Neural mechanisms underlying memory in Drosophila melanogaster

Paola Vargas Gutierrez

Keble College



University of Oxford

A thesis presented for the degree of

Doctor of Philosophy

Trinity 2022

Acknowledgements

First of all, I would like to express my gratitude to my supervisor, Scott Waddell. Every conversation with Scott ended up loaded with interesting new ideas, questions and highlighted project branches to explore. I appreciate the intellectual freedom I was given to shape my projects. This DPhil came at one of the most challenging times, personally and globally; I appreciate all the support Scott offered me through this. I am sure this endeavour would not have been possible without it.

I am extremely grateful to the Sloane Robinson Fund from Clarendon and Keble College, and to CONACYT-Mexico for providing me with the financial means to study this DPhil. To Sarah Noujaim for her dedication to making students' experience in the department as enjoyable as possible, and for always providing guidance on the bureaucracies of doing a DPhil in Oxford. To Ruth Brain for being the hero of the CNCB; without her work everything would descend into chaos. I want to thank Johannes Felsenberg and Pedro Jacob for showing me the ropes when I first joined the world of fly learning and memory. I'm also thankful to Pedro for all the guidance and help throughout my DPhil. To Kristijan Jovanoski for the countless discussions on satiety and nutrient signalling and taking the time to show me 2-photon microscopy. To Nils Otto and Zeynep Okray for the encouragement and enriching conversations we shared while working together; and for always bringing a positive attitude into the lab. To Bhagyashree Senapati for her immense help with proofreading this manuscript, for her friendship, support and encouragement. To Annie Park for all the suggestions, inspiring ideas and patient repeated proofreading during this thesis writing; and for all the fun adventures outside lab, from writing to cycling or kayaking adventures, Annie always encouraged me to be better, and then laugh when I fall into the river. I am deeply

in debt with all members of the Waddell lab, not only for all the guidance and advice they offered during the past 4 years, but also for all the fun moments along the way.

I was lucky to share my DPhil years with incredible people in the CNCB. I am privileged to have befriended a lot of these brilliant minds, some of whom I shared the pain of doing a DPhil, some who will be friends for life and all of whom made this whole experience a lot more enjoyable.

To all my friends, in particular Eri Ichijo, Hania Fiaz and Mauricio Aguilar, for providing invaluable emotional support to push through the hardest parts of this DPhil. I am forever grateful to my parents, Lucy and Victor, for always encouraging me to follow my dreams, even if that meant leaving to the other side of the world. To my siblings, Juan Carlos and Andrea, and cousin, Ady, for always looking out for me and encouraging me at all times. Finally, I'd like to thank Chris, for proofreading this thesis, and who no matter how hard things got was always there to comfort me and bring joy into my life.

Declaration

I, Paola Vargas Gutierrez, declare that this DPhil has been funded by the Sloane Robinson Clarendon Fund given by Clarendon and Keble College; and by CONACYT Mexico. The work presented here has been done under the supervision of Prof. Scott Waddell as main supervisor and Dr. Zeynep Okray as secondary supervisor. Experiments presented throughout Chapter 5 are solely my work. Experiments in Chapter 4 were performed in collaboration with Dr. Pedro Jacob, Dr. Zeynep Okray, Stefania Vietti-Michelina, Dr. Johannes Felsenberg and Prof. Scott Waddell; which have been published in Current Biology (See annex B). This publication also includes functional imaging data which are not reported as part of this thesis. My contribution to this publication is presented as part of this thesis as follows:

- Figure 4.1 A, B, D
- Figure 4.2 A, C
- Figure 4.4 B
- Figure 4.11 A, B
- Figure 4.13 A



21/January/2023

Signature *Date*

1 | Mitigating Circumstances Statement

This statement intends to detail unforeseen situations that disrupted my DPhil studies and therefore the contents and length of this thesis. These mitigating circumstances had a huge impact in my performance, as well as in reshaping the structure and progression of this DPhil.

First, the outbreak of the COVID-19 pandemic. In March 2020, the UK went into its first national lockdown to prevent the spread of COVID-19. Following the national guidelines and regulation, the University of Oxford issued a statement to work from home. This lasted until July 2020. During this time, the pandemic prevented me from accessing the lab for long periods of time. All experiments were halted and the few visits to the lab were restricted to maintenance of reduced *Drosophila melanogaster* stocks. See COVID-19 log below in Section 1.1 for detailed impact during the first lock down. Once lockdown was lifted, experiments restarted, but the speed at which this happened was slowed down by the need to expand fly stocks for use. The impact of the COVID-19 pandemic continued to a lesser extent through the second and third national lockdowns. This, and other personal circumstances changed the pace and structure of my DPhil as I will detail below.

When I started my DPhil, I joined a project led by Dr. Pedro Jacob investigating the effect of stimulus pre-exposure in memory performance which is detailed in Chapter 4. Alongside, I started an independent project researching a potential non-apoptotic role for caspases in learning and memory of *Drosophila melanogaster*. Caspases are a conserved family of proteases best known for their role in orchestrating apoptotic programs. Importantly, it has been shown that caspases have a wide range of non-apoptotic roles, including cytoskeleton remodelling, cell proliferation, and neurite pruning during development, amongst others (reviewed in [1]). Interestingly, caspases and its regulators play a role in micro-plasticity and synapse remodelling during memory formation in vertebrates, possibly by mediating receptor internalisation and turnover during long-term synaptic depression[1–3]. Thus, I dedicated the first year of my DPhil to investigate a non-apoptotic role for caspases in *Drosophila*'s long-term memory processing. However, no clear memory phenotype was seen by either down-regulating expression of initiator caspase, Dronc, in the mushroom body via RNAi, nor by overexpression of the caspase inhibitors P35 or DIAP. This was the case for both long- and short-term sucrose memory performance. I did however observe a mild but significant disruption of immediate memory performance following a standard aversive conditioning protocol when Dronc was down-regulated in the mushroom body. A single aversive conditioning event in *Drosophila melanogaster* does not lead to long-term memory performance [4], making it difficult to speculate a role for caspases similar to that in vertebrates. This project was therefore set aside, and I soon began investigating the nutrient-dependent memory consolidation circuitry which is detailed in Chapter 5.

While I continued to work on the odour pre-exposure and the nutrient consolidation projects for the rest of my DPhil, my work also took a more collaborative approach as a result of the situations mentioned previously. Therefore, I started working on a project with Dr. Nils Otto to investigate the role of mushroom body output neurons (MBONs) interactions in state regulation and state-dependent memory retrieval. Previous work has implicated γ1-MBON as a hunger signalling neuron [5]. γ1-MBON feedbacks into other compartments of the mushroom body inhibiting avoidance driving MBONs, thus promoting approach [6] (see Section 2.3.2). At the end of 2020, a whole-brain electron microscopy connectome reconstruction was published, including a detailed connectivity of the mushroom body [7]. This work proposed a model of how MBON-MBON interactions could regulate state dependent memory retrieval though feedback loops. Given its connectivity, this model suggests that β1-MBON would serve as an antagonistic signal to γ1-MBON, providing a satiety signal. This would occur through the simultaneous inhibition of approach-driving α lobe innervating MBONs and inhibition of γ1-MBON, leading the disinhibition of avoidance-driving MBONs, leading to an overall avoidance. We therefore set to test this model. We observed a trend towards a diminished 24h memory performance when inhibiting synaptic transmission of β1-MBON in starved flies using one β1-MBON GAL4 driver but not another. However, these results were inconclusive given a noisy data set. Moreover, we observed that the drivers used were variable in the neuronal selectivity across flies, sometimes including β4-MBON labelling in addition to β1-MBON. Importantly, we failed to either induce memory retrieval in fed flies upon inhibition of these neuron, or suppress memory retrieval and learning upon activation of these neurons during acquisition. However, we do not believe this was enough evidence to rule out this model and it only highlighted the complexity of state regulation connectivity.

Half way through the third year of my DPhil, I also got involved in a project investigating the effect of multisensory conditioning on memory performance, led by Dr. Zeynep Okray and Dr. Pedro Jacob. Consistent with research in vertebrates[8, 9], they found that flies perform better after conditioning using complex multisensory stimuli. In this case, the stimuli used consisted of an olfactory and a visual component (odour and colour light). Interestingly, multisensory training enhances performance even when testing with only one of the modalities alone (i.e. odour or light retrieval). Further work showed that simultaneous stimuli pairing during training allows the binding of neurons responsive to visual and olfactory stimuli. Recruitment of bound neurons during either multi- or uni-modal retrieval allows enhancement of memory performance. As part of this work, I tested whether this enhancement could be explained by the sum of two associations to two different CS^{+}, one between the light and reward and another association between reward and odour. I found that simultaneous presentation of the odour and the light was required for this memory enhancement, which is in line with the sensory binding. Given that flies are phototactic [10], I also investigated whether light could be acting as a reinforcer, representing an overall better reward when combining light and sucrose. However, I failed to observe a light reinforced memory when pairing odour to light only. Moreover, I contributed in the data collection of two experiments investigate the role of ɣdorsal and α/βposterior KCs in light recall. Must of the data in this paper was collected by other authors. This work has been submitted for publication to Nature and is already available as a pre-print in BioRxive [11].

Despite the challenges presented during my DPhil studies, I have contributed important intellectual and experimental input and results to two pieces of published work. One being the multisensory project mentioned above, and the other being how pre-exposure to a stimulus leads to subsequent inhibition of memory retrieval,

underlying latent inhibition, which is detailed in Chapter 4. Furthermore, in Chapter 5, I have challenged existing work on nutrient-dependent memory formation and provided evidence to suggest that the idea of an emerging nutrient memory might be incorrect. Moreover, I propose that ɣ5n provides a behavioural inhibitory signalling potentially through a motivation switch such as satiety. However, more experiments are needed to confirm this being the case. Verifying that activation of ɣ5n still allows odour detection and discrimination would be a first step to support that lack of performance is not just a sensory disruption. More importantly, if ɣ5n was to be signalling satiety, inhibition these neurons should lead to an inappropriate expression of memory in satiated flies. I hope that the work presented here will serve as a reference and inspiration for works in the future.

1.1 COVID-19 impact log March-June 2020

MSD PGR Student Log – Impact of the Coronavirus (COVID-19) Public Health Emergency

The University and the Medical Sciences Division are aware that the current situation with the coronavirus (COVID-19) public health emergency will have a very significant effect on PGR students, including on their research projects. We are working on policies and procedures to deal with the impacts, but it will help us and you if there is clear information on how the situation has an impact on you as it progresses. We have therefore prepared this template log for you to use to help you to keep track of how the situation affects your research progression over time. If you keep this up to date it will provide important information for us to ensure that you receive any additional time and support you need at a later stage.

This log is designed to be completed by you, the student, but you should share this with your supervisor and work on content collaboratively if required. Supervisors can include their own comments in the additional details column if required. Please ensure it is clear where information is a supervisor contribution.

Clinically qualified students returning to clinical work: please complete the form and email it to Graduate School Administration graduate.school@medsci.ox.ac.uk. Please also ensure that your supervisors are copied and aware of your situation. We realise that you may not know the end date at this point so tbc/tba will be sufficient as the end date. You can complete the form again when you return and at that point you can work with your supervisors to assess the impact on your progress and research.

Please save this document with your name and student number so that we can easily trace it to you if necessary.

