies to the unpaired sugar reward for 5 min at 3 h after training leads to reduction of memory performance at 6 h (n ≥ 10) (A). Re-exposing flies to the sugar 3 h or 24 h after training leads to diminished memory retrieval in a 27 h test (n ≥ 10) (B). Exposing flies to sugar 3 h before training does not affect 6 h memory (n ≥ 25) (C). In a test against a novel odor approach to the CS+ is reduced after sugar re-exposure (n ≥ 12) (D). Artificially activating PAM DANs (R58E02-GAL4) for 30 min at 15 min or 24 h after sugar training diminishes 27 h memory. Similarly, activating PAM DANs 15 min after training reduces 1 h memory performance (n ≥ 9) (E). Activating LTM DANs labeled by R15A04-GAL4, 15 min or 24 h after sugar training, lowers 27 h memory. Similarly, activating LTM DANs 15 min after training diminishes 1 h memory retrieval (n ≥ 10) (F). Activating STM DANs (R48B04-GAL4) 15 min or 24 h after sugar training leaves 27 h memory unaltered. Activating STM DANs 15 min after training leads to a strong trend towards memory reduction in a 1 h test (n ≥ 9) (G). Activating PAM DANs 3 h after sugar training reduces 6 h memory retrieval (n ≥ 12) (H). When LTM or STM DANs are activated 3 h after sugar training, memory retrieval at 6 h stays intact (n ≥ 16) (I). The heart icon in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences (P < 0.05, t-test or ANOVA)

4.3 Activity from α1 PAM DANs after learning diminishes aversive memory.

In contrast to appetitive memories, single-trial aversive memories in flies don't get consolidated and fade over time. This decay, known as active forgetting, is mediated by DANs from the PPL1-cluster and β'1 PAM DANs (132, 197). Thus, we tested whether activating β'1 DANs diminishes aversive memory retrieval in our hands. For aversive conditioning we exposed starved flies to one odor (CS+) paired with electric shock, followed by an unpaired second odor (CS-). However, when we activated β'1 PAM DANs (MB025B-GAL4) 1.5 h after single-trial aversive training, 3 h memory was unaltered (Supplemental figure 1K). It is worth noting that the split GAL4 line we used here differs from that used in the study by Shuai *et al*., which can explain the discordant results. In our screen on appetitive memory, we didn't find a specific subset of DANs mediating the reduction of reward memory at 3 h. Nevertheless, we saw a trend towards memory reduction when activating α1 PAM DANs (Supplemental figure 1H). The α1 compartment is known for its role in consolidation and rigid memories (118, 154). Therefore, we tested whether activation of α1 PAM DANs would affect aversive STM. Indeed, when we activated α1 PAM DANs 1.5 h after aversive training, 3 h memory retrieval was abolished (Supplemental figure 1K). Therefore, we find that under our conditions α1 PAM DANs but not β'1 PAM DANs play a role in forgetting of aversive memories. Further, our experimental data suggest that α1 PAM DANs play a more general role in stability of memories not limited to appetitive memories.

4.4 Specificity of memory devaluation.

In line with our findings on sugar re-exposure induced memory devaluation, artificial training experiments in flies have shown that reactivating DANs used during training abolishes aversive and appetitive memories (132, 154).

Thus, we next tested whether devaluation by sugar re-exposure is reward specific. Flies were trained as previously using dry sucrose as a reward. At 3 h after training flies were re-exposed to either dried sugar or to standard food. Testing the memory at 6 h after training revealed that sugar-exposure reduces learned approach, whereas exposure to standard fly food shows normal appetitive memory retrieval (Figure 5A). However, re-exposing flies to dry arabinose, a sweet but non-nutritious sugar, at 3 h after sucrose training diminishes learned approach similar to sucrose re-exposure (Figure 5B). Therefore, it seems that reward-memory devaluation is to a certain degree reward-specific. Importantly, these experiments show that sugar-exposure induced changes in hunger level don't effect the memory performance in the test.

To further explore the link between learning and devaluation we switched to artificial training. First, we confirmed previous findings that reactivating specific DANs that have the capacity to

write an artificial memory diminishes learned behavior (154). In artificial training the sugar presentation is substituted by shifting flies to high 32°C temperature to activate DANs while presenting paired odor. To reactivate dopamine neurons flies were shifted to 32°C for either 1 min, 2 min or 30 min at 3 h after training (Figure 5C). Testing flies 6 h after training showed that reactivating PAM neurons reduces 6 h artificial memory expression. Though all treatments reduce memory retrieval, the strength of the phenomenon seems to depend on the reactivation length. Thus, we used 30 min reactivation for all following experiments. Importantly, Handling *per se* did not affect memory performance (Supplemental figure 2A). As thermogenetic activation, optogenetic activation induces an appetitive memory. Exposing optogenetically trained animals to the heat protocol does not affect learned approach confirming that high temperature does not diminish memory expression *per se* (Supplemental figure 2B). Thermogenetic reactivation of dopamine neurons either early or 24 h after the artificial training impaired memory expression at 24 h or 27 h, respectively (Figure 5D & E). Further, reactivating DANs for 2 min or 30 min at 3 h reduces memory retrieval when tested immediately after (Figure 5F, Supplemental figure 2C). Activating PAM DANs 3 h before the artificial training does not change 6 h memory performance, suggesting that, similar to the sugar pre-exposure, it is the post-training reactivation that reduces memory retrieval (Supplemental figure 2D). Finally, we tested whether our observations apply generally to memories reinforced with different dopamine subsets. To do so, we first assessed which subsets could implement a 6 h memory (Supplemental figure 2E, for labeled DAN subsets see supplemental table 1). Aside from R58E02-GAL4, 0273-GAL4 labels all PAM DANs and is known to implement reward memories when activated during training (198). Accordingly, we observe strong artificial memory scores at 6 h with this line. Additionally, 0279-GAL4 (β1 and β2 PAM DANs), MB043C-GAL4 (α1 PAM DANs) and MB315C (γ5 PAM DANs) can be used to train a 6 h memory (Supplemental figure 2E). We also observe 6 h memory performance when training with MB194B-GAL4 and R56H09-GAL4, both of which label multiple DAN subsets memory (Supplemental figure 2E). Notably, these lines include α1 PAM DANs which solely can drive appetitive memory formation. Among PPL1 DANs activating PPL1-γ1pedc (MB438B-GAL4) and PPL1-α3 (MB630B-GAL4) during training leads to an aversive 6 h memory.

In the MB network, MBONs downstream of KCs and DANs can drive either approach or avoidance behavior. Coincident activity of KCs and DANs during learning has been shown to lead to a depression of Ca^{2+} responses in the respective MBON (120, 121). Thus, MBONs downstream of DANs that implement an appetitive memory are expected to convey avoidance behavior. However, most insights come from stimulus free assays looking at self-activation behaviors (122). Understanding of MBON involvement in odor driven behaviors is incomplete. γ5, β'1, α1, and β1/β2 PAMs implement appetitive memory in our screen (Supplemental figure 2E). Yet, in the study by Aso *et al.* flies do not show consistent avoidance of activation of

downstream MBONs. Owald *et al*. demonstrated that inhibiting output from MBONs receiving input in the γ5 and β’2 compartments reverses innate odor avoidance (121). Further, odor-evoked activity in these neurons is depressed following appetitive conditioning. Both findings support our result that γ5 PAM DANs reinforce reward. Nevertheless, whether MBONs receiving input from α1 and β1/ β2 PAM DANs are involved approach or avoidance behavior is unknown. We conducted odor avoidance assays to test the capacity of MBONs to steer odor driven behaviors (Supplemental figure 2F, for MBONs labeled by driver lines see supplemental table 1). At high concentrations 3-octanol is aversive to flies. When given the choice between 3-octanol and air, wild type flies avoid the odor. Blocking output from avoidance coding MBONs these compartments should therefore lead to a reduction in odor avoidance. In accordance with published results, we observe diminished odor avoidance when restricting output from MBON-γ5β’2β in conjunction with MBON-β’2mp (MB210B-GAL4) (121). Similarly, blocking MBON-α1 (MB310C-GAL4) and MBON-β1>α(MB434B-GAL4) leads to a trend towards reduced odor avoidance (Supplemental figure 2F). Thus, these MBONs seem to convey avoidance behavior. Together with our artificial training results, these findings strengthen the notion that α1, β1/ β2 and γ5 PAM DANs can implement appetitive memories during learning. Additionally, we find, that blocking output from MBON-γ3 with MBON-γ3β’1 (MB083C-GAL4), MBON-α3 with MBON-α’2 (MB082C) and MBON-β’2d (SS95105-GAL4) leads to reduced odor avoidance (Supplemental figure 2F). Interestingly the α3 compartment is innervated by PPL1 DANs with aversive reinforcement potential. MBONs downstream of punishment-reinforcing DANs are expected to drive approach behavior. Blocking of these MBONs should therefore result in increased or at least unaltered odor avoidance. The result that MBON-α3 contributes to odor avoidance points towards more complex interrelationships in the network.

Of the artificial memories implemented by neurons labeled by different driver lines, we tested five lines for reduction after artificial activation of the same DANs (plus repetition of R58E02-GAL4). 3 h reactivation of all but two subsets diminishes learned approach tested at 6 h (Figure 5G).

The tested DAN subsets included the STM-DANs (R48B04-GAL4) and LTM-DANs (R15A04-GAL4) labeling GAL4 driver lines. Activating both DAN subsets during training in independent experiments produced a 6 h appetitive memory. However, reactivating the same DANs at 3 h only led to memory reduction in the case of STM-DANs (Figure 5G). To test for memory phase specific effects, we also assessed the impact of reactivation 15 min after training. Artificial 1 h memory implemented by STM-DANs was significantly reduced after reactivation (Figure 5J). In line with activating LTM-DANs after sugar learning, reactivating LTM-DANs 3 h after artificial training involving the same neurons did not significantly alter 6 h memory. Yet, 27 h artificial memory was impaired after reactivation either 15 min or 24 h after training (Figure 5H &I).

These results suggest that in addition to memory type specific effects, in the early phase after learning the memory is more general sensitive to disruption by DAN activity.

Together, these results confirm previous findings and support the conclusion that reactivation of PAM DANs perturbs consolidated artificial-reward memories independent of the timepoint of reactivation and leads to a permanent loss of learned approach behavior.