Student name (family, first name): Vargas Gutierrez, Paola **Student number: 1037017**

Department: Physiology, Anatomy and Genetics **Programme of Study: DPhil in Physiology, Anatomy and Genetics**

Supervisor: Prof. Scott Waddell

Event start date	Event end date	Brief description (try to keep to less than 10 words – use the additional details column to add detail if required)	Level of impact (low, medium, high)	Additional details
16/3/20	*25/3/20*	*Working from home with sporadic visits to the lab*	*high*	*I am at moderate risk due to existing medical condition. I reduced my visits to the lab. I go twice a week for fly maintenance under a rota system implemented by the centre. Have managed to do some experiment planning and literature review in the meantime.*
25/3/20	-	*Working from home*	*high*	*Brought important fly stocks home to maintain them there during the lockdown without the need to go to the lab and reducing the risk of losing crucial tools. I have set up a homemade incubator in my house to keep them.*
1/04/20	-	*Working from home*	*high*	*We have been having weekly lab meetings with the whole lab via videocalls in addition to 1-to-1 online meetings with Scott.*
7/04/20	-	*Working from home*	*high*	*We have decided to continue with the already stablished journal club organized by members of the lab and discuss recent articles. In addition to this, Scott has proposed a second journal club with a smaller group (including me) to reassess old literature in the light of more recent evidence published by the group. This week I will also be going to the lab to do fly maintenance of general lab stocks.*
14/04/20	-	*Working from home*	*high*	*Continue with lab meetings and journal clubs. We arranged another regular meeting group for women in the lab to prepare and discuss 1h presentations of topics in their respective area of expertise or interest. Topics that will be covered range from social behaviour in drosophila to single cell sequencing.*
21/04/20	-	*Working from home*	*high*	*Went to the lab to maintain some stock. Attended the online lab meeting and have been reading the papers for the journal clubs that will take place this week.*
May	-		*high*	*Still reading from home. Only go to the lab for maintenance of stocks during approved times in the lab rota*
June	*22-june*		*high*	*Waiting for approval to return to work to carry on with experiments. I have arranged a meeting with DGS and my supervisor to approve my return. Will have the meeting on June 24*
June 23				*The CNCB has now got approval to reopen. I will have a meeting tomorrow to approve my return as a student.*

Table of Contents

1 Mitigating Circumstances Statement **iv**

1.1 COVID-19 impact log March-June 2020 ix

2 Introduction **3**

2.1 Psychology of Learning and Memory 3

2.1.1 Learning Associations 3

2.1.2 From Learning to Behaviour 6

2.2 *Drosophila* as a Model Organism 8

2.3 Studying Learning and Memory in the Fly 11

2.3.1 *Drosophila* Mushroom Body Anatomy 12

2.3.2 Mushroom Body Circuitry in Learning and Behaviour . . 14

2.3.3 Aims 16

3 Methods **18**

3.1 Fly Strains 18

3.2 Behavioural Assay 19

3.2.1 Differential Associative Learning in the T-maze 19

3.2.2 Naïve avoidance 20

3.3 Quantifying Memory Performance 21

3.3.1 Statistical Analysis 21

3.4 Odour Delivery 22

3.5 Reinforcer Delivery 23

3.5.1 Sucrose Delivery 23

3.5.2 Artificial Neuronal Stimulation Delivery 23

3.5.3 Shock Delivery 24

3.6 Neuronal Inhibition 24

3.7 Context 25

4 Prior Experience Affects Expression of New Learning 27

4.1 Chapter Abstract 27

4.2 Introduction 28

4.3 Results 31

4.3.1 Odour Pre-exposure Effect in Memory Performance . . . 31

4.3.1.1 Odour Pre-exposure in Appetitive Conditioning 31

4.3.1.2 Odour Pre-exposure in Aversive Conditioning . 31

4.3.2 Odour Pre-exposure Leads to Latent Inhibition 34

4.3.3 LI in *Drosophila* is Context and Concentration Dependent 35

4.3.4 Repeated Unreinforced Odour Pre-exposure Produces Aversive Memory 40

4.3.4.1 Pre-exposure Memory Depends on Concentration but not Context 42

4.3.5 Neuronal Network Underlying Odour Pre-exposure Aversive Memory 44

4.3.5.1 Odour Pre-exposure Memory is Mushroom Body Dependent 44

4.3.5.2 Identification of Pre-exposure Reinforcing DANs 45

4.3.5.3 Role of PPL1-α3 in Aversive Reinforcement . . 49

4.3.6 Neuronal Network Underlying Behavioural Output 51

4.4 Discussion 54

5 Investigating the Emergence of Appetitive Long-term Memory 56

5.1 Chapter abstract 56

5.2 Introduction 58

5.3 Results 61

5.3.1 Optogenetic Activation of R15A04-GAL4 DANs Produces Long-term but not Short-term Memory 61
5.3.2 Dissecting the Absence of Short-term Memory 63
5.3.2.1 Odour Competition 64
5.3.2.2 Exclusive Activation of α1 PAM-DANs 65
5.3.2.3 Sucrose Memory is Inhibited via γ5n Activation 68
5.3.3 Long-term Memory Retrieval Inhibition via γ5n DANs Activation 74
5.4 Summary and Discussion 77

6 Discussion **80**
6.1 Conclusions 84

Appendix A Expression patterns of drivers used **102**

Appendix B Publication - Prior experience conditionally inhibits the expression of new learning in Drosophila **109**

List of Figures

2.1 Classical associative conditioning 4
2.2 Binary expression system GAL4-UAS 10
2.3 Mushroom Body Anatomy 13
2.4 Model for associative learning in the Mushroom Body 16

3.1 Differential associative learning in the T-maze. 20

4.1 Odour pre-exposure has differential effects on associative conditioning 33
4.2 Odour pre-exposure transiently inhibits memory retrieval 35
4.3 LI is sensitive to changes in context 37
4.4 Non-aversive odour concentrations do not induce LI 39
4.5 Odour pre-exposure leads to a transient aversive memory 41
4.6 Pre-exposure memory depends on concentration but not context . 43
4.7 Formation of pre-exposure memory requires Kenyon Cell activity 45
4.8 Reward and punishment reinforcing memories are involved in pre-exposure memory 47
4.9 Pre-exposure memory requires PPL1 DANs during acquisition . . 48
4.10 Pre-exposure memory requires $\alpha 3$ and $\gamma 2\alpha'1$ DANs 49
4.11 PPL1-$\alpha 3$ are dispensable for aversive shock conditioning 51
4.12 Blocking $\alpha 3$ and $\gamma 2\alpha'1$ MBONs disrupts pre-exposure memory . . 52
4.13 $\gamma 2\alpha'1$-MBON is required for retrieval of pre-exposure memory . . 53

5.1 R15A04-GAL4 driven expression of UAS-GFP 60
5.2 Activation of R15A04-GAL4 labelled PAM-DANs induce LTM . 62
5.3 Inverted sequential odour presentation and testing CS^+ vs novel odour do not reveal CS^+-specific immediate valence 65

5.4 PAM α1 labelling by the MB043C-GAL4 driver 67
5.5 Activation of α1 leads to both short- and long-term memory performance 68
5.6 Partially paired activation of γ5n suppresses immediate memory performance 70
5.7 Memory is recovered 24h after γ5n neuronal activation 72
5.8 Paired activation of VT006202-GAL4 throughout sucrose and odour presentation 73
5.9 Unpaired activation of γ5n inhibits long-term memory retrieval . . 75
5.10 Paired activation of 0804-GAL4 does not lead to competing short-term memory 76

A.1 MB247-GAL4 expression pattern 103
A.2 R58E02-GAL4 driver expression pattern 103
A.3 TH-GAL4 labels multiple subsets of Dopaminergic cells in the fly brain 104
A.4 MB504B-GAL4 labels PPL1 DANS 105
A.5 C601GAL4;MBGAL80 driver 105
A.6 MB630B-GAL4 driver 106
A.7 G0239-GAL4 106
A.8 MB058B-GAL4 driver 107
A.9 MB296-GAL4 driver 107
A.10 MB077B-GAL4 driver 108
A.11 Drivers for PAMs labelling γ5 compartment 108

List of Abbreviations

ARM . . . . . Anaesthesia-Resistant Memory

ASM . . . . . Anaesthesia-Sensitive Memory

CR . . . . . Conditioned Response

CS . . . . . Canton S (wild-type strain)

CS$^+$ . . . . . Conditioned Stimulus paired to outcome

CS$^-$ . . . . . Conditioned Stimulus not paired to outcome (control)

DANs . . . . . Dopaminergic Neurons

KC . . . . . Kenyon Cell

LI . . . . . Latent Inhibition

LL . . . . . Latent Learning

LTM . . . . . Long-Term Memory

MCH 4-Methylcyclohexanol

MO . . . . . Mineral Oil

MB . . . . . Mushroom Body

MBON . . . . . Mushroom Body Output Neuron

Oct . . . . . Octanol

PAM . . . . . Protocerebral Anterior Medial DAN cluster

PI . . . . . Performance Index

PPL1 . . . . . Protocerebral Posterior Lateral 1 DAN cluster

STM . . . . . Short-Term Memory

US . . . . . Unconditioned Stimulus (outcome)

Abstract

In a complex and constantly changing environment, animals are required to guide behaviour in a dynamic way to adapt to the current environment as well as the organism's needs. Although the ability to remember past events allows animals to predict future outcomes and thereby guide behaviour, an input does not always produce stereotypical behaviours. Factors such as conflicting information from parallel memories, cue specificity and context will impact the predictability of an outcome across time. Similarly, the animal's internal state influences whether the predicted outcome should be pursued at a given moment. Moreover, biological relevance of the memory, such as nutrient-reinforced associations, are thought to affect the lability of the memory and therefore how accessible it will be. Integration of predictability, motivation and memory stability allows a dynamic retrieval of appropriate memories to guide behaviour to properly balance their internal state.

Previous research in *Drosophila* has yielded detailed mechanisms describing the neural circuitry and molecular correlates involved in memory processing, but we lack a full understanding of how factors affecting memory retrieval become integrated to produce changes in behaviour. The work presented throughout this thesis uses *Drosophila melanogaster* to investigate the neuronal and behavioural mechanisms and how these factors regulate behavioural outcomes across time.

In this work, repeated stimulus presentations were used to address how stimulus exposure prior to learning affects the formation and expression of new memories across time. Our results show that odour pre-exposure leads to an aversive memory which competes with subsequently acquired memories, leading to latent inhibition of memory expression. Changes in context between pre-exposure and testing allow conflict resolution, retrieval of the appropriate trained memory and

inhibition of pre-exposure memory.

The second experimental chapter examines nutrient-reinforced long-term memory and the dynamics of its retrieval across time. Interestingly, neuronal stimulation studies have suggested that emergence of long-term nutrient-reinforced memory is independent of short-term memory, suggesting a delayed association between stimuli and nutrient-reward. However, the results presented here suggest that this apparent emergent memory results from an inhibition of short-term memory, potentially due to a motivational switch produced by co-activation of retrieval-inhibiting neurons.

Taken together, the work presented throughout this thesis delves into how different mechanisms, from memory integration to motivational suppression and context, lead to a temporal inhibition of memory retrieval to shape a changing behaviour across time.

***Keywords*—** Differential associative learning - Retrieval - Mushroom body - Dopamine

2 | Introduction

2.1 Psychology of Learning and Memory

2.1.1 Learning Associations

Knowing how to effectively guide behaviour is critical for survival. Learning provides a means for animals to create associations between environmental cues and potential outcomes. These associations, or memories, allow animals to create predictions about the environment and respond appropriately in order to balance their internal state.

Seminal studies of associative learning at the beginning of the 20th century by Pavlov and Thorndike studied animals' ability to link environmental cues and behavioural responses to outcomes [12, 13]. These studies established the principles of classical and instrumental associative conditioning (reviewed in [14]). Classical conditioning indicates that after pairing of a Conditioned Stimulus (CS^+) and an Unconditioned Stimulus (US), animals develop a Conditioned Response (CR) to the CS^+ similar to that produced by the US. Upon subsequent exposure to the CS^+, animals use this information to anticipate meaningful events and respond to the upcoming outcomes before experiencing them. This was showcased by Pavlov's dogs salivating upon the sound of a bell after repeated pairing of the sound to food availability. In this case, the CS^+ (bell) acquires a valence similar to that of the US paired to it (food delivery), producing a CR (salivation, Figure 2.1). On the other hand, instrumental conditioning and the law of effect proposed by Thorndike, established that behaviours that are followed by rewarding outcomes are more likely to be repeated, while those followed by unpleasant outcomes will be less likely to reoccur [13]. If after association, the CS^+ predicts an appetitive or

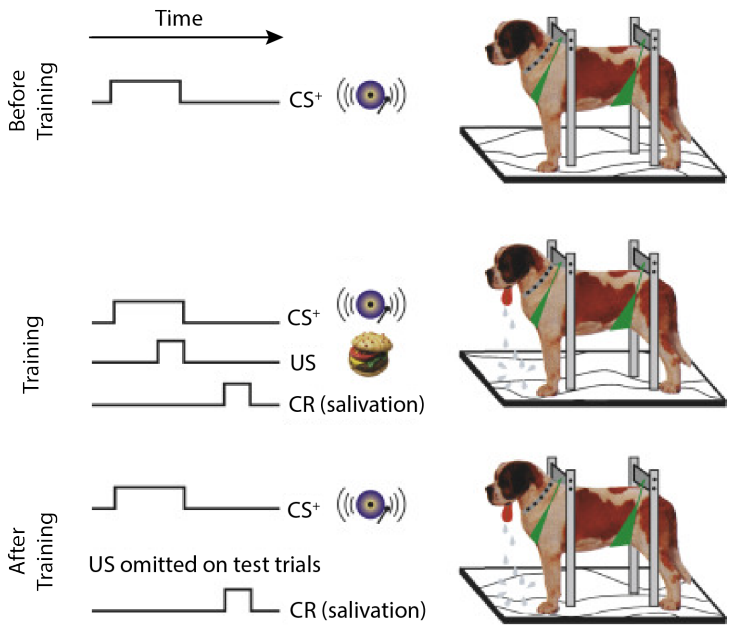


Figure 2.1: Classical associative conditioning

Schematic of how the response to a stimulus can change upon associative learning. In the top panel, before training, the bell (CS+) does not produce a response. During training (middle) the bell (CS+) is presented simultaneously with a food reward (US) leading to a salivation. Simultaneous pairing of CS and US creates an association between them. After training (bottom), this association will result in a conditioned response of salivation (CR) upon experiencing the same CS without the US. Modified from [15]

pleasant US, the animal might respond by approaching it. In contrast, a stimulus that has been paired to an aversive outcome is more likely to produce an avoidance or freezing response.

Years of studies have suggested that multiple factors influence the likelihood of the formation of a strong association (reviewed in [14, 16–18]). First, a CS^+ must be salient and distinguishable from the background context. This way, animals are more likely to pay attention to the stimulus. However, as we will see in Chapter 4, context will also play a major role in guiding behaviour by modulation of memory retrieval. Second, the CS^+ should be informative about the likelihood of an outcome or a change of state. This implies a role for temporal contingency between

the stimulus and the outcome in memory formation. If the CS^+ is followed by a US, the CS^+ might become a predictor of the outcome. However, if the CS^+ occurs after the US, as it does in backward conditioning, the stimulus might become predictive of termination of the US; leading to an opposing synaptic potentiation or depression response [14, 19–22]. This way, the same US-CS^+ pairing can lead to opposite behaviours or to a lack of association by changing the time contingency between the stimuli and outcome. In taste aversion learning, mammals and insects are capable of associating an ingested stimulus to a gastrointestinal illness outcome, leading to the future decrease in approach and consumption of the associated stimulus [23–26]. Interestingly, although this effect is still restricted in time and is subject to interference, taste aversion learning seems to occur when the illness occurs minutes or hours after consumption [27, 28]. Moreover, animals fail to strongly associate gastrointestinal illness to visual and auditory cues, suggesting that a generalised consumption->illness causal association allows post-ingestion learning [24]. As we will discuss in Chapter 5, a delayed association is also thought to occur with nutrient-dependent reinforcement, shaping the long-term memory phase of sucrose-reinforced appetitive learning [29, 30].

Once learned, associations will guide behaviour, allowing animals to maximise their fitness for as long as the memory is accessible. The accessibility of a memory will depend on multiple factors such as memory stability, interference and retrieval [14]. Because different memories decay on different timescales, not all memories influence behaviour for the same amount of time. For example, nutrient quality associated memories, persist for longer than those associated with sweet taste [31]. Long-lasting memories therefore have an effect on behaviour for an extended period of time compared to fast-decaying ones.

It is important to note that an animal can experience a CS^+ on several occasions throughout its life time, potentially leading to a varied range of paired US.

The multiple outcomes will not necessarily match previous experiences, and may sometimes conflict and interfere with the expression of prior associations. Moreover, as we will see in Chapter 4, sometimes there might not be an explicit outcome at all. Gathering more information about past outcomes allows animals to integrate multiple events to form a more accurate prediction about the potential future outcomes. Having differing memory dynamics associated to the same CS^+ allows a stimulus with conflicting information to shape behaviour dynamically across time [32].

Presumably, in a complex environment, multiple stimuli and contexts will be presented together. Therefore, it is not enough for an animal to execute a behaviour based on a single memory disregarding other information. This new memory has to be integrated to the memory repertoire, so the whole ensemble of memories can orchestrate an appropriate response. Behavioural guidance should therefore account for multiple presentations of a stimulus and its varied outcomes, but also consider the diverse range of stimuli and contexts present and their corresponding associations. This can be exemplified in *Drosophila*, where conditioning one stimulus to shock and another to sucrose results in an additive approach to a sucrose conditioned stimulus plus the avoidance of the shock-paired stimulus [33].

2.1.2 From Learning to Behaviour

But what determines the behavioural response? A big proportion of the initial studies of memory focused on memory acquisition as the main drive of memory performance. That is, the stronger the association, the more likely an animal will act on this association; the more relevant the memory, the more time it will influence behaviour [17]. However, as research in the field has progressed, this view has changed. It is now undeniable that behaviour is not determined exclusively by a cumulative summation of memory strengths. Memory retrieval tunes

behavioural action by operating through a combination of factors including the internal state of needs, hedonic and physiological motivation as well as the environmental availability [14, 16, 34]. The role of internal state in memory retrieval is clearly exemplified by the seeking of food-associated cues. Animals will approach these cues when hungry and ignore them when satiated [5, 6]. This does not mean animals have forgotten that the cue predicts food, but rather have become ambivalent to the cue. Internal state in this case plays a major role in memory expression and by consequence memory performance by producing a shift in motivational drive [5]. Environmental availability of resources is often indicated by context. We can imagine, how in the context of a restaurant, a cheesy odour would be perfectly appetitive and attractive for most people; however, this same stimulus in a shoe shop is likely to produce disgust. Context in this example provides a framework for selection of a memory to retrieve and guide different behaviours when presented similar cues [35, 36].

Integrating the robustness of multiple memories with the animal's internal state and context is critical for the retrieval process and for dynamic guidance of behaviour. Flexibility of behavioural determination allows animals to adapt to a constantly changing environment by filtering out irrelevant associations and allowing the animals to recall and act solely on what is relevant for a given situation. However, it is not completely clear how each of these factors are represented at a neuronal circuit level and how they are integrated. We know long-term memories impact behaviour for longer, but we do not fully understand the rules that lead to an event being consolidated and remembered for longer while others are soon forgotten (or simply not retrieved). Understanding how memories are encoded and integrated in the brain and the biological rules underlying their retrieval will shed light on how experience and state shape behaviour.

2.2 *Drosophila* as a Model Organism

The work presented here, uses the fruit fly, *Drosophila melanogaster* as a model organism to investigate the underlying neuronal circuits and behavioural correlates of these processes. *Drosophila melanogaster* are relatively inexpensive and easy to rear in large numbers under laboratory conditions. Its life cycle is much faster than those of mammalian animal models; with only 10 days at 25°C for an embryo to develop into a fully fertile adult, allowing multi-generational population studies. Most importantly, scientists have developed an extensive toolkit for *Drosophila*, that is endowed with many genetically encoded tools to manipulate, monitor and regulate neuronal activity and genes (reviewed in [37]. There is considerable conservation of genetic and physiological correlates with mammals that make it a good model to study molecular and cellular processes. Moreover, in terms of learning and memory, *Drosophila* has been shown to display reliable conditioning responses after a single training session, making it much simpler and cleaner to analyse immediate processes of learning at a molecular and behavioural level.

Arguably one of the most powerful genetic tools of the fruit fly is the implementation of genetically encoded binary expression systems, such as GAL4/UAS. These binary systems allow a controlled expression of target transgenes within a restricted tissue. The GAL4/UAS system works through an enhancer-specific expression of a yeast-derived transcription factor - GAL4, which will bind to an Upstream-Activating Sequence (UAS), promoting transcription of the downstream target (e.g. a reporter gene, an RNAi, a secondary transcriptional regulator, a molecular sensor, etc.) (Figure 2.2) [38]. The creation of independent GAL4 and UAS lines allows a wide mix-and-match approach to reproducibly drive the expression of genetic tools within the same cells. The addition of inducible tran-

scriptional repressors and activators, such as the temperature-sensitive GAL80^{ts} or hormone-dependent transcription, allows a layer of temporal restriction of expression [39, 40]. Moreover, to further spatially restrict expression of target genes, the Split GAL4s were developed, which is a modification of the GAL4/UAS technique [41, 42]. A collection of these driver lines has been made available allowing targeted gene expression of a countable number of cells, sometimes even at single-cell resolution [43–45]. This work takes advantage of the GAL4/UAS system and the Split GAL4 modification to target the expression of optogenetic and thermogenetic tools and manipulate neuronal activity, through activation and inhibition via expression of light-inducible Channelrhodopsins CsChrimson and GtACR or expression of temperature-sensitive Dynamin *Shibire*^{ts1} to block synaptic transmission [46–49]

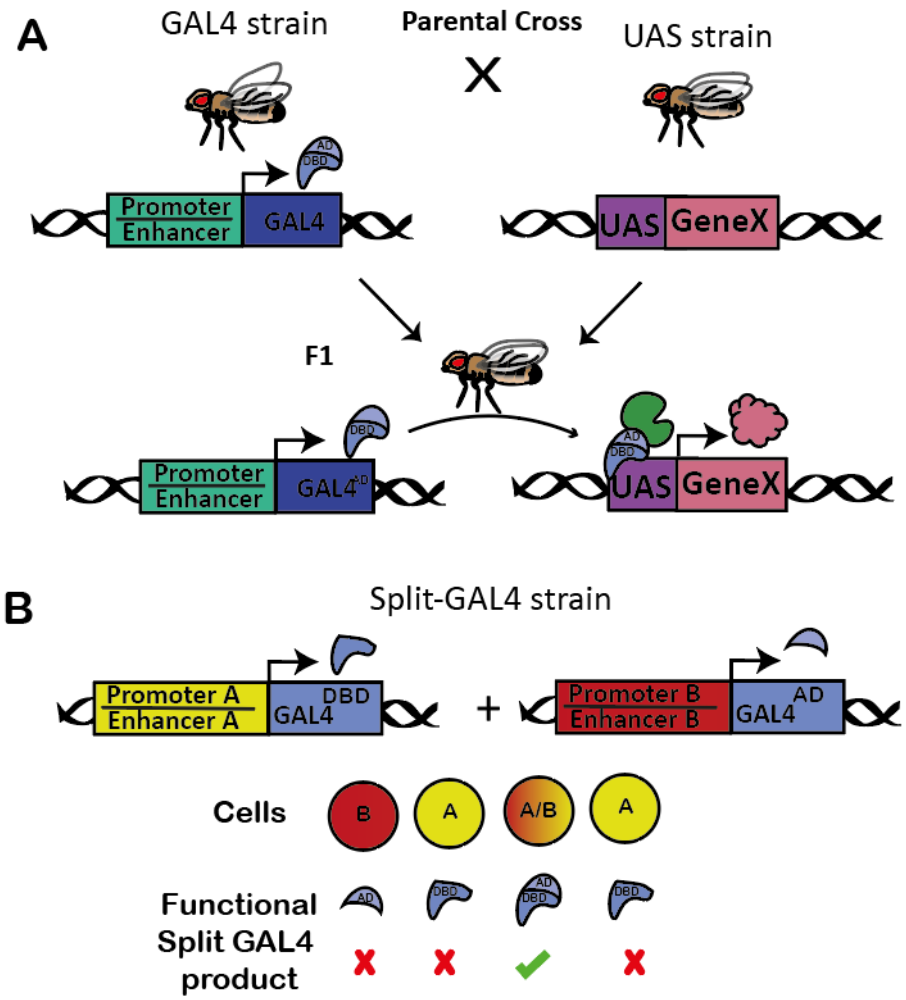


Figure 2.2: Binary expression system GAL4-UAS

A) Schematic of a GAL4-UAS system crossing scheme. A parental fly from the GAL4 strain contains a transgene with a tissue specific enhancer or promoter (green box) upstream the transcription factor GAL4 sequence (blue box). These GAL4 transcription factor is formed of a DNA binding domain (DBD) and an activation domain (AD) to recruit transcription machinery (green blob). GAL4 will be expressed only in the tissues where the promoter/enhancer is expressed. The other parental fly from the UAS strain contains a target gene (pink box) downstream of a Upstream-Activating Sequence (UAS, purple box). The offspring from crossing both strains would result in flies containing both transgenes. Tissue specific expression of GAL4 would then be able to bind the UAS sequence and promote expression of the target gene (pink blob). B) Restriction of expression can be achieved by Split-GAL4. In which the sub-units of the GAL4 transcription factor are expressed under different promoters. Promoter A (yellow box) will express the DBD and promoter B (red box) will express the AD. Only cells with active transcription of those promoters will carry both sub-units leading to a functional GAL4 protein.