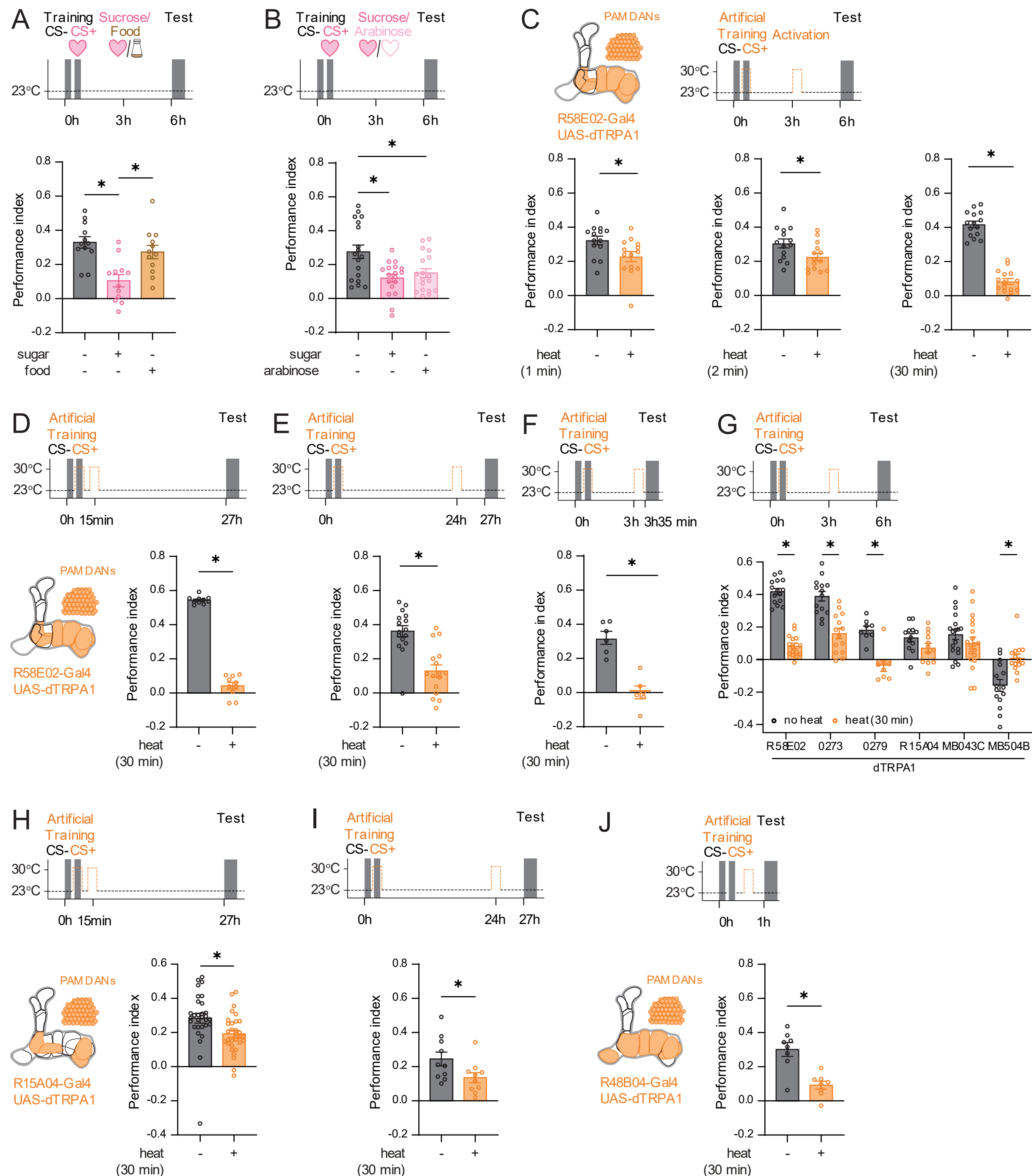


Figure 5: Specificity of memory devaluation. While re-exposing flies to sugar for 5 min at 3 h after training leads to memory devaluation, feeding flies regular fly food instead does not affect memory performance at 6 h (n ≥ 11) (A). Similarly to re-exposing flies to sucrose, providing them with arabinose (a sweet-only sugar) 3 h after training leads to reduction of memory performance at 6 h (n ≥ 17) (B). Artificially activating PAM DANs for 1 min, 2 min or 30 min at 3 h after artificial training (dTRPA1 expression under R58E02-GAL4) diminishes memory retrieval in a 6 h test (n ≥ 14) (C). There seems to be a link between length of activation and the strength of the phenotype. Therefore, 30 min reactivation was used in subsequent experiments. Artificial reactivation of R58E02-labeled neurons to 15 min or 24 h after artificial training (using the same line) diminishes 27 h memory retrieval (n ≥ 10) (D & E). Memory is gone immediately (3 h 35 min test) after reactivation at 3 h after training (n ≥ 6) (F). When reactivating the same set of PAM DANs used during artificial training 3 h later, 6 h memory performance is diminished for four lines (R58E02-GAL4, 0273-GAL4, 0279-GAL4, MB504B-GAL4) out of 6 lines tested. When the used lines include α1 PAM DANs (R15a04-GAL4, MB043C-GAL4) memory is maintained (n ≥ 8) (G). Activating LTM DANs labeled by R15A04-GAL4 15 min or 24 h after artificial training with neurons labeled by the same line reduces 27 h memory (n ≥ 10) (H & I). Activating STM DANs (R48B04-GAL4) 15 min after R48B04 artificial training diminishes 1 h memory (n ≥ 8) (J). The filled heart icon in the timelines depicts sucrose exposure, the empty heart icon depicts arabinose exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA)

To explore the interplay of different reinforcement circuits across training and reactivation we combined existing tools to generate flies expressing the thermogenetic activatable cation channel dTRPA1 under UAS control and additionally the light-activatable CsChrimson under lexAOP control (97, 98). Using these flies, one set of DANs can be used to establish an artificial memory, whereas a second set of DANs can be used to later reactivate a different set of neurons in a controlled manner (Figure 6A). First, we used R58E02-GAL4 and -LexA to express *dTRPA1* and *CsChrimson*. Temperature and light activation in these flies can write a memory. Reactivation with the respective other tool, light or temperature, diminishes the reward memory (Figure 6B &C). In the next step we tested whether writing a memory with R58E02-DANs is affected by R15A04-LTM DANs. However, the activation of R15A04-DANs at 3 h and test at 6 h, or reactivation at 15 min or 24 h hours with test at 27 h leaves the R58E02-DAN memory unaltered (Figure 6D&E). Similarly, activation of R48B04-DANs 3 h after training doesn't affect 6 h R58E02-DAN memory (Figure 6F). Conversely, R15A04 and R48B04-DAN memories are both diminished by R58E02-DAN reactivation (Figure 6G&H). To exclude effects from the used tool, we repeated training with R58E02-GAL4 labeled DANs and activation of R15A04-DANs using the respective other tool for training and subsequent activation. This experiment revealed the same result as before (Supplemental figure 3). Thus, rather than erasing specific memory phases, larger subsets of dopamine neurons seem to affect memories written by smaller subsets. To further test this hypothesis, we trained with 0273 GAL4 labeled PAM DANs (120-130 neurons) and reactivated R58E02-DANs (90 neurons) (3, 13, 16). Reactivating R58E02-lexA labeled cells following training reduces approach behavior slightly but not significantly compared to the control (Figure 6I). However, in the inverse experiment, training with R58E02-lexA labeled cells and reactivating the larger 0273-DAN cluster, the memory is completely abolished (Figure 6J). Thus, it seems that memory performance is diminished when the unpaired activation includes at least the same or more neurons than those involved during training for the respective memory phase. This idea goes in line with the result that none of the PAM clusters can individually reduce sugar memory when activated after training (Supplemental figure 1). Interestingly, when we expose flies to real-world sugar after training with R58E02-GAL4 labeled DANs, the memory is unaltered, suggesting that R58E02-GAL4 activation and sugar induced activity are not matching (Figure 6K).

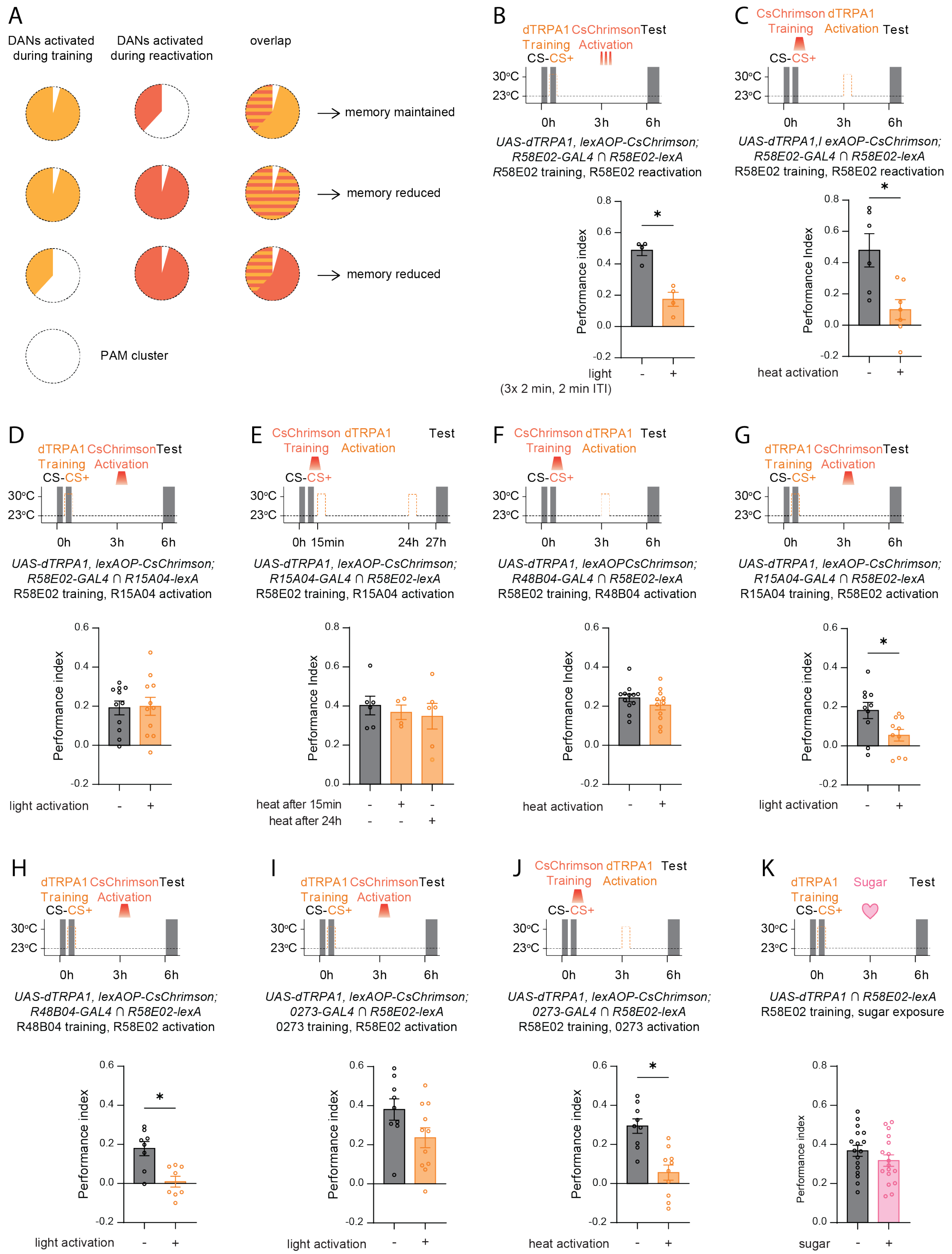

A
DANs activated during training
DANs activated during reactivation
overlap
memory maintained
memory reduced
memory reduced
PAM cluster
B
dTRPA1 Training
CsChrimson Activation
Test
UAS-dTRPA1, lexAOP-CsChrimson; R58E02-GAL4 ∩ R58E02-lexA
R58E02 training, R58E02 reactivation
light (3x 2 min, 2 min ITI)
C
CsChrimson Training
dTRPA1 Activation
Test
UAS-dTRPA1,l exAOP-CsChrimson; R58E02-GAL4 ∩ R58E02-lexA
R58E02 training, R58E02 reactivation
heat activation
D
UAS-dTRPA1, lexAOP-CsChrimson; R58E02-GAL4 ∩ R15A04-lexA
R58E02 training, R15A04 activation
light activation
E
UAS-dTRPA1, lexAOP-CsChrimson; R15A04-GAL4 ∩ R58E02-lexA
R58E02 training, R15A04 activation
heat after 15min
heat after 24h
F
UAS-dTRPA1, lexAOPCsChrimson; R48B04-GAL4 ∩ R58E02-lexA
R58E02 training, R48B04 activation
heat activation
G
UAS-dTRPA1, lexAOP-CsChrimson; R15A04-GAL4 ∩ R58E02-lexA
R15A04 training, R58E02 activation
light activation
H
UAS-dTRPA1, lexAOP-CsChrimson; R48B04-GAL4 ∩ R58E02-lexA
R48B04 training, R58E02 activation
light activation
I
UAS-dTRPA1, lexAOP-CsChrimson; 0273-GAL4 ∩ R58E02-lexA
0273 training, R58E02 activation
light activation
J
UAS-dTRPA1, lexAOP-CsChrimson; 0273-GAL4 ∩ R58E02-lexA
R58E02 training, 0273 activation
heat activation
K
dTRPA1 Training
Sugar
Test
UAS-dTRPA1 ∩ R58E02-lexA
R58E02 training, sugar exposure
sugar
Performance index