2.3 Studying Learning and Memory in the Fly

Drosophila melanogaster has become one of the most popular animal models for scientific research. Therefore, it does not come as a surprise that researchers have examined at high molecular and circuit-level resolution how behaviour works using the fly for decades [50–52]. Because flies can perform complex behaviours with a simpler brain, this has allowed scientists to pinpoint behavioural, molecular and network principles of memory formation, consolidation and retrieval that would otherwise have been harder to achieve with a more complex model [52–57]. It is later the task of comparative studies to use these principles to aid in further understanding of memory across species. Therefore this work does not attempt to explain or generalise mechanisms across all phyla but rather understand how the *Drosophila* nervous system can produce complex behaviours, such as experience-based behavioural guidance.

Pioneering studies of learning and memory using *Drosophila* showed that adult and larval flies can be conditioned to associate olfactory cues with aversive or appetitive US, such as electric shock and sugar, to guide behaviour [33, 53, 58, 59]. The use of cold-shock anaesthesia, pharmacological intervention and mutant studies allowed the identification of distinct phases of memory [4, 54, 55, 60]. These phases are subdivided according to lability and their effect on behaviour. Non-consolidated Anaesthesia-Sensitive Memory (ASM) is easily disrupted upon cold-shock anaesthesia; ASM guides behaviour in the short term, after which they rely on consolidated memories. Anaesthesia Resistant Memory (ARM), as the name suggests, is not disrupted by anaesthesia and it is considered as a type of consolidated memory. Another type of consolidated memory is long-term memory (LTM). LTM differs from ARM on the requirement of protein synthesis for maintenance of the memory, it is also believed to be longer lasting than ARM.

Different acquisition and training protocols lead to distinct memory compositions [4, 55, 60]. Moreover, these studies demonstrate that flies can learn independent memories that guide behaviour in an additive way. This is, when using differential associative paradigms, if one stimulus (CS^+1) is paired to sucrose and another (CS^+2) to electric shock, the flies will approach the sucrose-predicting CS^+1 (and avoid the shock-predicting CS^+2) as an additive function of both memories [33]. This property of memory integration was later seen to apply not only to independent memories of different stimuli, but also to parallel memories linked to the same stimulus [32, 61]. Moreover, these studies allowed for the identification of the Mushroom Body (MB) as a critical brain region for learning in insects, as well as a detailed anatomical description of this region [7, 45, 62].

2.3.1 *Drosophila* Mushroom Body Anatomy

The Mushroom Body (MB) in Drosophila is an essential brain structure for olfactory learning and memory [62]. The MB is formed by a parallel array of Kenyon Cells (KCs), of which there are three main anatomically distinct types: α/β, α'/β' and γ. Both α/β and α'/β' KCs bifurcate their axons at the peduncle and create the vertical (α and α') and horizontal (β and β') lobes of the MB, whereas γ KCs do not bifurcate and form the horizontal γ lobe (Figure 2.3, left) [63–65].

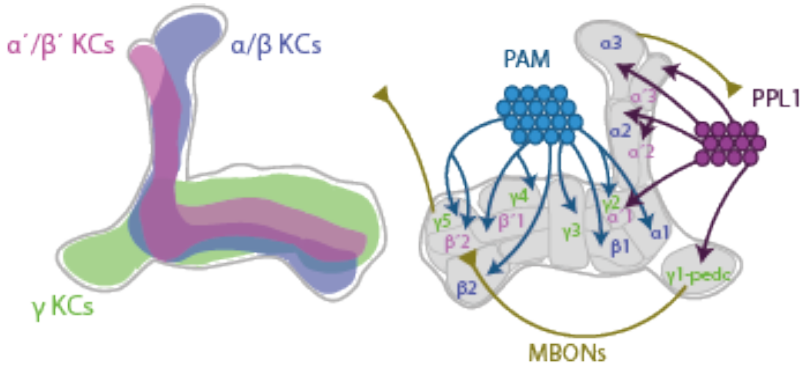


Figure 2.3: Mushroom Body Anatomy

Schematic representation of the Mushroom Body architecture. The left hemisphere illustrates the Kenyon Cell composition of the MB. Cell bodies are not shown. The axons of α/β KCs are depicted in blue, α′/β′ in pink and ɣ in green. The right hemisphere of the MB illustrates some of the input and output cells innervating the MB. PPL1 DANs in purple target mostly the vertical lobe, while the horizontal lobe is mostly innervated by PAM DANs in blue. Dendrites of MBONs innervate each of the compartments overlapping with DAN axons and project to different regions in the brain. Depicted here are three different types of MBONs which target outside the MB, within the MB or feedback into input DANs. Each KC-DAN-MBON compartment is considered a functional learning subunit.

The MB is divided into 15 anatomically restricted regions where axons of dopaminergic neurons (DANs) and dendrites of MB output neurons (MBONs) overlap and tile across the MB lobes, forming largely independent functional compartments (figure 2.3 right) [7, 45, 66]. DANs projecting to the MB lobes are broadly divided in three: those with cell bodies in the Protocerebral Anterior Medial cluster (PAMs), DANs with cell bodies in the Protocerebral Posterior Lateral 1 cluster (PPL1s) and those in the Protocerebral Posterior Lateral 2ab cluster (PPL2). The PAM DANs innervate eight of the compartments of the horizontal lobes (β1, β2, β′1, β′2, ɣ2, ɣ3, ɣ4, ɣ5) as well the vertical lobe compartment α1. Meanwhile, PPL1 DANs innervate the vertical compartments (ɣ1-pedc, α′1, α′2, α′3, α2 and α3) [7, 45]. Finally, PPL2 DANs project to the MB calyx, where KC dendrites reside, and are thought to convey memory strength rather than valence [67, 68].

Each compartment is also innervated by the dendrites of MBONs, which themselves send downstream projections to different regions of the fly brain. These targets include higher order neuropils outside the MB, where MBON activity promotes specific motor and behavioural responses such as approach and aversion [56]. However some MBONs, like MBON-γ1-pedc or MBON-β1 feedback onto the mushroom body to modulate MBON activity in other compartments to tune neuronal activity based on other factors, such as hunger state [5–7, 61]. In contrast, MBONs such as MBON-α3 and MBON-α1 feedback onto DANs to modulate their activity [7, 69]. This connectivity provides potential for multiple layers of regulation of neuronal output. Because many of the anatomical intricacies of the MB are known, this provides an ideal opportunity to determine how different features of learning are encoded on a mechanistic level. Therefore, the functional connectivity of the MB is of great interest to understanding the computations of how coincidence between olfactory stimulation from a CS^+ and reinforcement signals from a US can be integrated with other retrieval signals and lead to a change in behaviour.

2.3.2 Mushroom Body Circuitry in Learning and Behaviour

In the MB, odour identity is represented as sparse activation of KCs, which receive secondary olfactory information from the primary olfactory sensory neurons, via the projection neurons of which the axons are located in the calyx of the MB (Figure 2.4A) [65, 70–72]. PAM and PPL1 DANs provide reinforcement information. However, there is clear behavioural distinction between the two clusters. Co-activation of PAM DANs together with odour presentation leads to learned approach to the odour; while co-activation of the neurons in the PPL1 cluster during odour presentation leads to learned aversion. PAMs are therefore thought to provide reward-reinforcing signals, while PPL1 conveys a representation of pun-

ishment, such as shock [47, 73, 74]. Moreover, different subsets of DANs are thought to convey different properties of a reward, with some neurons reinforcing sweet components and others conveying the nutrient component of sucrose reward [30].

Similarly, MBONs can be broadly divided in two behaviourally defined groups, as neuronal activation of individual MBONs will either make flies approach (approach-directing MBONs) or avoid (avoidance-directing MBONs) the stimulation. Thus, learned behaviour is thought to be dictated by a balance between the output of approach and avoidance MBONs [6, 56, 57]. In naïve flies, an odour activates a subset of KCs which will activate MBONs with a relatively similar strength, leading to a net zero approach/avoidance (Figure 2.4A). During learning, coincidence activity of KCs and DANs leads to a synaptic depression of the overlapping KC-MBON connections (Figure 2.4B). In this way, learning produces a shift in the MBON-dependent motivational balance, prompting the overall network strength to favour approach over aversion, or vice versa, upon subsequent odour presentation (Figure 2.4C) [57].

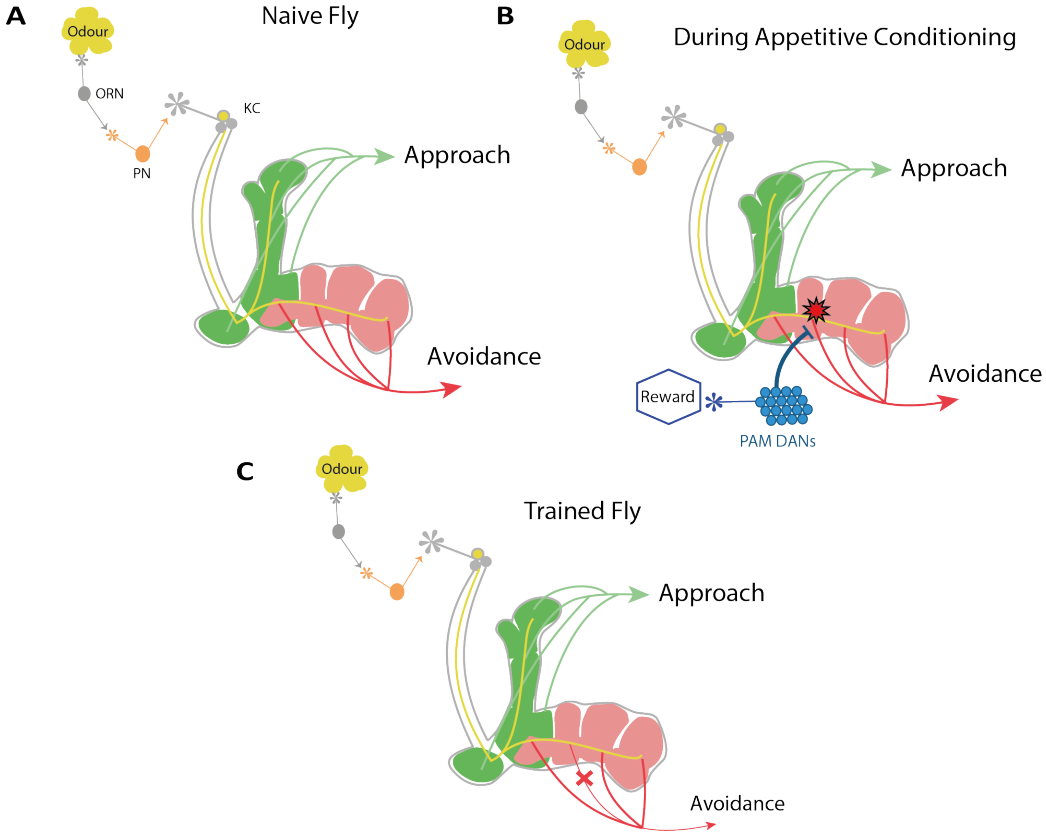


Figure 2.4: Model for associative learning in the Mushroom Body

A) In naïve flies, odour is detected by olfactory receptor neurons (ORN) which get conveyed to the Kenyon Cells (KC) in the mushroom body via projection neurons (PN). KCs activate both approach (green) and avoidance (red) driving MBONs, leading to a net zero approach/avoidance. B) During appetitive training, coincidence activity between odour encoding KCs and reinforcement through Dopaminergic neurons leads to MB compartment specific plasticity. C) In trained flies, the overall balance between approach an avoidance is skewed through inhibition of avoidance driving MBONs.

2.3.3 Aims

This thesis will focus on how integration of competing memories and motivational shift through state regulation can influence memory retrieval to modulate and shape behaviour across time. Using the fruit fly as a model organism allows us to use tools for investigation and manipulation of the temporal modulation of

memory performance at a circuit level. We examined quantitative behaviour and memory processing in the context of latent inhibition (Chapter 4) and nutrient-dependent memory formation (Chapter 5). These two phenomena share a diminished immediate memory performance followed by the emergence of performance later in time. As I will present throughout this thesis, these arises form two different mechanism. In Chapter 4, we provide evidence to show that latent inhibition in *Drosophila melanogaster* arises from context dependent integration of a competing memory generated by odour pre-exposure. Moreover, we show that latent inhibition results from an interference at the level of memory retrieval, and not through reduced attention impacting learning as it had been previously suggested. In Chapter 5, I show that the lack of short-term memory performance seen in previous reports describing nutrient-dependent memory is not originated from the temporally delayed emergence of a nutrient-reinforced memory. Instead I show that parallel co-activation of DANs innervating the γ5n compartment of the mushroom body inhibits memory retrieval, potentially through a shift in motivational state. Moreover, I propose PAM-γ5n activation can provide a motivational shift to inhibit memory retrieval of sucrose reinforced memories.

3 | Methods

3.1 Fly Strains

Flies were raised in a 12:12h light:dark cycle at 25°C on standard cornmeal food (100 g/l anhydrous d-glucose, 47.27 g/l organic maize flour, 25 g/l autolyzed yeast, 7.18 g/l agar, 12.18 g Tegosept (antifungal) in 8.36 ml of ethanol, per liter of fly food). Male flies from the GAL4 strains were crossed to virgin females from the UAS-CsChrimson or UAS-*Shibire*^{ts1} or UAS-GtACR1 strains at a ration of 3:1. Except for the 0804-GAL4, where females of the GAL4 strain were crossed to males of the UAS-CsChrimson. Wild-type strain Canton S (CS) were crossed to the different GAL4 drivers or UAS transgene strains as parental controls for heterozygosity and genetic background contribution. Crosses were left to lay eggs in standard yellow food for 2-3 days before being flipped to fresh food bottles. Mixed sex population of 4-8 day-old flies were aliquoted into groups of 100 flies and used for experiments.

Prior to all experiments requiring starvation, flies were aliquoted into groups of 100 flies which were kept in vials containing 1% agar. Flies were kept in the starvation vials for 19-24 hours at 23°C to promote food seeking behaviour.
For optogenetic manipulations using UAS-CsChrimson or UAS-GtACR1, flies were aliquoted and kept in food vials containing 1% all-trans retinal (100 mMolar concentration in 100% EtOH, Sigma-Aldrich) for 2-3 days before being moved to starvation vials as indicated above.
CS, 0804-GAL4, VT006202-GAL4, R35B12-GAL4 and c061-GAL4;MBGAL80 flies were obtained from the Waddell Lab central stocks. MB043C-GAL4 (BDSC:68363), R15A04-GAL4 (BDSC:48671), MB077B-GAL4 (BDSC:68316), MB504B-GAL4 (BDSC:68329), R58E02-GAL4 (BDSC:41347), G0239-GAL4 (BDSC:12639),

MB630B-GAL4 (BDSC:68334), MB058B-GAL4 (BDSC:68278), MB296B-GAL4 (BDSC:68308) and MB247-GAL4 (BDSC:50742) were obtained from Bloomington Stock Centre. For reference on expression patterns for each driver line see Annex A, or refer to Huetteroth et al 2015 [75], Otto et al 2020 [76] and Aso et al. 2014 [45]. Flylight collection from HHMI Janelia Research Campus provides a broad collection of confocal images of drivers expressing GFP that were used for confirmation of patterns of expression [43, 77].

3.2 Behavioural Assay

3.2.1 Differential Associative Learning in the T-maze

To study learning and memory in flies we used a previously described differential associative olfactory learning behavioural assay using a T-maze (Figure 3.1)[55]. For each N, 2 groups of 100 flies were trained and tested in parallel in two training chambers. During training, flies were presented with two odours: a control odour alone (CS^-) and a second odour (CS^+) which was paired to the presence of a US such as sucrose, shock or artificial neuronal activation. Each group was presented with CS^- for two minutes followed by a 30 second pulse of clean air and then two minutes of CS^+. CS^- was presented before CS^+ for all appetitive training, unless otherwise stated, to prevent any associations with metabolic processing from the appetitive training, such as digestion and nutrient sensing, that persist to become associated with the CS^-. During aversive training CS^+ was presented ahead of CS^- to prevent the CS^- acquiring a predictive valence. In all experiments, pairs conforming each N were conditioned with counterbalanced odours to control for odour preference. After training, flies were tapped into an elevator and moved to the testing chamber. Once there, flies were kept in the dark for two minutes to discriminate between CS^+ and CS^-. At the end of this time flies were collected and

a performance index was calculated (see 3.3). Flies in the T-maze will approach an odour that predicts reward and avoid an odour that predicts punishment.

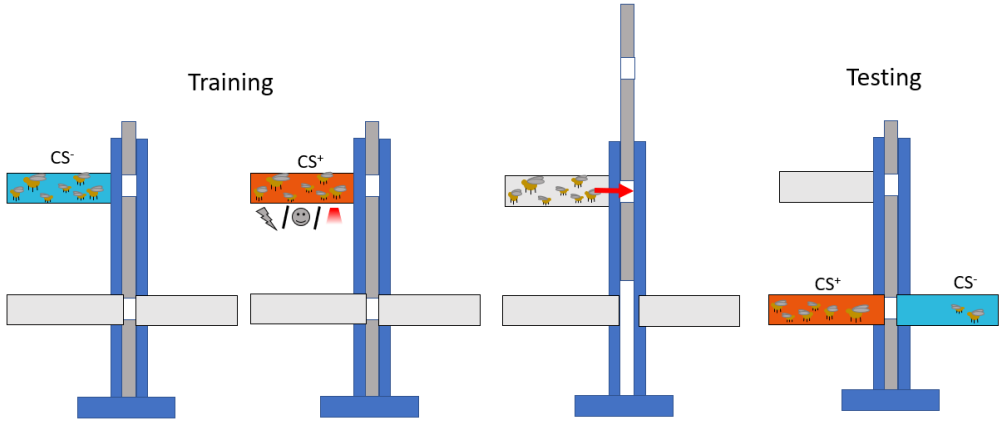


Figure 3.1: Differential associative learning in the T-maze.

Naïve flies are first placed in the training chamber (top) where they will be presented with two conditioned odours, CS^- (blue) and CS^+ (orange). The CS^+ is normally presented with an outcome such as punishment (shock), reward (sucrose) or neuronal activation (light). Flies will then be transferred into the testing chamber (bottom) where they will be presented with the choice of CS^+ and CS^-. While naïve flies have a balanced preference between these odours, after training, flies approach CS^+ if they formed and retrieve a rewarding memory and avoid it if they formed an aversive memory.

3.2.2 Naïve avoidance

To assess flies' naive odour preference to different concentrations of the odour, groups of 100 flies were transferred to the testing chamber of a t-maze. In one side of the t-maze, flies were presented with air; in the other arm, they were presented with the odour at either high or low concentration. Flies naive odour preference was calculated the same way as performance index, with air substituting CS^- and the odour substituting CS^+, see equation 3.1

3.3 Quantifying Memory Performance

We used Octanol (OCT) and 4-methylcyclohexanol (MCH) for the CS^+ and CS^-. The two groups of flies trained in parallel were conditioned with reciprocal odours to control for odour bias. After testing, flies were collected in tubes according to the odour which they chose at the end of the two minutes of testing. Flies were counted manually after at least 4 hours in the freezer to ensure they were dead. The performance index (PI) of each pair of groups is averaged to generate one N. The PI was calculated for each group using the following formula:

$$PI = \frac{\#\ of\ flies\ in\ CS^{+} - \#\ of\ flies\ in\ CS^{-}}{total\ \#\ of\ flies} \tag{3.1}$$

3.3.1 Statistical Analysis

Normality was tested using the Shapiro-Wilk normality test. For normally distributed data, ordinary one-way ANOVA test was performed to compare a group to two or more groups. For non-normally distributed data, Kruskal-Wallis test was used instead. Unpaired t-test was performed when comparing memory performance between an experimental group and its handling control. One-sample t-test was used to confirm memory performance different from 0, including for CsChrimson activation controls. In figures 5.5, 5.6, 5.7, 5.8 and 5.10, activation controls MB504B>CsChrimson and/or R58E02>CsChrimson were excluded from the group analysis and only used to confirm effective CsChrimson activation. For the emergence of artificial LTM in Chapter 5 Figure 5.2, a two-way ANOVA was performed main effect and interaction between genotype and time factors are reported. 2-way ANOVA also used in the low/high concentration naïve avoidance experiments in Chapter 4 (Figure 4.4. Tukey's, Dunnett's,

Dunn's and Sidak's post-hoc multiple comparison tests were performed as follows: Tukey's and Dunn's when comparing all groups to each other for normally and not-normally distributed data, respectively. Dunnett's when comparing one group to more than one other column. Sidak's was used when a selection of multiple columns was compared to their controls. When a statistic is not explicitly described, p>0.1. All graphs were created using GraphPad Prism and Adobe Illustrator. Statistical analyses were performed using GraphPad Prism.

3.4 Odour Delivery

Odours were delivered via air bubbled under vacuum through a mineral oil solution containing an odorant. The odours were diluted in 8ml of mineral oil (MO, Sigma-Aldrich) at the concentration of $1{:}10^{-3}$ for all experiments except for when low concentration is specified, where $1{:}10^{-6}$ was used. The odours used during these experiments were octanol (OCT, Sigma-Aldrich, 99%), 4-methylcyclohexanol (MCH, Sigma-Aldrich, 98%), indole-3-acetic acid (IAA, Sigma-Aldrich) and ethyl butyrate (EB, Sigma-Aldrich). For the novelty test experiment in Chapter 5, CS^- was always paired with IAA and CS^+ was paired with either OCT or MCH to counterbalance for odour preference.

Flies were always tested to discriminate between OCT and MCH. Odour concentrations were balanced for odour preference between MCH and OCT such that naïve flies would distribute 50/50 between the two odour choices. Training and odour pre-exposure will shift the balance between OCT-MCH preference through learning and/or familiarity and novelty and therefore be reflected as the change in the PI (see 3.3).

For MO exposure in Chapter 4, instead of odour, air was bubbled through the mineral oil solvent without any odours. For clean air delivery during training, no MO

was used, environment air was allowed to pass through using the vacuum source, without any bubbling.

3.5 Reinforcer Delivery

3.5.1 Sucrose Delivery

For appetitive conditioning in Chapters 4 and 5, flies were transferred to a transparent plastic T-maze tube lined with a dried filter paper, where they were first presented with a control odour, CS^- without any reinforcer for 2 minutes, followed by 30 seconds of clean air and then 2 minutes of CS^+. During the CS^+ presentation, flies were transferred to a T-Maze tube lined with a filter paper which had been pre-soaked in a 5M sucrose solution and left to dry over 2 days at room temperature prior to testing. Flies were free to sample and consume the sucrose throughout the time they were exposed to CS^+ during the training.

3.5.2 Artificial Neuronal Stimulation Delivery

Neuronal activation was performed using the light sensitive red-shifted channel rhodopsin CsChrimson, which depolarises the neuron upon light presentation leading to neuronal activation [48]. To do this, we followed previously described activation setup where heat sinks were fitted with three red LEDs [700mA, centered at 630nm] and mounted around the T-maze arms. Stimulation was triggered by light presentation delivered in pulses at a rate of 500Hz [48, 76]. According to each experiment, light presentation was paired or unpaired to odour delivery. As stated in the text, at times, light was presented in addition to sucrose delivery. Activation controls were performed to verify efficient CsChrimson induced activation. Odour paired activity from PPL1 and PAM DA neurons is fundamental

for aversive and appetitive memory [30, 78]. We therefore used PPL1 and PAM GAL4 drivers (MB504B and R58E02, respectively) as activation controls by pairing odour to the artificial neuronal stimulation via CsChrimson. These controls are necessary to show that the light is penetrating and reaching the flies, in normal transparent tubes, but also even in the presence of filter paper, as is the case for figures 5.6,5.7, and 5.8.