Figure 6: Reactivation of the same or more neurons than activated during training leads to memory devaluation. Schematic illustration of the paradigm, the full circle depicts the entire PAM DAN cluster (A). Driving the expression of the thermos-sensitive *dTRPA1* and the light-sensitive *CsChrimson* under UAS or lexAOP control, with different driver lines (GAL4 or lexA) allows to train with one subset of neurons (indicated in orange) and activate a different (indicated in red) set later. Activating the same set or more PAM DANs after training leads to memory reduction. On the contrary, activating a smaller subset than involved in training leaves the memory unaffected. When expressing *dTRAP1* and *CsChrimson* both in R58E02 labeled neurons the memory is reduced when training is done thermogenetically and reactivation optogenetically (n ≥ 4) (B) or the other way around (n ≥ 6) (C). In (C) optogenetic activation consisted of 3 trials, 2 min activation each (634 nm, 500 Hz, 19.5 lm/W) with an inter trial interval (ITI) of 2 min. In all other experiments optogenetic and thermogenetic activation after training lasted for 30 min. Activating R15A04-lexA labeled neurons (LTM DANs) 3 h after training with R58E02-GAL4 labeled neurons leaves 6 h memory unchanged (n ≥ 11) (D). Likewise activating LTM DANs 15 min or 24 h after R58E02 training does not affect memory retrieval at a 27 h timepoint (n ≥ 6) (E). Further, activating R48B04-GAl4 labeled neurons (n ≥ 11) (STM DANs) 3 h after R58E02 training leaves 6 h memory performance intact (n ≥ 10) (F). Both R15A04 and R48B04 memories at 6 h are reduced if R58E02-lexA labeled lines are reactivated 3 h after training (n ≥ 8) (G & H). Though activating R58E02-lexA labeled neurons after training with the larger DAN subset labeled by 0273-GAL4 leads to a tendency towards memory reduction at 6 h, this trend is not significant (n ≥ 10) (I). Activating 0273-GAL4 labeled neurons 3 h after training, leads to diminished R58E02 memory at 6 h (J). Exposing flies to sugar for 5 min at 3 h after artificial training with R58E02-GAL4 labeled neurons leaves 6 h memory unchanged (n ≥ 16) (K). In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA)

4.5 Sugar memory re-evaluation and memory reduction by PAM DAN activity are two distinct mechanisms.

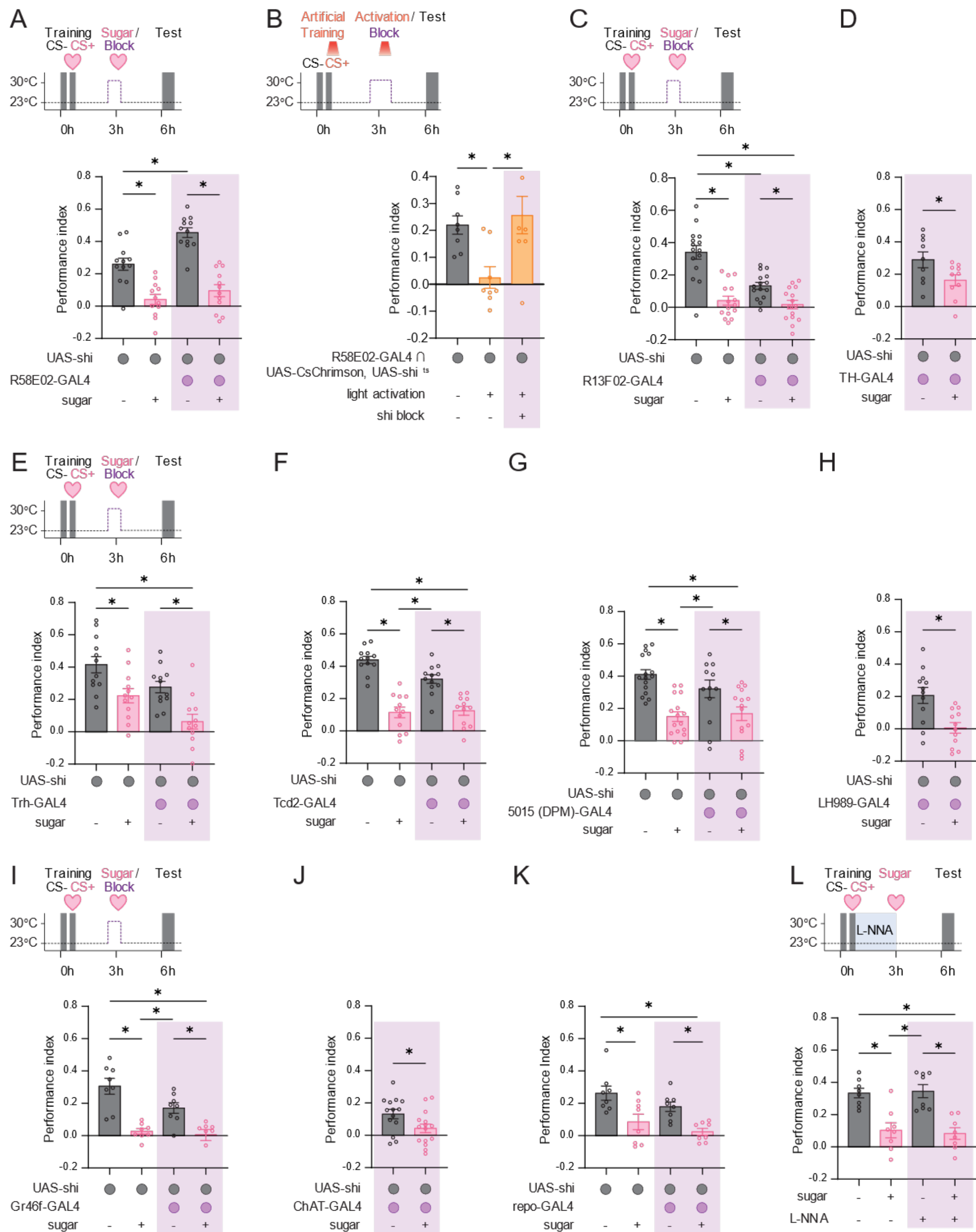
PAM DANs signal reward-related information to the mushroom body system. Further our experiments show that the reactivation of PAM DANs after sugar learning leads to sugar-reward memory reduction. Therefore, we next tested whether PAM DANs are necessary for the sugar-exposure induced memory devaluation. To interfere with neuronal function, we expressed the temperature-sensitive dynamin mutant *shibire* (*shi^ts^*) in PAM DAN neurons labeled by R58E02-GAL4 (99). Shifting flies to restrictive high temperature reversibly interferes with presynaptic small vesicle release (for simplicity reasons referred to as “blocking” in the rest of this thesis). Flies expressing *shi^ts^* in PAM DANs were trained in permissive low temperature and were shifted to high temperature before and during the sugar re-exposure. Maybe surprisingly, testing flies at the permissive temperature revealed that blocking PAM DANs leaves sugar induced memory devaluation unchanged (Figure 7A). Flies can sense the nutritious value of sugar after feeding (199). To exclude prolonged reward signaling during digestion, we prolonged blocking of PAM DANs until 30 min after sugar re-exposure and obtained the same result as with shorter blocking (Supplemental figure 4A). Further blocking PAM DANs with an even broader driver line (0273-GAL4) confirms that blocking vesicle release from PAM DANs does not impact memory devaluation (Supplemental figure 4B). Considering heterogeneity of PAM DANs, different subpopulations could exert antagonizing effects. Thus, blocking all PAM DANs could lead in sum to no change. The α1 compartment seems to store particular stable memories, aversive memory retrieval is reduced and appetitive memory expression shows a tendency of reduction when activating PAM-α1 after training (154) (Supplemental figure 1H &K). Therefore, we blocked these DANs specifically during sugar re-exposure. However, memory devaluation was not reversed when blocking α1 PAM DANs (Supplemental figure 4C). Since activation of PAM DANs after training diminishes

memory performance and *shi*^{ts} does leave activity within the neuron intact, we asked whether memory reduction through artificial activation is mediated by *shi*^{ts} independent PAM DAN activity. Hence, we co-expressed the light inducible cation channel CsChrimson together with *shi*^{ts} in PAM DANs labeled by R58E02-GAL4. When we reactivated PAM after artificial training, learned avoidance was diminished (Figure 7B). However, when PAM DAN neurons were reactivated at the restrictive temperature, the memory stayed intact (Figure 7B). This result demonstrates that reduction mediated by activation of PAM DANs depends on *shi*^{ts} dependent signaling. In conclusion, despite the similar consequences on learned behavior the reduction of memory by artificial DAN activation or by sugar re-exposure are different mechanisms. Interestingly, blocking R58E02-labeled neurons in handling controls increases memory performance at 6 h (Figure 7A). Thus, though PAM DANs seem not to contribute to re-exposure induced devaluation their activity seems to bidirectionally control memory strength: PAM DAN activation diminishes memory expression while blocking baseline activity of PAM neurons increases expression of learned approach.

Our experiments demonstrate that blocking vesicle release from PAM DANs with *shi*^{ts} does not affect re-exposure induced memory devaluation. Thus, either *shi*^{ts} independent PAM DAN signaling or other pathways are mediating memory devaluation. To test the latter, we set out to check different neuronal groups that are known to be part of the MB network. Namely: PPL1 DANs and other dopaminergic neurons of the fly brain (so called PPL2-, PD1-, and PPM1,2&3-DANs) (MB504B-GAL4, TH-GAL4), Kenyon Cells themselves (R13F02-GAL4), octopaminergic neurons (Tdc2-GAL4), serotonergic neurons (Trh-GAL4) and DPM neurons (5015-GAL4) (Figure 7C-G). Of note, solely blocking KC reduces memory performance in the test, suggesting that output from KC at 3 h is involved in memory maintenance (Figure 7C). One explanation could be a slightly prolonged consolidation window (over the expected 1.5 – 2 h) since output from α'/β' KCs is known to be crucial during the consolidation phase (104). Genetic controls showed the same 6 h memory scores as the experimental group (Supplemental figure 4D). We also tested involvement of Lateral horn output neurons PD2a1/b1 (LH989-GAL4) which have been shown to be involved in aversive memory retrieval and are predicted to connect to neurons from the PPL1/ and PAM cluster (101, 193, 200) (Figure 7H). Further we probed a large number of the peripheral sweet sensing neurons targeted by Gr64f-GAL4 that are necessary for sugar preference (201) (Figure 7I). Last we expressed *shi*^{ts} using a driver line broadly labeling cholinergic neurons (ChAT-GAL4) (Figure 7J). Overall, none of the experiments resulted in maintenance of the memory despite sugar re-exposure. (Figure 7C-J and Supplemental figure 4A-C).

As reported in Aso *et al*. 2019 subclasses of DANs express nitric oxide synthase (NOS) and produce nitric oxide (NO) as a neurotransmitter upon learning (166). Plasticity induced by NO co-transmission onto KCs leads to the formation of a memory of opposing valence to the

memory established by dopamine. Thus, blocking NO signaling increases aversive memory expression. We therefore tested whether NO is involved in sugar-mediated re-evaluation of the appetitive memory. As previously reported by Aso *et al.*, we used L-NNA to pharmacologically block NOS (166). First, we tested whether blocking NO-signaling increases reward learning as suggested. In a first approach we fed flies with food containing 100 mM L-NNA for 24 h prior to the starvation that precedes reward training. Indeed, when comparing flies fed on L-NNA to the controls in a 6 h memory test, they show an enhanced reward memory expression (Supplemental figure 4E). Nevertheless, sugar re-exposure 3 h after training led to reduction of memory in L-NNA fed and control flies (Supplemental figure 4E). These experiments demonstrate that blocking NO signaling 24 h before training increases reward learning but leaves re-exposure-driven memory devaluation intact. Next, we tested the effectiveness of L-NNA-application for shorter time phases. We placed flies into vials containing a filter-paper soaked in saturated L-NNA solution, during the starvation period at 3 h before the training. Again, compared to the controls, these flies showed enhanced 6 h memory performance (Supplemental figure 4F). Consequently, we tested whether interfering with NO-signaling directly before sugar re-exposure affects memory devaluation. Flies were trained and presented with L-NNA immediately afterwards until the sugar re-exposure at 3 h. In the 6 h test, there was no difference in memory reduction between the two groups (Figure 7L). Thus, NO signaling is not involved in memory devaluation upon sugar re-exposure. Of note, memory expression was not enhanced when flies only received L-NNA after learning, showing that NO specifically affects learning and not consolidation or retrieval (Supplemental figure 4E &F).



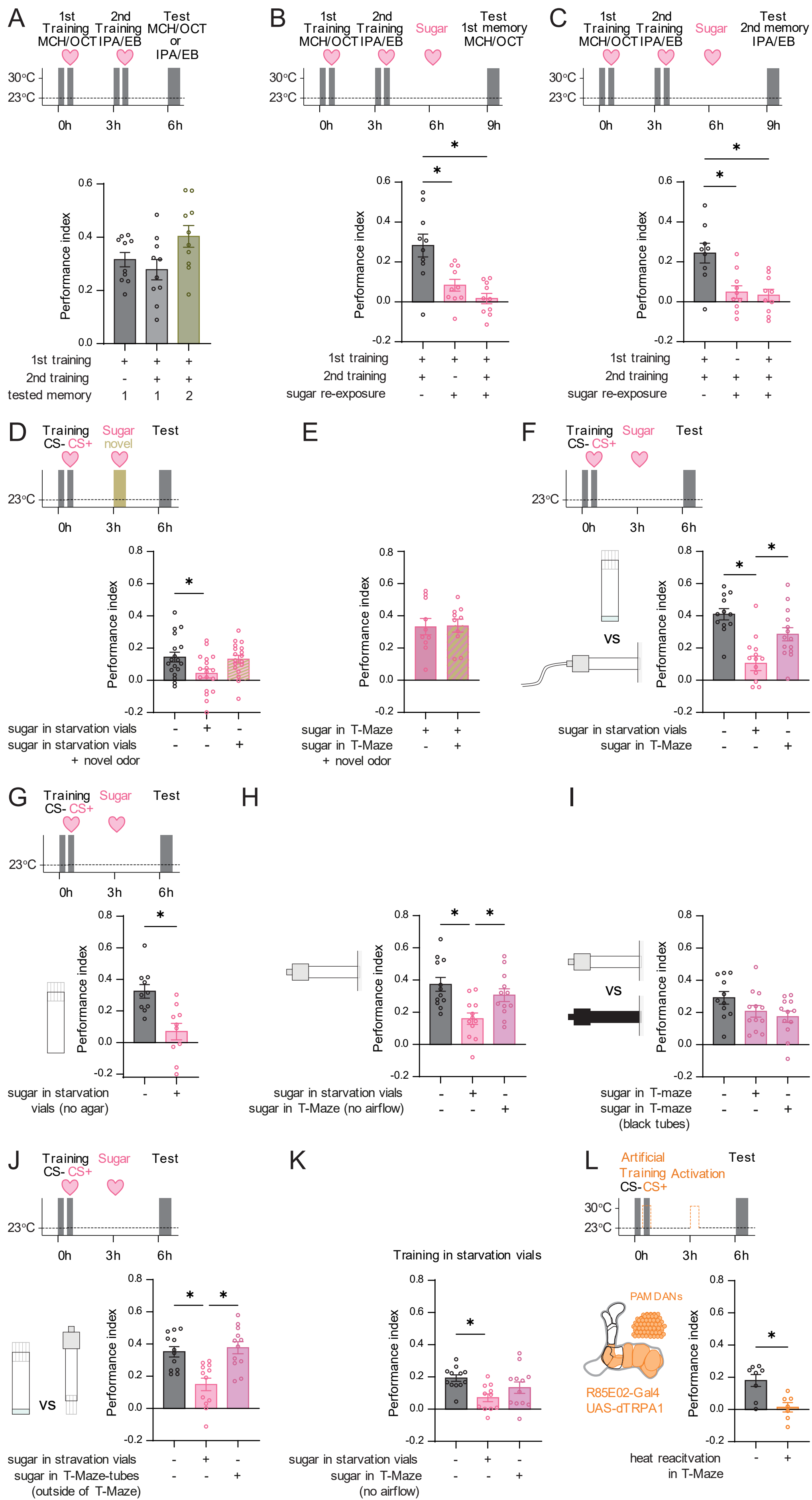
<u>Figure 7</u>:Sugar memory re-evaluation is independent from PAM DAN small vesicle release. When blocking R58E02-GAL4 labeled neurons (using *shi*^{ts}) during sugar re-exposure, sugar-mediated memory devaluation still occurs (n ≥ 12) (A). Only blocking PAM DANs for 35 min at 3 h after training enhances memory expression at 6 h (n ≥ 12) (A). When output from PAM DANs is restricted while artificially reactivating neurons at 3 h, DAN activity-mediated memory reduction is impaired (n ≥ 6) (B). Blocking none of the tested neuronal population during sugar re-exposure sugar-mediated memory devaluation (C-K). Candidates tested: KCs (R13F02-GAL4) (n ≥ 15) (C), PPL1-, PPL2-, PAL-, PPM1-3-DANs (TH-GAL4) (n ≥ 10) (D), serotonergic neurons (Trh-GAL4) (n ≥ 11) (E), octopaminergic neurons (Tcd2-GAL4) (n ≥ 12) (F), pair of DPM-neurons (5015-GAL4) (n ≥ 12) (G), PD2a1/b1 LHONs (LH989-GAL4) (n ≥ 11) (H), sweet gustatory receptor neurons (Gr64f-GAL4) (n ≥ 7) (I), cholinergic neurons (ChAT-GAL4) (n ≥ 14) (J) and glial cells (repo-GAL4) (n ≥ 8) (K). Similarly, Inhibiting NOS activity (L-NNA) between training and sugar re-exposure doesn't impact sugar-mediated memory devaluation (n ≥ 8) (L). The heart symbol in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences (P < 0.05, t-test or ANOVA)

4.6 Contextual control of sugar memory devaluation.

Re-exposure to training related cues, including contextual information, can induce update of learned information. Given that in natural settings more than one cue can be associated with a meaningful event, it is a promising strategy to use reward re-exposure to target all related memories. Therefore, we tested whether sugar re-exposure in flies can affect multiple memories associated with the reward. To implement two distinct memories, flies were trained in two training sessions that were spaced by 3 h. During these sessions, two distinct odor pairs were used in the first and the second training. Testing 3 h after the second training shows that both memories are retrievable (Figure 8A). However, when flies were re-exposed to the sugar 3 h after the second training and tested again 3 h later, both memories are diminished (Figures 8B &C). These experiments show two things; first re-exposure to the reward can devaluate multiple associations and second, that sugar presentation during the second training does not affect the memory established during the first training. The latter is consistent with the general notion that new learning can protect memory update (Figure 8D). However, in the previous experiments sugar re-exposure was conducted in vials similar to those used for starvation and different to tubes used for the T-maze. Thus, to directly compare, we conducted an experiment where flies were trained and re-exposed 3 h later to either the sugar only or the sugar paired to a novel odor within the T-maze setting. Maybe surprisingly, we found that in both groups the learned approach was intact, suggesting that sugar re-exposure in the T-maze does not lead to memory devaluation (Figure 8E). To test whether the context of sugar re-exposure is important we trained flies and either re-exposed them to sugar in starvation vials or within the T-maze. Indeed, flies that received the re-exposure in starvation vials outside the T-maze show reduced memory scores whereas flies re-exposed to the sugar in the T-maze have intact 6 h and 24 h memories (Figure 8F and Supplemental figure 5A). Therefore, it seems reasonable to conclude that sugar re-exposure induced memory devaluation is gated by the context. We conducted several experiments in which we systematically tried to vary components such as light, airflow orientation, and texture of the context in the T-maze as well as in starvation vials (Figure 8G-J, Supplemental figure 5). Nonetheless, whenever sugar re-exposure was conducted in T-maze tubes, sugar memory remained intact. In contrast, when sugar is delivered outside of the T-maze tubes memory seems to be devalued. To test whether this context dependency relates to the meaning assigned to the T-maze environment during training, we trained flies in the vials used for sugar re-exposure. However, we didn’t observe a shift in context dependency. As before, re-exposure to sugar in starvation vials leads to memory devaluation whereas flies exposed to sugar in T-maze tubes show no reduction in memory retrieval (Figure 8K). This data exclude the interpretation that the training context could provide additional information that could protect the memory from being devalued. Nevertheless, starvation and testing context are consistent over the two experiments.

Therefore, it rather seems that sugar memory devaluation is either bound to the starvation context or depends on the context relationship between sugar re-exposure and test.

Interestingly, when we repeated these experiments for artificial memories using R58E02-GAL4 labeled PAMs during training and reactivation, we observe no context dependency (Figure 8 L). In fact, we find that, independent of the setting, when PAM DANs are reactivated artificially memories are diminished. This includes reactivation paired with a novel odor (Supplemental figure 5C). These results further support our conclusion that sugar induced devaluation and DAN-driven memory update are distinct processes.


A
1st Training MCH/OCT
2nd Training IPA/EB
Test MCH/OCT or IPA/EB
30°C
23°C
0h
3h
6h
Performance index
1st training
2nd training
tested memory
B
Sugar
Test 1st memory MCH/OCT
9h
sugar re-exposure
C
Test 2nd memory IPA/EB
D
Training CS- CS+
Sugar novel
Test
sugar in starvation vials
sugar in starvation vials + novel odor
E
sugar in T-Maze
sugar in T-Maze + novel odor
F
VS
sugar in T-Maze
G
sugar in starvation vials (no agar)
H
sugar in T-Maze (no airflow)
I
sugar in T-maze
sugar in T-maze (black tubes)
J
sugar in stravation vials
sugar in T-Maze-tubes (outside of T-Maze)
K
Training in starvation vials
sugar in T-Maze (no airflow)
L
Artificial Training Activation
PAM DANs
R85E02-Gal4
UAS-dTRPA1
heat reacitvation in T-Maze

<u>Figure 8:</u> Sugar-mediated memory devaluation is context dependent. When sugar is trained with two sets of odors (second training 3 h after the first training), flies can retrieve both memories in a test 3 h after the second training ($n \geq 10$) (A). Sugar re-exposure after two sets of training leads to devaluation of both memories ($n \geq 10$) (B & C). New learning (presentation of a novel odor together with sugar during re-exposure) prevents sugar-mediated memory devaluation ($n \geq 16$) (D). Sugar re-exposure in the T-maze leaves the memory intact whether or not a novel odor is present ($n \geq 10$) (E & F). Re-exposing flies to sugar in vials without agar leads to memory devaluation ($n \geq 10$) (G). Memory is maintained if sugar is presented in the T-maze without airflow or in black tubes ($n \geq 10$) (H & I). Re-exposure to sugar in T-maze tubes outside of the T-maze does not initiate devaluation of the memory ($n \geq 12$) (J). When flies are trained in starvation vials, the memory is still devaluated when sugar re-exposure happens in the same vials and maintained if sugar is presented in the T-maze ($n \geq 11$) (K): Artificial reactivation of PAM DANs in the T-maze does lead to diminished memory independent of the context ($n \geq 8$) (L). The heart symbol in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA).

5 Discussion

A crucial aspect of treating disease involving maladaptive memories is to target underpinning associations. To date, many approaches are inefficient and unreliable since underlying principles are not sufficiently understood. In this study we apply a simple learning paradigm using *Drosophila melanogaster* to assess the processes involved in memory updating. We find that re-exposure to the reward after olfactory conditioning devalues previously learned cue-reward associations in a context dependent manner. Similarly, reactivation of reward-reinforcing PAM DANs leads to diminished memory retrieval. Though apparently similar it seems that these phenotypes emerge through two distinct mechanisms.