3.5.3 Shock Delivery

For aversive conditioning in Chapter 4, flies were transferred to a T-maze tube lined with a wired copper grid. Flies received 1 min of odour (CS^+) paired with twelve 1.5 second, 30V or 90V (as indicated) electric shocks every 5 seconds, followed by 45 seconds of clean air and then 1 minute presentation of a second odour without reinforcer (CS^-).

3.6 Neuronal Inhibition

Shibire is a dynamin fundamental for vesicle recycling. Studies have shown that the temperature-sensitive dominant allele *Shibire*^{ts1} can inhibit synaptic. When used at restrictive temperature (>29°C), *Shibire*^{ts1} prevents endocytosis, blocking dynamin-dependent synaptic transmission [49]. Prior research has shown that *Shibire*^{ts1} can incapacitate flies immediately after transferring them to a restrictive temperature, with a greater effect with longer times at restrictive temperature [49, 79, 80]. To inhibit neuronal transmission, we expressed a temperature sensitive Dynamin, *Shibire*^{ts1} using the drivers specified in each experiment. For consistency with previous research in learning an memory of adult *Drosophila*[6, 81, 82], and to make sure neurons were inhibited at the required time, in our experiments using UAS-*Shibire*^{ts1}, flies were transferred and incubated at 33°C for 30

minutes before proceeding with behavioural assays and kept at that temperature as indicated for the relevant experiments. Temperature controls were performed for experiments in which UAS-*Shibire*^{ts1} was used. These experiments replicated those done at restrictive temperatures but at permissive temperatures of 23°C. For all experiments reported here, temperature controls performed as parental controls, confirming performance is due to temperature inactivation of Shibire. This data is however not shown.

Although *Shibire*^{ts1} acts relatively quickly, flies require required between 2-5 minutes to begin showing signs of recovering [79], for which it can not be used to restrict neuronal inhibition to specific parts of the protocol without risk of disrupting other physiological processes. Optogenetic silencing provides a tool to allow temporal restriction of neuronal inhibition. GtACR1 is an anion Channelrhodopsin which promotes a rapid neuronal hyperpolarization upon green light presentation (peak at 530nm, 700mA)[83]. This can quickly be reversed by stopping light presentation [83–85]. Therefore, to temporally restrict neuronal inhibition, we expressed UAS-GtACR1 in the cells of interest using the drivers indicated in each experiment. Odour presentation was paired with continuous illumination from green LEDs [700mA, centered at 530nm] mounted around the arms of the T-maze.

3.7 Context

Context is referred to the T-maze tube environment in which flies experience pre-exposure, training or testing. For experiments using optogenetic activation, flies were trained in clear tubes, except when activation was paired to sucrose presentation; in which case tubes were lined with sucrose paper (see 3.5.1). Experimental controls for neuronal activation are provided. For sucrose conditioning, flies were

trained in tubes lined with filter paper (see 3.5.1) and tested in clear tubes. For all shock experiments, flies were trained in copper tubes (see 3.5.3). For both appetitive and aversive training, flies were tested in clear tubes, unless otherwise stated. In Chapter 4 we tested the effect of context by changing the conditions in which flies were pre-exposed, trained or tested through different experiments. This is indicated in the text and figures as follows: Context A, standard plastic T-maze tubes were used without any lining; for Context A*, the same T-maze tubes were lined with filter paper. For Context B, shock T-maze tubes lined with copper grid were used. These shock tubes were not wired and no current was passing through the grid.

4 | Prior Experience Affects Expression of New Learning

4.1 Chapter Abstract

Stimuli acquire valence based on subsequent outcomes. This first experimental chapter will explore how a stimulus can acquire valence without explicit presentation of a reinforcer. We will focus on how multiple presentations of a stimulus can generate competing memories and how these are integrated to generate dynamic behaviour across time. In particular, this chapter addresses how prior exposure to an olfactory stimulus can alter the expression of subsequently acquired memories associated to that stimulus. We found that presentation of an odour alone, without any explicit reinforcer transiently inhibits expression of subsequent appetitive memories and enhances aversive memories in *Drosophila*. Our data suggest that this transient effect results from creation of a short-lived aversive memory during the odour pre-exposure phase. Integration of the two memories with different decay rates allows dynamic recovery of the second memory while the initial transient memory decays. Conflict resolution between the aversive and appetitive memories can be achieved by a change in context between the pre-exposure, training, and testing phases, allowing the appetitive memory to be expressed before the aversive pre-exposure memory decay time. This chapter provides evidence of the neural circuitry involved in the creation of the short-lived aversive memory produced by the unreinforced odour pre-exposure.

4.2 Introduction

In nature, animals are likely to experience a given stimulus on multiple occasions. In order for the predictions to accurately guide behaviour, animals must assess the valence of a given stimulus' outcome. Outcomes following a cue might be straight forwardly rewarding, such as tasty food, or aversive, such as punishments or electric shocks. However, outcomes can be quite complex combining appetitive and aversive reinforcers. Animals can evaluate and integrate multiple components with opposite valences within a complex outcome to modulate behaviour across time [25, 32, 86, 87]. Moreover, valence of the outcomes following a stimulus presentation might change over time; an odour that one day predicted food, can another time predict a predator. Animals' ability to integrate multiple stimulus presentations to reflect the variety of outcome valences is critical to be able to generate a more accurate prediction and therefore guide decision making in a dynamic way.

The time contingency of a cue and its outcome is critical for memory formation [14, 20–22]. Occasionally, stimuli are experienced without any explicit outcome. Interestingly, the unreinforced presentation of a stimulus can acquire a valence despite the lack of an explicit outcome, as the absence of an outcome would be the outcome. Presentation of a stimulus without its predicted outcome from previous associative events leads to the extinction of that memory. For example, animals trained in an aversive paradigm when subsequently presented with the CS^+ in absence of the expected punishment will interpret this event as rewarding despite the lack of an explicit reward. This prediction error is regulated by reward-reinforcing dopaminergic neurons, leading to a competing appetitive memory which is integrated in parallel to behaviourally neutralise the previously formed aversive association [61, 88, 89]. However, the original memory is only

inhibited by this integration and not erased, as evidenced by spontaneous recovery of the performance seen in extinction experiments [90]. Moreover, a stimulus which has neither a predicted nor an explicit outcome associated to its presence, can acquire a valence when it follows another association or outcome. An example of this is the valence acquired by the CS^- following an aversive spaced training protocol; where despite CS^- not being explicitly associated to anything, it occurs so close in time to the CS^+ that it acquires a positive valence, consistent with a safety memory [81]. Multiple presentations of a stimulus provides animals more information about the potential outcomes and therefore leads to a learned change in behaviour. In the short term, repeated presentations of a stimulus are also known to change the animal's response through sensitisation and habituation [91, 92].

In the associations mentioned above, the non-reinforced stimulus acquires a valence relative to either the termination of another stimulus association (safety memory) or to an expected outcome (extinction). Unreinforced exposure to a stimulus prior to training would lack the relative valence from an expectation as well as from an alternative stimulus valence; thus it was thought to affect behaviour mostly through sensitisation or habituation [92–95]. However, experiments exposing rats to a maze without any reward saw an enhanced performance once trials begin to be rewarded compared to mice without pre-exposure to the maze [96, 97]. This suggested that animals learn information about the environmental cues in the absence of a reinforcement; this Latent Learning (LL) is not expressed unless it is required to receive the reward. Interestingly, other experiments found that stimulus pre-exposure leads to a transient inhibition of the memory performance of subsequent associative events. This phenomenon is known as Latent Inhibition (LI) [98, 99]. Remarkably, LI is sensitive to context changes, suggesting animals are associating the stimulus to the environment [100]. However, it is unclear

whether this learning interferes with the animal's ability to learn new associations to the pre-exposed stimulus, by ignoring new information (across no-association and association trials); or whether this memory is integrated to compete with the retrieval of subsequent memories in an additive way.

To determine by which mechanism LI operates, we can assess whether later spontaneous recovery of memory performance occurs, which would suggest that learning occurring during the pre-exposure inhibits memory performance rather than the learning. Mammals require multiple conditioning trials for reliable memory performance, making it difficult to disentangle whether this recovery comes from mitigating acquisition or retrieval. Appetitive associative learning in the fly occurs after a single training session, which allows us to dissect this phenomenon. In this chapter we examined how LI arises in *Drosophila* and whether stimulus pre-exposure can acquire a valence without explicit reinforcement. These results provide an important framework of how information is integrated across time and how valence is encoded in the brain in absence of a reinforcer.

4.3 Results

4.3.1 Odour Pre-exposure Effect in Memory Performance

LI has not been seen using a single pre-exposure event [101]. We investigated the effect of repeated odour pre-exposure on differential associative learning performance using two conditioning protocols: appetitive and aversive conditioning. Repeated exposure to an odour has been demonstrated to change responses to that odour through familiarity and habituation, however, LI in flies has not been demonstrated previously [92].

4.3.1.1 Odour Pre-exposure in Appetitive Conditioning

During a standard appetitive differential conditioning paradigm, flies are presented with a 2 min presentation of an odour alone (CS^-) followed by 30s of clean air before experiencing two minutes presentation of a second odour (CS^+) paired with sucrose presentation. To determine the effect of pre-exposure on appetitive memory, wild-type CS flies were pre-exposed to the CS^+ twice for 2 min with a 15 min inter-pre-exposure interval before being trained using an appetitive conditioning paradigm (Figure 4.1A). Consistent with previous LI studies, when flies were pre-exposed to the CS^+ prior to conditioning, memory performance was impaired compared to flies that were instead pre-exposed to the odour's solvent, mineral oil (MO) (Figure 4.1A). Interestingly, when flies were pre-exposed to the future CS^-, they performed better than those exposed to MO (Figure 4.1B).

4.3.1.2 Odour Pre-exposure in Aversive Conditioning

We next asked whether CS^+ and CS^- pre-exposure consistently inhibited and enhanced, respectively, memory performance in 30V shock-reinforced aversive

training protocols. Surprisingly, we found that pre-exposure to either CS^+ or CS^- in aversive conditioning had opposite effects to those in appetitive training. CS^+ pre-exposure led to an enhancement of aversive memory performance (Figure 4.1C), while flies pre-exposed to CS^- showed a reduced memory performance compared to flies pre-exposed to MO (Figure 4.1D). For simplicity, we mostly focused on appetitive memory performance inhibition to study the effect of odour pre-exposure on LI.