5.1 Reward-exposure-mediated memory update.

Maladaptive forms of memory can promote harmful behaviors, such as substance abuse. In these cases, it is beneficial to modify the problematic memory. Memories can be changed in active update processes that require exposure to a reminder in conditions that lead to a mismatch from the expected outcome (prediction error). Typically, the non-reinforced presentation of the conditioned stimulus (CS) is used as a reminder cue. This can lead to memory update in one of two ways: extinction or reconsolidation. Larger prediction errors, such as from extended or repeated CS exposure, lead to extinction learning (28). Since the omission of expected reward or punishment is aversive or appetitive, respectively, a new memory of opposing valence to the original memory is learned. Together these memories balance each other out and lead to a weakened behavioral outcome (26, 31). However, the old memory can regain dominance in different ways: the original memory usually resurfaces over time (recovery)(1). Repeated exposure to the US can renew the behavioral response (renewal) or the memory is reinstated by experiencing a context very similar to the original learning situation (reinstatement)(26, 29). These effects are a major challenge in clinical application.

A shorter CS presentation can trigger reconsolidation, during which the memory becomes destabilized and is vulnerable to change (28, 40). While the memory can be retrieved throughout the reconsolidation process, it must be restabilized in a protein synthesis dependent manner to persist over time. Therefore, interfering with the restabilization step, e.g. by blocking protein synthesis, leads to memory erasure (202). Thus, reconsolidation offers a promising alternative to extinction-based approaches in the treatment of maladaptive memory disorders. In both cases, using the CS as a reminder cue has the caveat that only the respective CS-US association is weakened. Other cues associated with the US can still elicit the harmful behavior (56). In contrast, the US is part of the connection to all associated CS. Indeed, it has been shown that using the US as a reminder can initiate reconsolidation of multiple related memories (57). When we exposed flies to the same sugar reward after sugar learning, we find that trained flies show diminished memory retrieval (Figure 4A-D). Though

this finding reminds of an extinction-like phenotype, we don't observe any sign of recovery as a hallmark of extinction (26). Instead, the memory seems to be permanently lost since it doesn't recover after 24 h (Figure 4B). Alternatively, memory retrieval could be permanently suppressed by additional plasticity established in other parts of the circuit. However, it is unclear how and where this additional information could be integrated into the network. During the devaluation session, there is neither an odor cue present nor an omission of reward that would serve as an aversive teaching signal for the animal. Future functional imaging experiments will address this question and reveal whether the initially established plasticity is reversed after sugar re-exposure.

In vertebrates, re-exposure to the US has been shown to initiate reconsolidation of multiple CS-US associations. Interfering with memory restabilization after presenting a US reminder impaired freezing response to two separately trained tones (56). However, if the two tones were paired with different types of shocks (eyelid or foot shock), the presentation of each type of shock could only destabilize the respective memory (56). This experiment showed that there is a specificity regarding the US used in the reminder session. In line with this study, we observed reduced retrieval for two different odors that were independently learned when only the reward was presented again after learning (Figure 8B &D). However, presenting a different food reward (cornstarch based flyfood), does not impact memory retrieval, mirroring US specificity (Figure 5A). In contrast to published studies and perhaps surprising, the reward memory in flies is diminished without further intervention. This phenotype can be explained by the potential of reconsolidation to change memories. Therefore, what we present here appears to be a reconsolidation-like process during which novel experience is integrated in a way that weakens the memory. Exploiting this novel paradigm to investigate the underlying processes can contribute to the understanding of general rules in reconsolidation memory update.

5.2 Separable mechanisms can abolish reward memory

In *Drosophila*, artificial activation of PAM DANs is often used as a substitute for a naturalistic reward experience. Neurons from the PAM cluster have been shown to be crucial in reward learning since blocking vesicle release from PAM DANs can impair the formation of different reward memories such as food, water, or sexual rewards (124, 135, 203, 204). Activating these neurons during conditioning can substitute for a real-world reward and assign positive valence to a cue. In line with the role of DANs in reward reinforcement and in accordance with a recently published study, we find that activation of PAM DANs after sugar training leads to memory loss (155) (Figure 4E &H). Surprisingly, blocking PAM DANs after learning leads to the opposite, an increase in memory performance (Figure 7A). Accordingly, PAM DANs seem to be good candidates to mediate sugar-driven memory devaluation. However, when blocking output from PAM DANs during sugar re-exposure the memory reduction is intact (Figure 7A, Supplemental

figure 4A &B). Thus, in contrast to forgetting through DAN activity, memory devaluation by sugar re-exposure is independent of the *shibire*-mediated neurotransmitter release from PAM DANs. We observed further differences between the paradigms: exposing flies to a novel odor during sugar re-exposure (new learning), protects the memory from being diminished (Figure 8D). Still, presenting a novel odor while activating PAM DANs leads to memory impairment (Supplemental figure 5C). Furthermore, memory devaluation by sugar re-exposure is context dependent while erasure by artificial PAM DANs is context-independent (Figure 8I).
Therefore, we conclude that these processes depend on two separable mechanisms (Figure 9). The first involves ongoing activity in PAM DANs, which may influence the homeostasis between memory maintenance and forgetting of reward memories. Similar to active forgetting of unconsolidated aversive memories, this ongoing PAM DAN activity might be influenced by the fly's state (19). The exact nature of this state and its involvement remain to be explored. The second mechanism involves re-exposure to the actual sugar reward, representing an acute life event for the flies that leads to memory update. As a result, the learned association between the cue and the reward is devalued. This process does not depend on PAM DAN output.

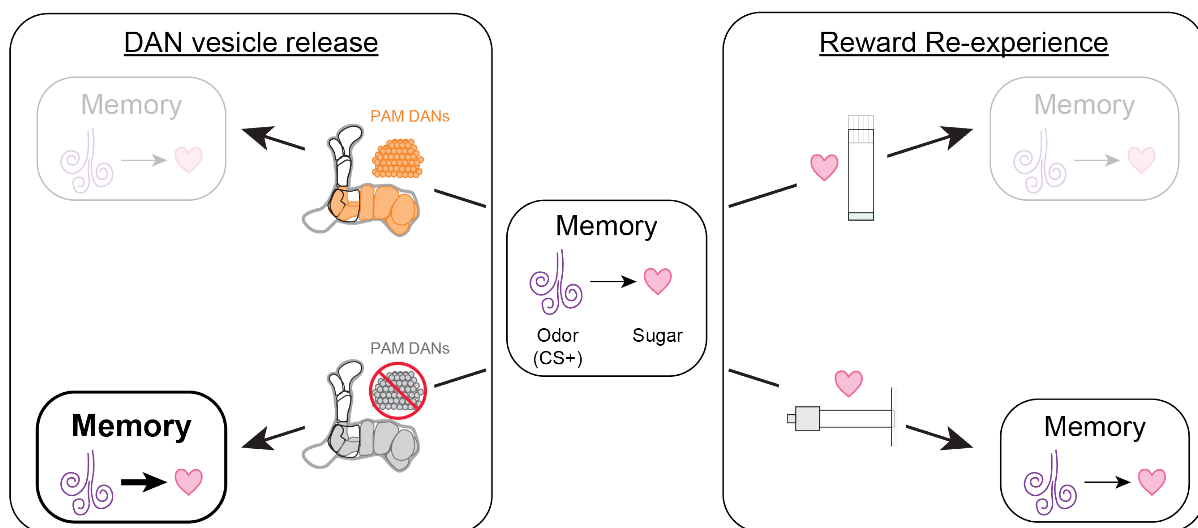


Figure 9: Two separate mechanisms reduce reward memory expression. Artificial activation of PAM DANs after appetitive learning leads to impairment in a later test. Correspondingly, inhibition of PAM DANs between training and test leads to enhanced memory performance (left panel). Independent from PAM DANs' small vesicle release, sugar re-exposure can lead to memory devaluation in a context dependent manner (right panel).

5.3 DAN-mediated balance between maintenance and forgetting of a memory.

The fate of learned information is defined by processes that support memory maintenance and those that favor forgetting (18). Recent work has suggested that these two seemingly antagonistic operations are active processes (132). Activity from distinct dopaminergic neurons seem to be able to bias the memory to be either stabilized or promote its decay (19). Aversive memories do not get consolidated after a single training session and fade within the first hours after learning. Inhibiting aversive coding PPL1 DANs can delay this effect, while

activating the same neurons leads to rapid memory loss (132). It seems that increased activity (walking) of the fly translates into higher ongoing activity in PPL1 DANs (19). Over time this constant input leads to reversal of plasticity in MBONs established during learning, a process known as active forgetting (19). Additionally, a different study reports the involvement of β'1 PAM DANs in active forgetting of aversive memories (197). However, our results do not show an effect of β'1 PAM DAN stimulation on aversive memory (Supplemental figure 1K). This discrepancy could be explained by the use of different driver lines. Instead, activation of α1 PAM DANs after one trial aversive training diminishes memory performance in our experiments (Supplemental figure 1K). The α1 compartment of the MB is involved in consolidation (118). Perturbing α1 signaling could therefore disrupt memory maintenance. In line with the concept of active forgetting, activating PAM DANs after learning impairs memory retrieval without recovering within 24 h after appetitive conditioning (132) (Figure 4E). Yet, appetitive memories in the fly require only one training trial to become long lasting (Supplemental figure 1A). Activating PAM DANs at a later timepoint after training leads to forgetting of the stabilized memory, which cannot be explained by a disruption of consolidation processes. Rather than a permanent loss of memory, studies on consolidated aversive memories suggest a transient form of forgetting following exposure to threatening stimuli-mediated by PPL1 DANs. Artificially activating PPL1 DANs after multiple training trials leads to temporary retrieval deficit (20). In contrast to active forgetting, plasticity in the MBONs is not changed even when the memory cannot be expressed on a behavioral level. Memories recover after some time, but forgetting lasted up to 14 days depending on activation strength of PPL1 DANs (20). Similarly, PPL1 DANs are involved in the hunger state dependent expression of food memories. Activity from PPL1-γ1pedc DANs transiently counteracts memory expression when the animal is in a fed state (151). Therefore, DAN activity not only influences maintenance of an unconsolidated memory but also modulates retrievability of memories in coherence with the animal's state. Modulation of memory expression could also explain increased retrieval after block of PAM DANs (Figure 7A). Experiencing a period in which reward is limited to the minimum (due to block of reward DANs), could tune the system to a state in which retrieval of any memory guiding towards reward is favored, in contrast to situations where reward is abundant anyway. Whether sugar memory expression would recover after longer time subsequent to PAM DAN activation is unknown. Since flies need to be starved for sugar learning and memory retrieval, later testing timepoints require in between feeding to keep flies alive. To avoid refeeding effects, we refrained from testing later timepoints. However, we observed a gradual effect in forgetting depending on the length of DAN reactivation (Figure 5C). Shorter reactivation sessions diminished retrieval but to a lesser extend compared to longer DAN reactivations. Thus, it might be that there is a linear relationship between memory devaluation and DAN reactivation or that short DAN activation leads to transient forgetting that is recovered within

2.5 h between reactivation and test. Testing flies at different timepoints including shortly after DAN activation together with functional imaging experiments will be necessary to answer this question.

How can DANs exert opposing functions? Since PAM DANs are heterogenous in their function, different processes can be mediated by different neurons. Indeed, transient forgetting relies on PPL1-α2α'2 DANs that are not directly involved in learning, thus separating learning and forgetting on a neuronal level (20). However, active forgetting involves the same DANs (PPL1-γ1pedc>α/β and PPL1-γ2α'1) as aversive learning (132). We couldn't identify a specific subset of PAM DANs diminishing reward memory expression (Supplemental figure 1H). Our data suggest that the same neurons involved in training must be reactivated later to reduce memory performance (Figure 6). Thus, the same neurons fulfill antagonistic functions. In the fly, four different dopamine receptors are known (77). Learning requires Dop1R1 in KCs and its downstream signaling via Gs and the adynyly cyclase rutabaga (125, 136, 137, 205). On the contrary, Berry *et al.* showed that active forgetting of aversive memory is mediated by the receptor cascade of Dop1R2 (DAMB) which activates Gq and the IP_3 pathway (132, 158). The two receptors show different sensitivities in dopamine binding and are differently co- regulated (126, 158). Therefore, dopamine input can activate different downstream receptor cascades, depending on the concentration and coincidence with other signals. Functional divergence can also be achieved through different transmitter systems. DANs have been shown to co-release NO and dopamine during learning. Such NO signaling leads to the formation of a memory of opposing valence dampening the effect of dopamine (166). Blocking NO signaling before but not after sugar training, indeed leads to an enhancement in appetitive learning (Supplemental figure 4E &F). Furthermore, a recent study reported the involvement of GABAergic co-transmission from DANs in the forgetting of appetitive memories (155). Thus, decreased retrievability of the sugar memory after activation of PAM DANs could be mediated by co-release of other transmitters than dopamine.

5.3.1 Time phase dependency

Memories are assigned different time phases. Early after learning they exist as so-called short-term memories (STM) followed by a phase of mid-term memory until a few hours after training and later become stabilized as long-term memories (LTM). In *Drosophila* STM and LTM are stored in different lobes of the MB (116, 117). Further, separate PAM DANs subpopulations are involved in reinforcement for either STM or LTM (STM-DANs (R48B04-GAL4), LTM-DANs (R15A04-GAL4)) (138, 139) (Supplemental figure 1E &F). Therefore, it seems that STM and LTM exist as separate systems. Hence, we wondered whether they could also be separated on a level of forgetting. When activating the broad cluster of PAM DANs after training STM, and LTM memory was diminished independently of the timepoint of activation (Figure 4E &H). Though stimulation of STM-DANs 15 min after training, led to a strong trend towards reduction

of STM, it left LTM unaffected (Figure 4G). Similarly, activating STM-DANs late (24 h) after training did not diminish LTM expression (Figure 4G). Interestingly, driving LTM-DANs either early or one day after learning reduced LTM performance (Figure 4F). Perhaps surprisingly, early activation of LTM-DANs also reduced STM retrieval (Figure 4F). Both STM- and LTM-DANs have only weak capacities to reinforce mid-term memory (6 h) (Figure 5G, Supplemental figure 1E &F). Accordingly, mid-term memory was only diminished by activation of the broad DAN cluster but stayed intact after STM-DAN or LTM-DAN activation 3 h after training (Figure 4I).

Together, STM is reduced independent of the activated subpopulation of DANs whereas LTM is only diminished when the driver line used for activation includes LTM-DANs. Intermediate memory was not affected by either STM- or LTM-DAN activation. Thus, our data confirms a model in which STM and LTM act on different memory circuits and we find a certain degree of specificity in forgetting. In line with memories being vulnerable shortly after learning, it suggests an open time window in which disturbing signaling processes by DAN activation in general leads to forgetting.

5.4 DAN vesicle release independent memory devaluation

Unpaired re-exposure to sugar after sugar learning leads to diminished memory retrieval (Figure 4A-D). However, this process seems to be distinct from forgetting mediated by PAM DANs. When output from PAM DANs during sugar re-exposure is restricted, devaluation still occurs (Figure 7A, Supplemental figure 4A &B). This suggests that other neurons or mechanisms might be involved. In our endeavor to investigate the underlying circuits of the phenomenon we probed different neuronal populations impinging on the MB network. We tested the requirement of output from aversive reinforcing PPL1 DANs, as well as additional DAN clusters of the fly brain labeled by the TH-GAL4 driver line (i.e. PPL2-, PD1-, PPM1,2&3- and PAL-DANs) (Figure 7D). Further we blocked KCs, octopaminergic neurons labeled by Tdc2-GAL4, most of the serotonergic neurons (Trh-GAL4), DPM neurons, a pair of LHONs (PD2a1/b1), cholinergic neurons labeled by chAT-GAL4 as well as gustatory receptor neurons labeled by Gr64f-GAL4 and glia cells (Figure 7C &E-K). Our results showed that none of these genetic manipulations prevented memory devaluation. We used genetic expression of *shibire*, a dynamin mutant interfering with recycling of small vesicles at the presynapse (99). As a result, the presynaptic neuron depletes its vesicle pool and transmitter release is impaired. Nonetheless, signaling via other systems like gap junctions, neuropeptides or gaseous molecules remains functional. NO is a gaseous neurotransmitter that has been shown to be co-released from PPL1 and PAM DANs (166). Co-transmission of NO during learning leads to the formation of a memory that opposes the dopamine-mediated memory (166). When we pharmacologically blocked NO synthesis before appetitive training memory performance was

enhanced (Supplemental figure 4E &F). Hence, we tested whether NO release is involved in sugar memory devaluation. However, when we blocked NO synthesis during sugar re-exposure, memory retrieval at the test was still reduced (Figure 7L). Thus, we exclude NO as the crucial player in reward memory devaluation. Since blocking neuronal output by expressing *shibire*^{ts} leaves activity of targeted neurons intact we tried to deploy a different approach. The optogenetically silencing channel GTACR1 blocks neuronal activity upon green light exposure (206). Unfortunately, exposure to strong green light seemed to have interfering effects on the phenotypes we observed. Therefore, we could not draw conclusions from the results we obtained (data not shown). Thus, until now we cannot exclude the involvement of one of the tested neurons via a *shibire*- or NO-independent signaling pathway. To investigate whether other signaling pathways are involved, future experiments will utilize the inward-rectifier potassium channel Kir2.1 to silence candidate cells (207, 208). Additionally, other pathways that we have not probed may also be responsible for sugar-mediated memory devaluation. The precise mechanism by which the perception of sugar is translated into a reinforcement signal at the level of the MB is not yet understood. This lack of knowledge makes it challenging to test the involvement of other potential candidates in an informed manner. Moreover, these activity patterns are likely influenced by the animal's internal states, such as hunger. Recent data suggest that hunger-state-dependent hormonal signaling modulates responsivity of DANs (209). Other similar mechanisms might exist that are still unknown. Therefore, such long-range inputs could also play a role in sugar-mediated memory devaluation.

Overall, the circuit mechanisms underlying sugar-mediated memory devaluation remain unclear to date. However, our negative results are themselves a fascinating finding, highlighting the system's complexity and indicating parts of the circuitry that are not yet understood. Nevertheless, the presented paradigm provides a valuable opportunity to further investigate the neuronal circuitry underlying US-mediated memory update.

5.5 Context gates reward memory devaluation

Exposure to the unpaired reward after olfactory conditioning reduces memory retrieval. However, whether devaluation of the learned association occurs depends on the context of reward re-exposure. Presenting flies sugar in vials used for starvation diminishes memory performance. Contrarily, when flies receive the sugar in the tubes usually used in T-maze experiments, the memory is maintained (Figure 8F, Supplemental figure 5A). This context dependency aligns with previous studies that have highlighted the importance of context for memory retrieval (210, 211).

To understand the significance of different contexts for the flies, one must consider the lifetime experiences of the animals. In the lab flies live at their preferred temperature (22-25°C) and

have infinite access to food until they are brought to experimental conditions. Only then, they undergo a procedure of starvation interrupted by training, sugar re-exposure and testing which can be considered major events in the fly's lifespan. Starvation is conducted in plastic vials with an agar pit on the bottom as a water source (starvation vials). The training context is marked by conditions (e.g. tubes and airflow) related to the T-maze. These environments can provide further cues that could be associated with the reward during learning. Therefore, the learning context, besides missing the major odor cue, could provide sufficient information so that the memory is still considered reliable. However, when flies were trained in starvation vials, memory devaluation still occurred in starvation vials, but not in the T-maze (Figure 8K). Thus, it is not the training context *per se* that protects the memory from being devalued. Considering the data, there are two other possible interpretations. Firstly, the T-maze context serves as the testing context in all experiments. By experiencing the sugar without odor memory retrieval could become context dependent. Therefore, behavior would only be shown when re-exposure and testing context are matching. Secondly, memory devaluation could depend on the starvation vials. Flies might be in an aroused state since the starvation context means stress in the form of nutrition deprivation. It has been shown that arousal states can lead to differences in behavioral expression of memories (212, 213). Further, sugar might be less expected in the starvation context where food is normally restricted. In this case it could elicit a larger prediction error, consequently leading to larger necessity for memory update. Moreover, the starvation context could change the motivation to learn about nutrition supply, since food is more valuable in situations where long starvation phases are expected. Testing these hypotheses requires changing either the starvation or testing context. Since establishing these protocols is out of the scope of this study, future work could address these questions.
To gain better control over the system, we explored various features that could define the context. However, none of the tested aspects resulted in a change in context dependency regarding starvation vials or T-maze tubes (Figure 8G-J, Supplemental figure 5B). When we changed the T-maze context by removing the airflow or using completely dark tubes, flies still maintained the memory (Figure 8H &I). Similarly, providing the sugar in unplugged T-maze tubes did not diminish memory retrieval in the test (Figure 8J). We also modified exposure in the agar vials in a way that could eliminate potential differences to the T-maze tubes. Nonetheless, sugar re-exposure in starvation vials lacking the agar pit or those exposed to flies for one day before, to introduce a fly-smell, still resulted in memory devaluation (Figure 8G, Supplemental figure 5B). Hence, at this point we cannot conclusively identify the critical components that flies sense in the different environments. It could be a particular visual cue, the slightly different diameter of the vials, a smell emanating from the different materials, or a combination of these features that is essential for flies to distinguish between the different contexts.

Though open questions remain, the context dependency of reward memory devaluation highlights the importance of the event history of an animal's lifetime in the processing and updating of memories.

5.6 Potential and limits of using the US as a reminder cue to activate memories in the clinics.

In exposure therapy, the associated cue is typically used as a reminder cue to initiate memory update. However, this approach only changes the targeted CS-US memory. Since meaningful events (US) are often associated with multiple cues, other cues can still elicit unwanted behavior. The US is part of all these associations. Therefore, using the US as a reminder cue holds potential to change multiple memories in the same session. In line with previous animal studies, we demonstrate that exposure to the US can trigger memory update in *Drosophila*. However, the question remains: how feasible is this approach for translation to clinical applications? In the case of fearful memories, it is unrealistic to imagine re-exposure to the US. The meaningful event in this case can be harmful. It is inconceivable to return traumatized veterans to war zones as part of clinical treatment. Nevertheless, substance abuse disorders might allow implementation. In rats, administration of the drug of abuse can destabilize reward memories. Subsequent interference with the restabilization step has been shown to successfully reduce spontaneous recovery, renewal, and reinstatement (58, 214). In some studies, reinforcement involving reminder sessions even seem necessary to induce memory update (59). Therefore, utilizing the US as a reminder cue, might more broadly lead to change of memories of different kind and strength. However, significant risks and ethical concerns cannot be overlooked as administering drugs to patients is potentially harmful and can lead to relapse. This issue might be addressed by using chemically similar substances without addiction potential, such as methadone or methylphenidate (58, 60).

In line with data about CS-mediated memory change, our data show the context dependency of US-mediated memory change (215, 216). Thus, contexts of the reminder session should also be intentionally chosen in clinical settings. Thereby, not only external environments but also internal states of an individual matter and should be considered.

Overall, there seems to be potential for using the US-based approach in clinical treatment. However, further research is needed to apply this technique in a targeted manner. Animal models will be crucial for gaining a deeper understanding of the underlying mechanisms before implementing therapeutic procedures for humans. In this study, we provide a simple behavioral paradigm using the fruit fly as a well-studied model organism. Using this paradigm along with the fly's predominantly stereotypical brain wiring and genetic accessibility, allows for further systematic and mechanistic investigations.

5.7 Outlook

The results from this study show that in flies re-exposure to sugar after appetitive training leads to devaluation of the memory. Similarly, activation of DANs from the PAM cluster leads to diminished memory retrieval in the test. However, our data reveals that those two processes are distinct and involve different neuronal mechanisms.