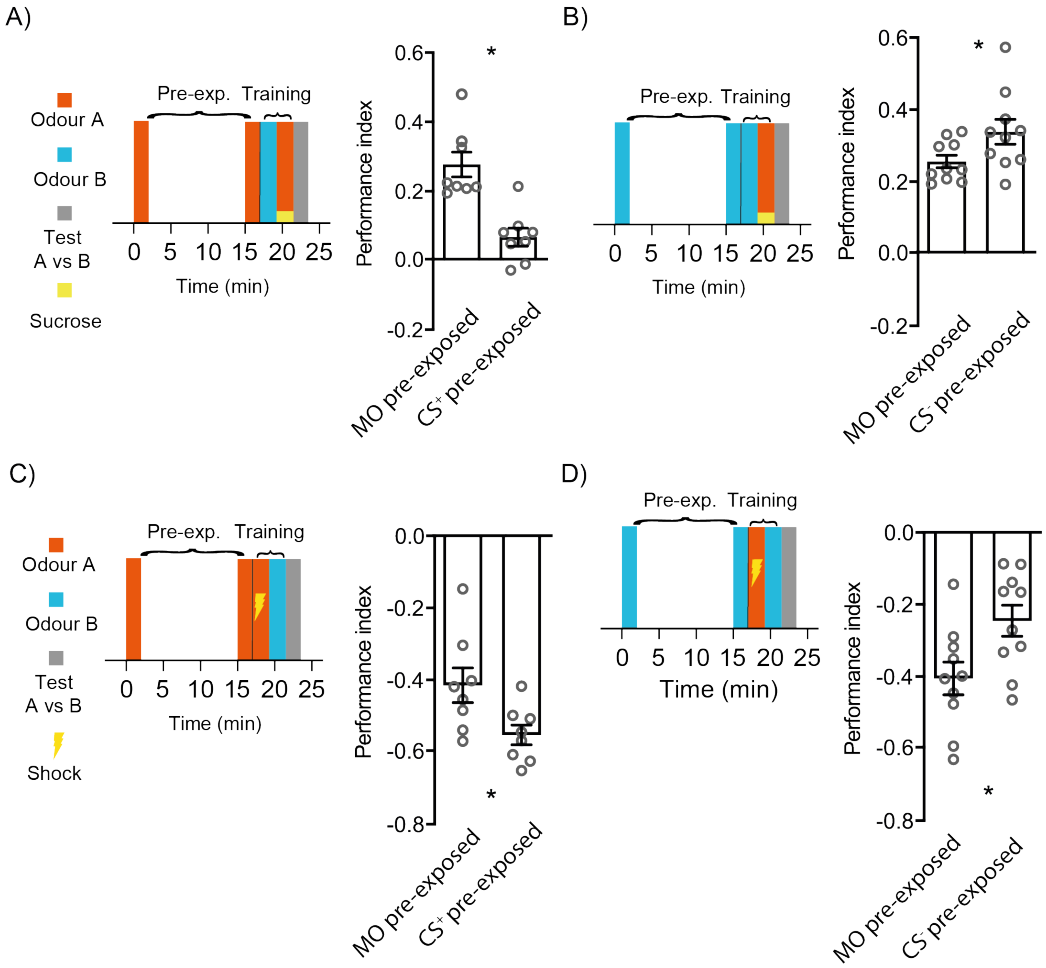


Figure 4.1: Odour pre-exposure has differential effects on associative conditioning A) Flies trained with appetitive conditioning and given double odour pre-exposure to odour A (future CS^+, orange, n=8) prior to training show impairments in performance compared to flies pre-exposed to mineral oil (MO, n=8; unpaired t(df=14) = 4.719, p = 0.0003). B) Flies trained with appetitive conditioning and given double odour pre-exposure to odour B (future CS^-, blue, n=10) show enhanced memory performance compared to flies pre-exposed to mineral oil (MO, n=10; unpaired t-test t(df=18) = 2.127, p = 0.0475). C) Flies trained with 30V shock-reinforced aversive conditioning and pre-exposed to odour A (future CS^+, orange, n=8) show an enhancement in memory performance compared to flies pre-exposed to mineral oil (MO, n=8; unpaired t-test t(df=14) = 2.485, p = 0.0262). D) Flies trained with aversive conditioning and pre-exposed to odour B (future CS^-, blue, n=10) show an impaired memory performance compared to flies pre-exposed to mineral oil (MO, n=10; unpaired t-test t(df=18) = 2.560, p = 0.0197).

4.3.2 Odour Pre-exposure Leads to Latent Inhibition

To determine how odour pre-exposure leads to LI, we investigated for how long odour pre-exposure inhibits memory performance by spacing out the time between the odour pre-exposure and the training. When the second pre-exposure occurred 2h before appetitive training, memory performance was impaired but partially recovered compared to flies pre-exposed to MO (Figure 4.2A). Subsequently, when odour pre-exposure and testing were spaced by 4h, memory performance was indistinguishable from the control (Figure 4.2B). Taken together, these data indicate that pre-exposure to the CS^+ odour leads to latent inhibition of appetitive memory.

In line with LI, the effect of odour pre-exposure on performance vanishes with time. We therefore asked whether this arose as a consequence of suppressing acquisition or retrieval. To resolve this, we trained flies immediately after pre-exposure but waited 4h before testing. Because pre-exposure suppression of appetitive memory decays at 4h, we reasoned that if pre-exposure was affecting acquisition, memory performance would be disrupted 4h after testing. Moreover, if pre-exposure was affecting memory retrieval, performance would be recovered at the 4h mark. Consistent with an inhibition of retrieval, memory performance of flies pre-exposed to the CS^+ was indistinguishable from those flies pre-exposed to MO (Figure 4.2C). These results suggest that following this pre-exposure protocol, flies' ability to learn remains intact and they are able to integrate the outcomes of prior experiences at the level of retrieval.

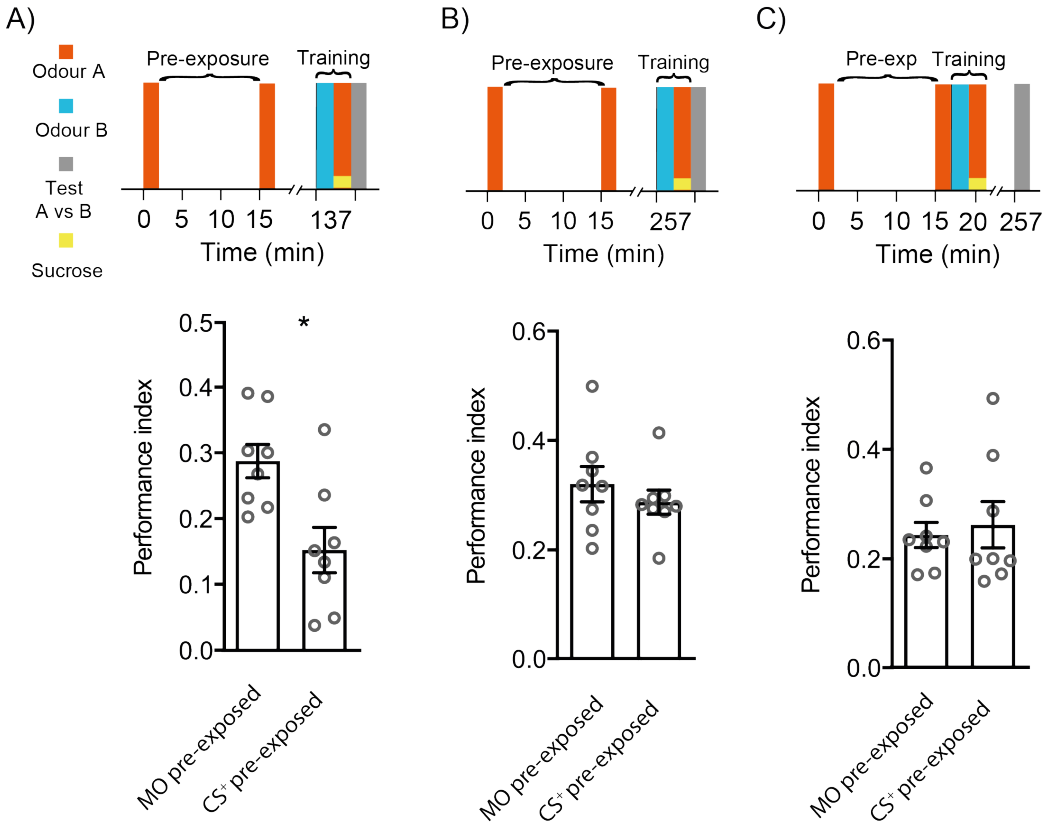


Figure 4.2: Odour pre-exposure transiently inhibits memory retrieval
A) Spacing training from the pre-exposure phase by 2h showed a significant reduction in memory performance compared to flies exposed to MO in the same protocol ($t(df=14) = 3.155$, $p = 0.007$). B) Memory performance in flies pre-exposed to the odour was recovered and not significantly different from flies pre-exposed to MO when training occurred at 4h after pre-exposure ($t(df=14) = 0.8368$, $p = 0.4167$). C) Flies that were trained immediately after odour pre-exposure and tested at 4h recovered memory performance which was indistinguishable from flies pre-exposed to MO ($t(df=14) = 0.3877$, $p = 0.7041$). A)-C) unpaired t-test, all groups n=8.

4.3.3 LI in *Drosophila* is Context and Concentration Dependent

As mentioned previously, LI in mammals is sensitive to context changes as well as to the properties of the pre-exposed cues. To determine if odour pre-exposure

leads to LI akin to that seen in mammals, we changed the contexts in which flies were either pre-exposed, trained or tested and assessed the impact on LI. The context was modified by using different tubes for each phase; we used either clear tubes (A), clear tubes lined with filter paper (A*) or copper-wired tubes (B) (Figure 4.3A). Given the methodological limitations (see 3.5.1), all appetitive training was done in the A* context.

For all previous and future appetitive conditioning experiments, with the exception of the ones mentioned in this section, flies were presented with a AA*A context, where they were pre-exposed in clear tubes (A), trained in clear tubes lined with filter paper (A*) and tested in clear tubes (A). As we saw before, flies that went through the LI protocol in this context sequence had impaired memory performance compared to those pre-exposed to MO (Figure 4.1A). We saw a similar inhibition when the context was consistent between pre-exposure and training and similar to that of testing (A*A*A, Figure 4.3B). However, when the context in the pre-exposure differ greatly from that in the training and testing (BA*A), LI was not seen (Figure 4.3C). Restoring the consistency between pre-exposure and testing was enough to recover the LI phenotype (Figure 4.3D).

Interestingly, while it seems that consistency between pre-exposure and testing is critical for LI in appetitive learning, this did not seem to be the case for the performance enhancement seen in aversive conditioning following odour pre-exposure. For the previous aversive experiments, flies were pre-exposed and trained in a different context from testing (BBA), which led to an enhancement of aversive memory performance compared to flies pre-exposed to MO (Figure 4.1C). However, this enhancement was not seen when context between pre-exposure and testing was consistent with each other but different from that of training (ABA, Figure 4.3E).

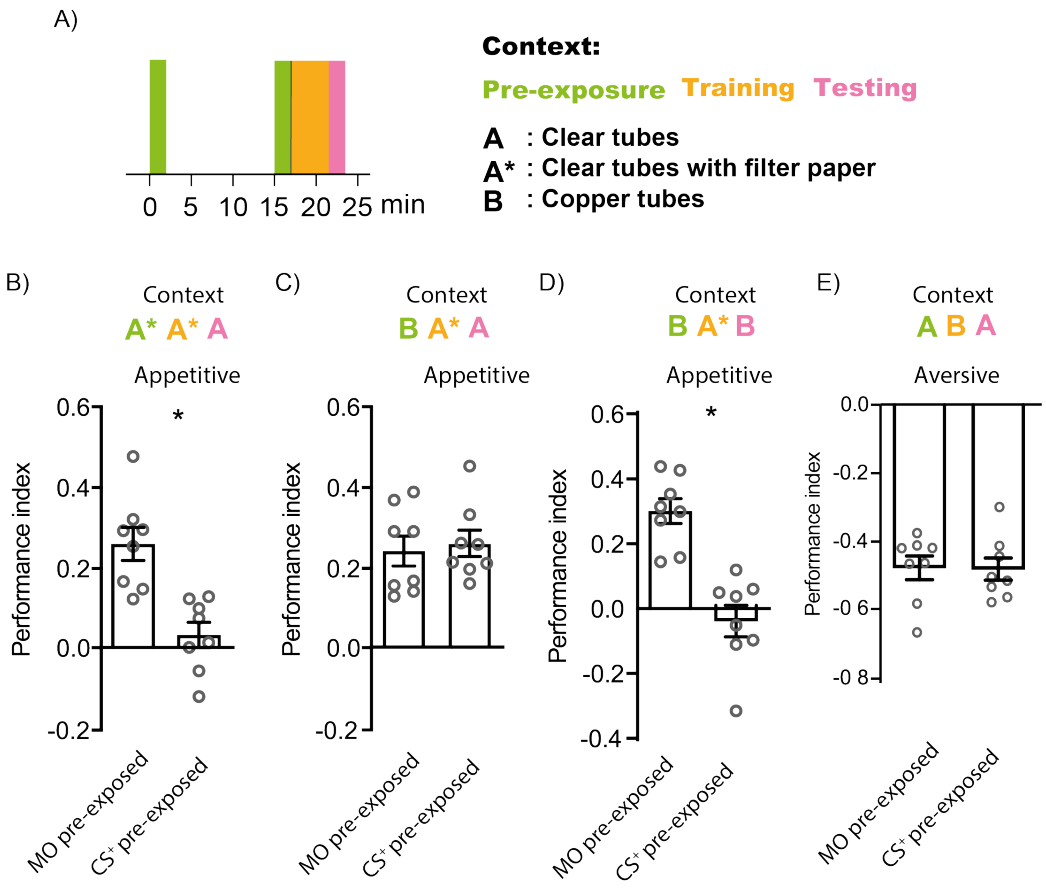


Figure 4.3: LI is sensitive to changes in context

A) Schematic of the pre-exposure/training/testing phases in which context was changed. Context in these phases was either clear tubes -A-, clear tubes with filter paper -A*- or copper tubes -B-. The order and colour determine which phase of memory the context refers to. Green for pre-exposure context, yellow for training context and pink for testing context. The pre-exposed odour corresponded to the CS^+ during training. B) When flies were pre-exposed to the odour and trained in a similar context to testing -A*A*A-, memory performance was significantly impaired compared to flies pre-exposed to MO in the same context (unpaired t-test $t(14) = 4.376$, $p=0.0006$). C) Memory performance was indistinguishable between flies pre-exposed to the odour and flies pre-exposed to MO when pre-exposure context differed from the testing and training -BA*A- (unpaired t-test $t(14)=0.3902$, $p>0.5$). D) Memory inhibition in odour pre-exposed flies was evident compared to MO pre-exposed flies when flies were pre-exposed and tested in the same copper tubes context ($t(14) = 5.449$, $p<0.0001$). E) Memory enhancement in aversive conditioning is disrupted by changes in context. A)-E) unpaired t-test. All groups n=8