Studies on aversive memories report permanent or transient forgetting processes in case of unconsolidated or consolidated memories, respectively (20, 132, 155, 197). Thereby, plasticity in downstream MBONs is reversed when the memory is permanently forgotten (19). Contrarily, transient forgetting rather seems to be a retrieval deficit with the plasticity being maintained (20). Appetitive memories in flies get consolidated after one training trial (104). We find that activation of PAM DANs after training leads to reduction of memory retrieval independent of the memory phase (Figure 5C-F). Further, the memory does not recover 24 h after an early reactivation, suggesting that the memory is permanently gone (Figure 5D). It has been shown that sugar learning leads to the depression of odor-evoked Ca^{2+} responses in MBON-β′2mp (121). In our next steps, we will conduct functional imaging experiments to investigate whether this plasticity is reversed following a) activation of dopamine neurons and b) sugar re-exposure. Additionally, it will be interesting to explore if there is a difference in reversal when activation or re-exposure occurs inside versus outside the consolidation window.

To understand the circuit mechanisms underlying sugar-mediated memory devaluation, we tested several neuronal populations impinging on the MB. However, blocking any of these neurons during sugar re-exposure did not impaired memory devaluation (Figure 4). We used the temperature-sensitive dynamin mutant *shi*^{ts} to block output from targeted cells. Though *shi*^{ts} restricts small vesicle release, activity within neurons remains functional. Therefore, in future experiments, we will express the inward-rectifier potassium channel Kir2.1 in a time restricted manner to investigate involvement of *shi*^{ts} independent signaling pathways in targeted neurons.

Sugar re-exposure in the T-maze context leaves the memory unaltered (Figure 8F). However, it is still possible that the memory undergoes reconsolidation without being significantly updated. Without interference or update towards forgetting, a memory undergoing reconsolidation remains retrievable. Therefore, interference with the restabilizaiton step is necessary to observe reconsolidation. Preliminary experiments indicate that exposing flies to sugar in the T-maze context, after training in the same context, leads to memory reconsolidation (data not shown). However, it is unclear whether the sugar, the context, or both serve as the reminder triggering memory destabilization. To clarify this, we will conduct experiments where flies are trained in a different context and then exposed to sugar in the T-

maze context. If reconsolidation occurs under these conditions, it would suggest that sugar re-exposure triggers reconsolidation in the T-maze context, with different consequences than outside the T-maze context.

These outlined future experiments will enhance our understanding of the neural mechanisms underlying the devaluation phenomenon presented here. It will be exciting to uncover the consequences of sugar re-exposure-mediated memory devaluation on a circuit level.

6 Literature

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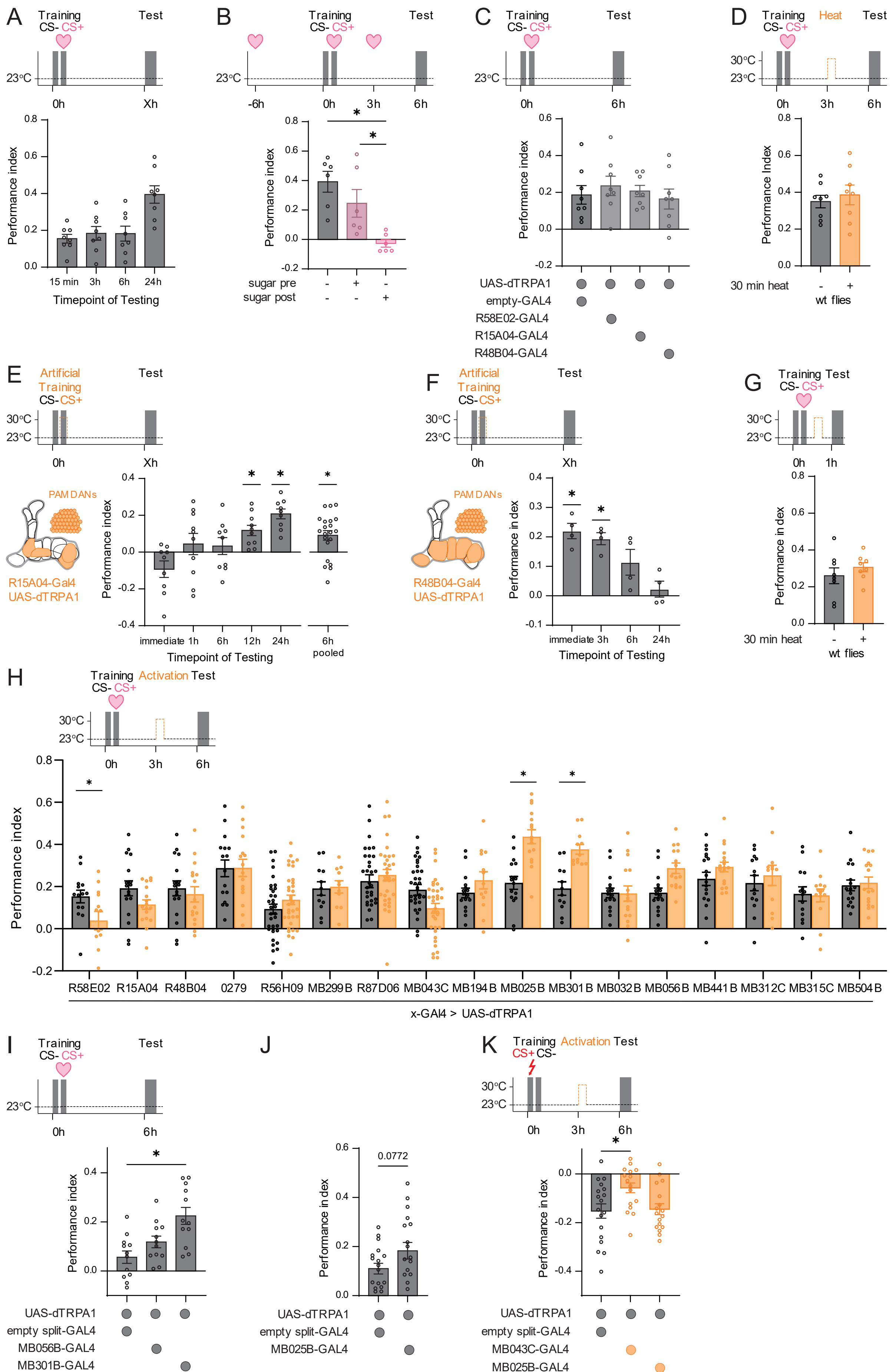
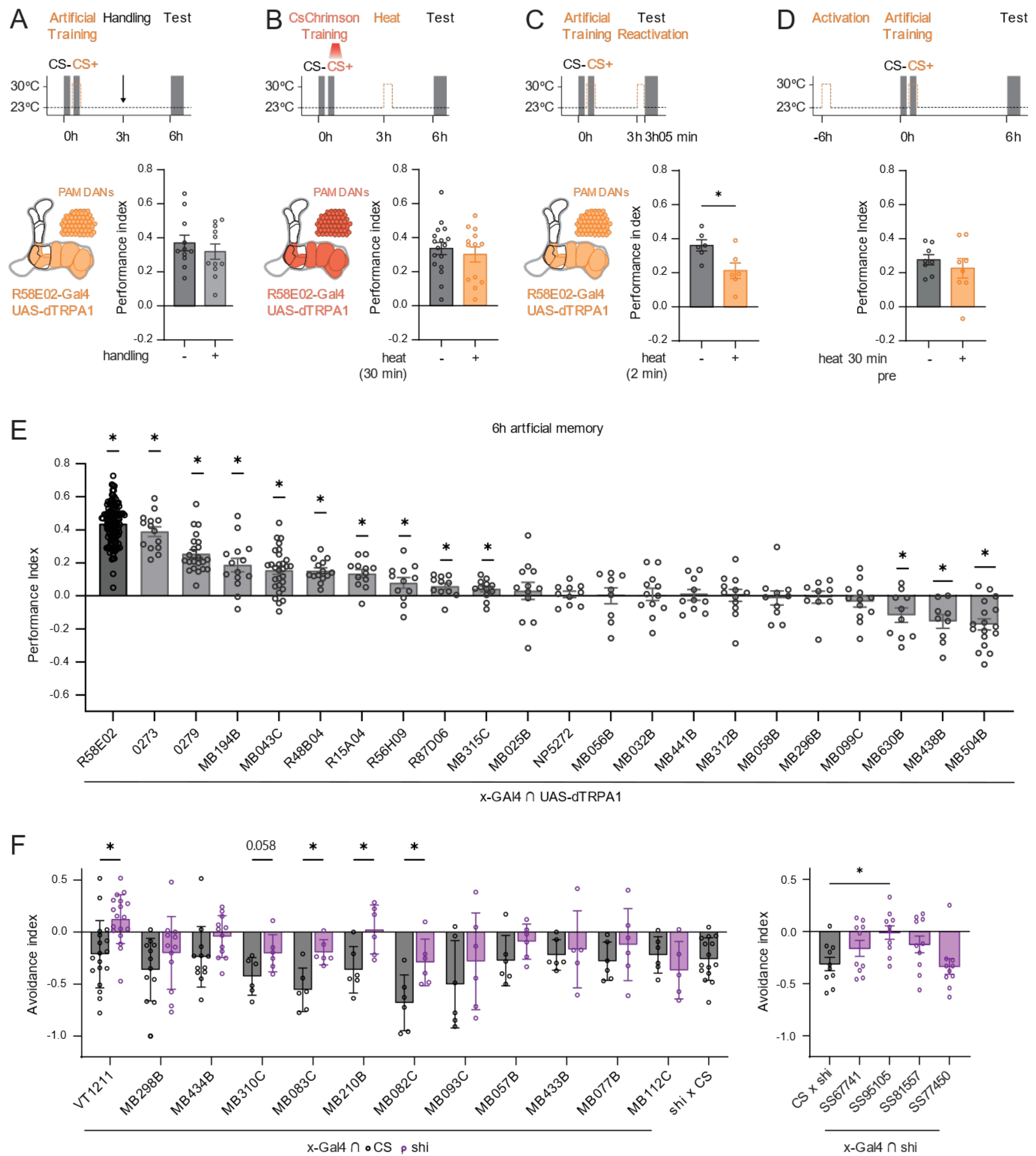
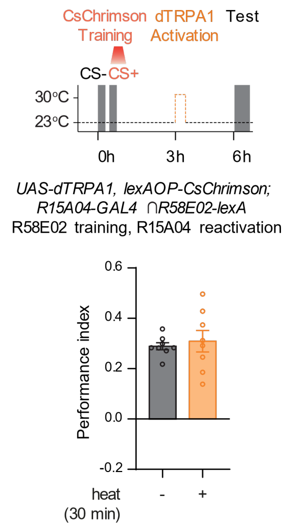
7 Supplement



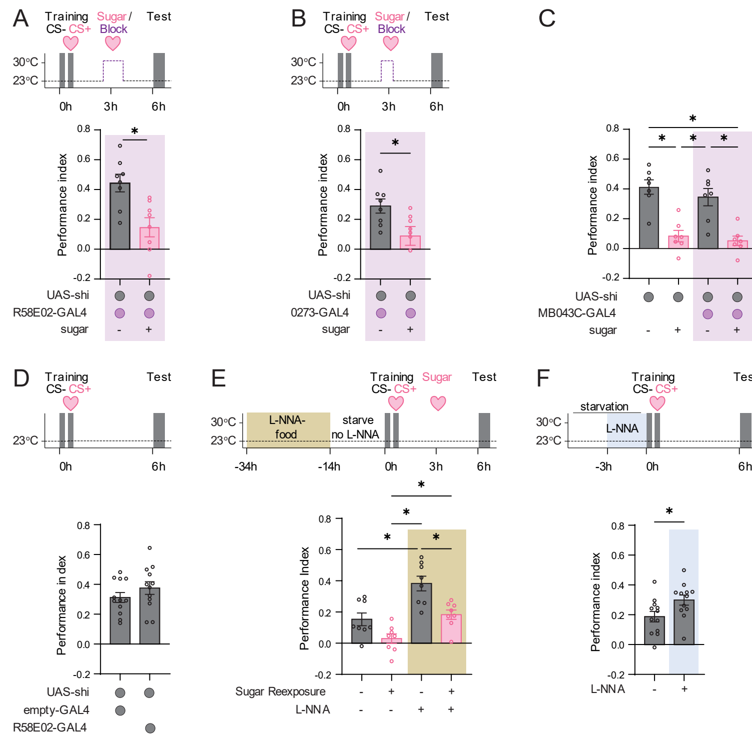
Supplemental figure 1: Sugar memories are long lasting after one training trial. Tests were conducted at 15 min, 3 h, 6 h and 24 h after sugar training (A). Exposing flies to sugar 3 h after, but not 3 h before, sugar training diminishes 6 h memory retrieval (B). Flies expressing *UAS-TRPA1* in R58E02- GAL4, R15A04-GAL4 and R48B04-GAL4 labeled neurons show same performance in sugar learning as controls crossed to empty-GAL4 (C). Exposing wt flies to heat 3 h after training does not affect 6 h memory performance (D). Artificial training with R15A04-GAL4 labeled neurons exclusively leads to appetitive LTM expression. At 6 h memory was not expressed in this particular data set. However, pooling 6 h memory performance with datapoints from other data sets shows robust memory expression (last bar "6 h pooled") (E). Contrarily, training with R48B04-GAL4 labeled neurons implements an appetitive STM at different timepoints after artificial training (F). Exposing wt flies to heat for 30 min at 15 min after sugar training does not influence memory retrieval at 1 h (G). Reactivation of subsets of PAM-DANs 3 h after sugar training, 6 h test. Only activation using the broad driver line R58E02-GAL4 leads to reduced memory performance at 6 h (H). For 6 h sugar memory, genetic controls of MB056B-GAL4, MB301B-GAL4 and MB025B-GAL4 show significantly increased or a tendency for increased memory performance (I&J).Reactivation of MB043C-GAL4, but not MB025B-GAL4, labeled neurons 1.5 h after aversive shock training leads to diminished memory retrieval at 3 h (K). The heart icon in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA). For labeled neurons by the respective driver line, see Supplemental table 1.



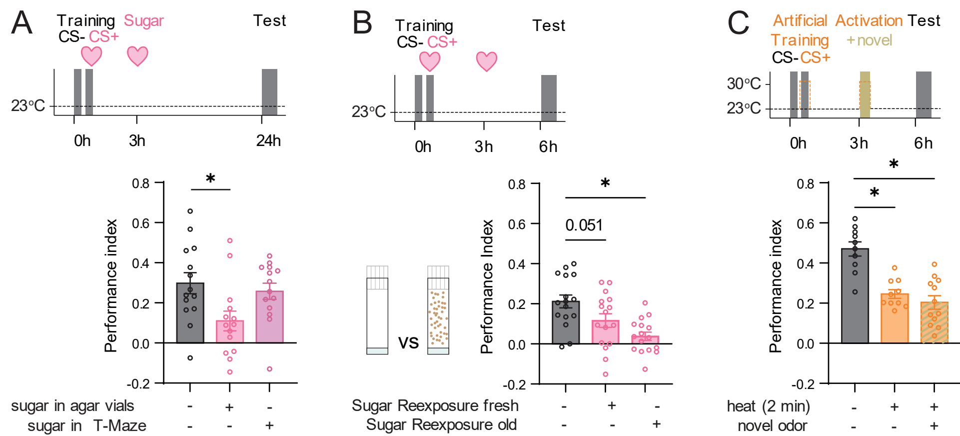
Supplemental figure 2: Handling flies 3 h after training (flipping into vials and back) does not alter 6 h retrieval of artificial R58E02 memory (n ≥ 10) (A). Exposure to heat (32°C) after optogenetic training (R58E02-GAL4) does not affect 6 h memory performance (n ≥ 13) (B). 2 min reactivation of R58E02-GAL4 labeled neurons at 3 h after training leads to reduced memory in an immediate test. Asterisk denotes significant difference (P < 0.05, t-test) (n ≥ 6) (C). Activation of R58E02-GAL4 labeled DANs 3 h before learning does not affect 6 h retrieval of artificial memory (n ≥ 8) (D). DANs labeled by some but not all GAL4 driver lines have the capacity to implement a 6 h memory (one sample t-test, asterisks denote significant difference to 0 (P < 0.05)) (n ≥ 8) (E). Screen of MBON involvement in naïve odor avoidance (F). Flies expressing *shi*^{ts} in MBONs and genetic controls were given the choice between air and 3-octanol at a restrictive temperature of 32°C. VT1211-GAl4 labeled neurons are known to be involved in odor avoidance (121) and were used as a positive control. Data for this group and the Canton S (CS) control were pooled over several experiments (F). Asterisks denote significant differences (P < 0.05, t-test (left graph) or ANOVA (right graph)) (n ≥ 6). In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. For labeled neurons by the respective driver line see Supplemental table 1.



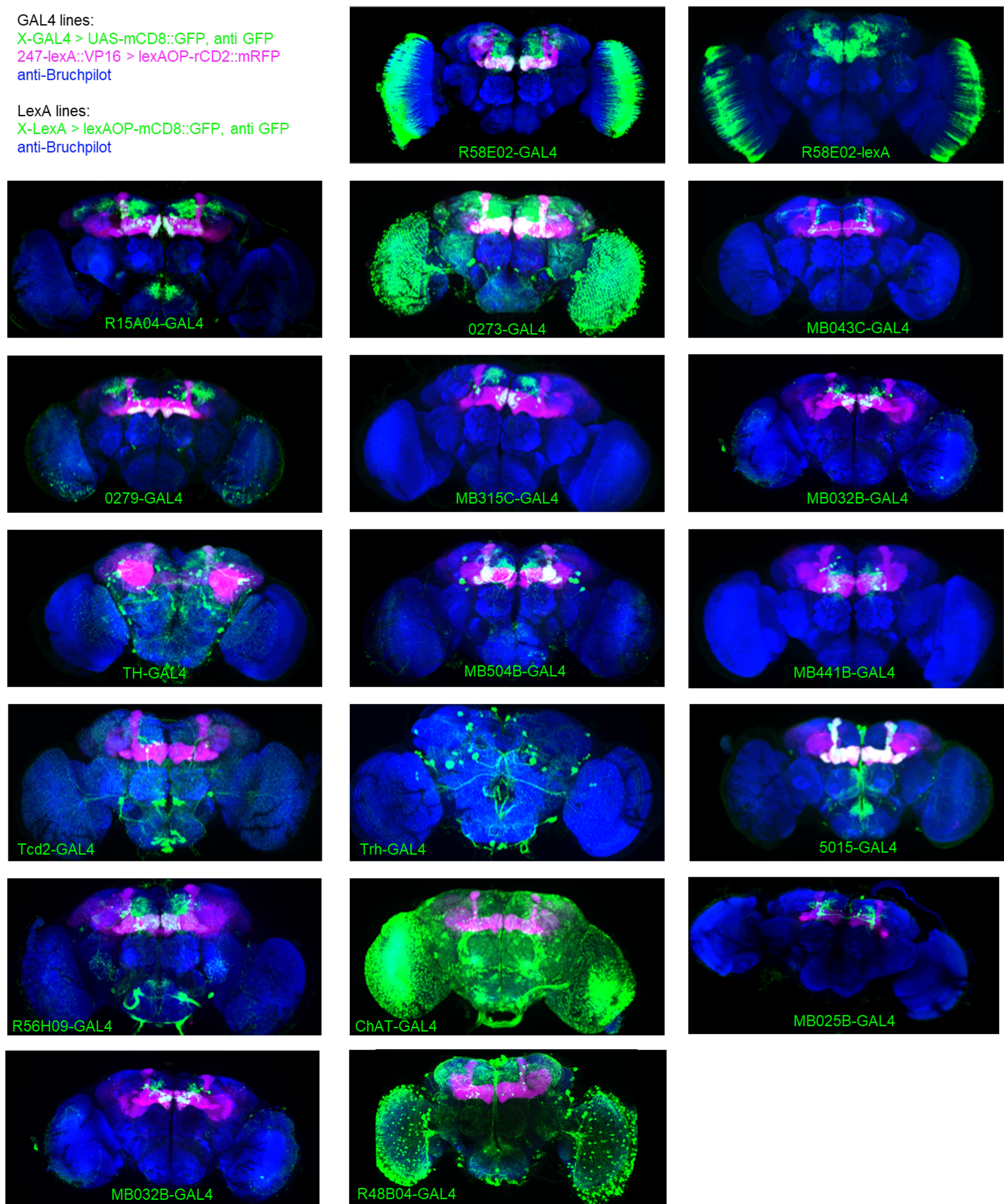
Supplemental figure 3: Thermogenetic activation of R15A04-GAL4 labeled neurons (LTM DANs) 3 h after optogenetic training with R58E02-lexA labeled neurons leaves 6 h memory unchanged (n ≥ 8) (t-test).



Supplemental figure 4: When blocking output from R58E02-GAL4 labeled neurons (using *shibire*^{ts}) during sugar and for 30 min after re-exposure, sugar-mediated memory devaluation still occurs (n ≥ 8) (A). Blocking PAM DANs labeled by 0273-GAL4 leaves sugar-mediated devaluation intact (n ≥ 8) (B). Blocking α1 PAM DANs labeled by MB043C-GAL4 does not impair sugar-mediated memory devaluation (n ≥ 8) (C). 6 h memory control for genotypes *R58E02-GAL4 ∩ UAS-shi*^{ts} or *empty-GAL4 ∩ UAS-shi*^{ts} reveal no difference (n ≥ 10) (D). Feeding flies L-NNA-containing food for 24 h before starvation leads to enhanced memory performance in a 6 h test but does not impair memory devaluation (n ≥ 8) (E). Feeding flies L-NNA 3 h before training in a saturated solution on filter paper enhances 6 h memory (n ≥ 10) (F). The heart icon in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA)



Supplemental figure 5: Re-exposing flies to sugar in the T-maze at 3 h after training leaves 24 h memory intact (n ≥ 12) (A). Exposing flies to sugar in starvation vials that had been exposed to flies before does not impair sugar-mediated memory devaluation (n ≥ 15) (B). Reactivation of PAM DANs diminishes 6 h artificial memory independently of presenting a novel odor (EB) during reactivating (n ≥ 10) (C). The heart symbol in the timelines depicts sugar exposure. In all figures, data represent the mean ± s.e.m. Individual n are indicated by circles. Asterisks denote significant differences ($P < 0.05$, t-test or ANOVA)



Supplemental figure 5: Expression patterns of selected GAL4 and lexA driver lines.

Supplemental table 1: List of driver lines indicating the labeled DANs or MBONs.

Drivers	Labelled neurons
R58E02	90 PAM DANs
0273	120 (all) PAM DANs
MB504B	PPL1-γ1pedc, PPL1-α'2α2, PPL1-γ2α'1, PPL1- α3
0279	PAM-β1, PAM-β2
R56H09	PAM-β′2m and PAM- γ5n
MB299B	PAM-α1
R87D06	PAM-α1 and PAM-β1
MB043C	PAM-α1
MB194B	PAM-β1ped
MB025B	PAM-β'1m, PAM-β′1 ap
MB310B	PAM-β2β′2a
MB032B	PAM-β′2m
MB056B	PAM-β′2p
MB441B	PAM-γ3
MB312C	PAM-γ4, PAM-γ4<γ1γ2
MB315C	PAM-γ5
MB194B	PAM-β1ped
NP5272	PAM-β2β′2a
MB058B	PPL1-α′2α2
MB296B	PPL1-γ2α′1
MB099C	PPL1-α'2α2, PPL1-γ2α'1
MB630B	PPL1-α3
MB438B	PPL1-γ1pedc
VT1211	MBON-γ5β'2a, MBON-β'2mp, MBON-β2β'2a
MB298B	MBON-γ4>γ1γ2
MB310C	MBON-α1
Mb083C	MBON-γ3, MBON-γ3β'1
MB210B	MBON-γ5β'2a, MBON-β'2mp
MB082C	MBON-α3, MBON-α'2
MB093C	MBON-α3, MBON-α'2
MB057B	MBON-β'1
MB433B	MBON-β1>α
MB077B	MBON-γ2α'1
MB112C	MBON-γpedc>α/β
SS67741	MBON-γ1γ2
SS95105	MBON-β'2d
SS81557	MBON-α'3
SS77450	MBON-γ4γ5