We tested whether properties of the odour influence its ability to induce LI. Depending on the concentration used, flies will adjust their behaviour to the odour. While high concentrations tend to be aversive, lower concentrations tend to be neutral or attractive. We confirmed this to be the case for the odours used in this study (OCT and MCH) and for two more odours used in olfactory conditioning (IAA and EB). We found that reducing the odour concentration by three orders of magnitude, from $1{:}10^{-3}$ to $1{:}10^{-6}$, would reverse the response from avoidance to approach (Figure 4.4A,B). We therefore tested whether pre-exposing, training and testing the flies using a lower concentration of the odours, $1{:}10^{-6}$ as opposed to $1{:}10^{-3}$ used for all other experiments, would induce LI. Surprisingly we found that flies did not show a reduced memory performance when low concentrations of the odour were used; their performance was indistinguishable from those pre-exposed to MO (Figure 4.4C).

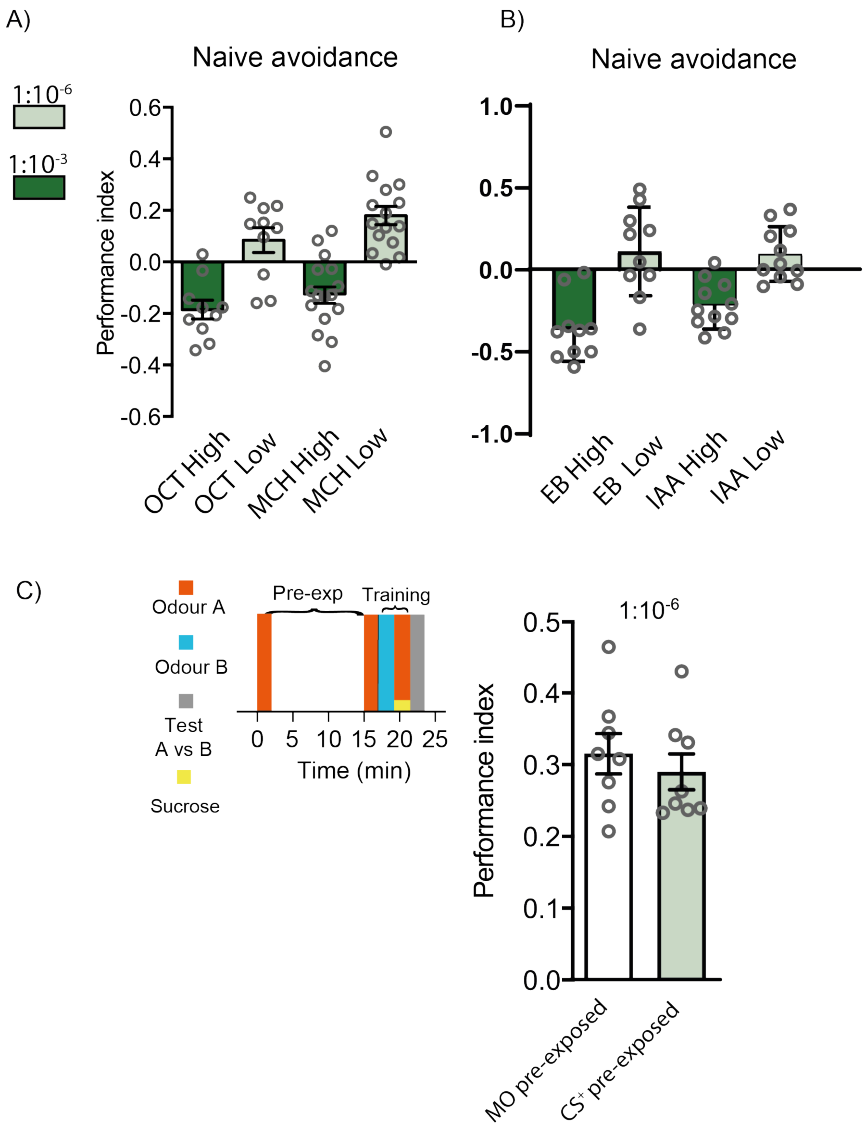


Figure 4.4: Non-aversive odour concentrations do not induce LI
A) Naïve odour avoidance to high ($1{:}10^{-3}$) and low ($1{:}10^{-6}$) concentrations of MCH and OCT. Flies avoid high concentrations but not low concentrations. Oct High n=10, OCT low n=10, MCH high n=16, MCH low n=16. B) Flies avoid high concentrations of EB and IAA (two-way ANOVA effect of concentration (1,38)=42.21, p<0.0001, effect of odour identity (1,38)=1.188 p=0.2826, Interaction (1,38)=1.828, p=0.1844) EB high and low n=10, IAA high and low n=11. C) Pre-exposure to low concentration of odour A (future CS^{+}) failed to inhibit memory performance of subsequent training, this was indistinguishable from flies pre-exposed to mineral oil (unpaired t-test t(df=14)=0.6741, p=0.5112).

4.3.4 Repeated Unreinforced Odour Pre-exposure Produces Aversive Memory

Taken together, these results indicate that latent memory inhibition induced by odour pre-exposure results form a temporary inhibition of retrieval and not from a disrupted learning. It is likely that flies are evaluating pre-exposure relevance based on the context in which they are pre-exposed or trained. However, it is not clear whether pre-exposure alone is able to inhibit memory retrieval through habituation or through a competing memory.

Importantly, repeated CS^+ pre-exposure led to opposite effects in appetitive an aversive conditioning, indicative of the presence of an additive aversive element from pre-exposure to high odour concentrations. This is supported by the opposing effects seen when pre-exposing the flies to the CS^-.

To test this, we investigated whether repetitive unreinforced odour presentation was enough to induce behavioural changes towards the pre-exposed odour when compared to a novel odour. We used three different exposure protocols: in exposure protocol 1 (E1), flies were presented with a single 2 minute exposure session before testing; in exposure protocol 2 (E2), flies were presented with a 4 minute presentation of the odour before testing; and in exposure protocol 3 (E3), flies experience the same pre-exposure protocol that was shown to produce LI, consisting of two exposure sessions of 2 minutes each (Figure 4.5A). After exposure, flies were tested with a choice between the pre-exposed odour vs. a novel odour. Interestingly, only flies in protocol E3 avoided the pre-exposed odour, while flies in E1 and E2 did not (Figure 4.5A).

This experiment suggests that multiple exposure events are necessary for pre-exposure to produce avoidance behaviour. Moreover, flies in E2 experience the odour for the same overall duration as flies in E3, indicating that this avoidance is

a conditioned response and not arising from a habituation effect.

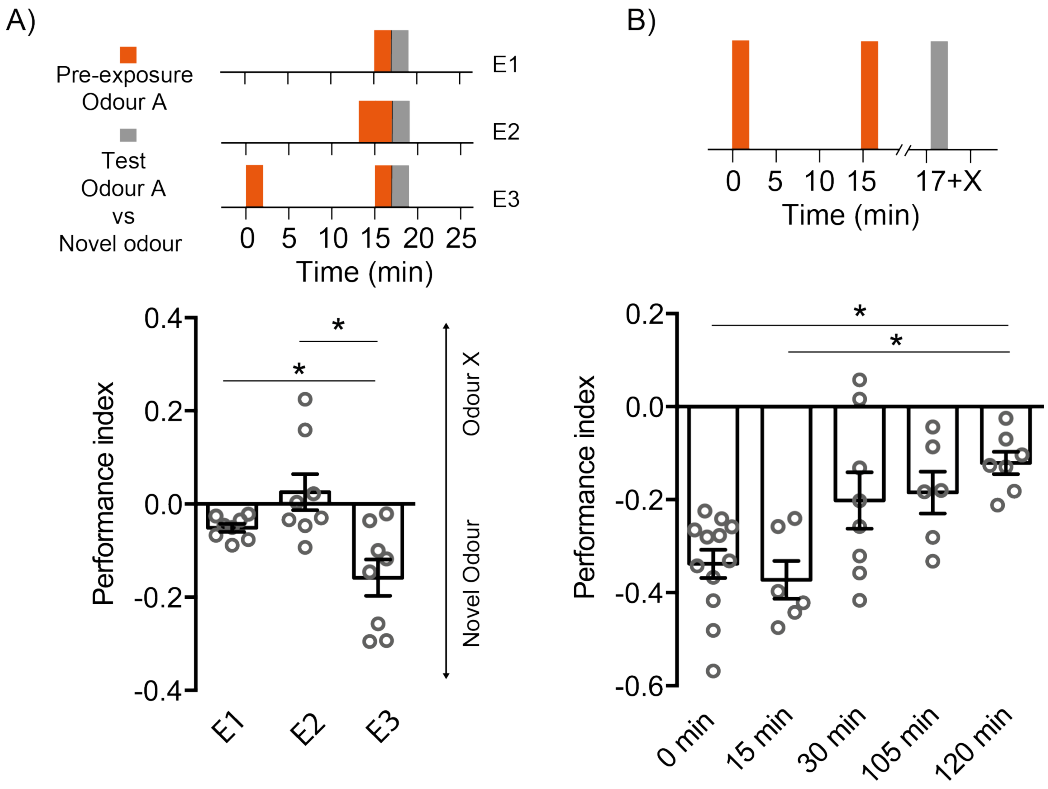


Figure 4.5: Odour pre-exposure leads to a transient aversive memory

A) Three different odour exposure protocols were tested. In E1, flies were exposed to 1x 2min exposure to odour A followed by testing preference between odour A and a novel odour. In E2, flies were exposed to 1x 4min odour A presentation followed by testing. In E3, flies received 2x 2min exposures to odour A separated by 15min inter-exposure interval. Only flies that went through the E3 exposure protocol showed an aversive memory against the pre-exposed odour compared to the other protocols (One-way ANOVA [2,21]=8.365, p=0.0023, for all groups n=8). Flies that went through E1 or E2 protocols failed to change the odour preference (Tukey's post hoc: E1 vs. E2, p=0.07; E1 vs. E3, p=0.0182; E2 vs. E3, p = 0.0048). B) Odour pre-exposure memory decays significantly by 2h after pre-exposure (one-way ANOVA F(4,34)=6.295 p=0.0007, Tukey's post hoc 0min vs. 120min, p=0.0036; 15 vs. 120 min, p=0.0040). A) and B). Non-explicit comparisons p>0.06.

4.3.4.1 Pre-exposure Memory Depends on Concentration but not Context

Consistent with the idea that aversive memory created during odour pre-exposure competes with the trained appetitive memory leading to LI, we found that pre-exposure memory decays at the same rate as LI (Figure 4.5B). Furthermore, pre-exposure to high concentrations of the odour lead to avoidance response to the pre-exposed odour, while lower concentrations did not (Figure 4.6A). This is in line with pre-exposure to high, but not low, concentration inducing LI of subsequent training (Figure 4.4B). In contrast to LI, we did not find pre-exposure memory to be sensitive to context changes between the pre-exposure and testing phases (Figure 4.6B).

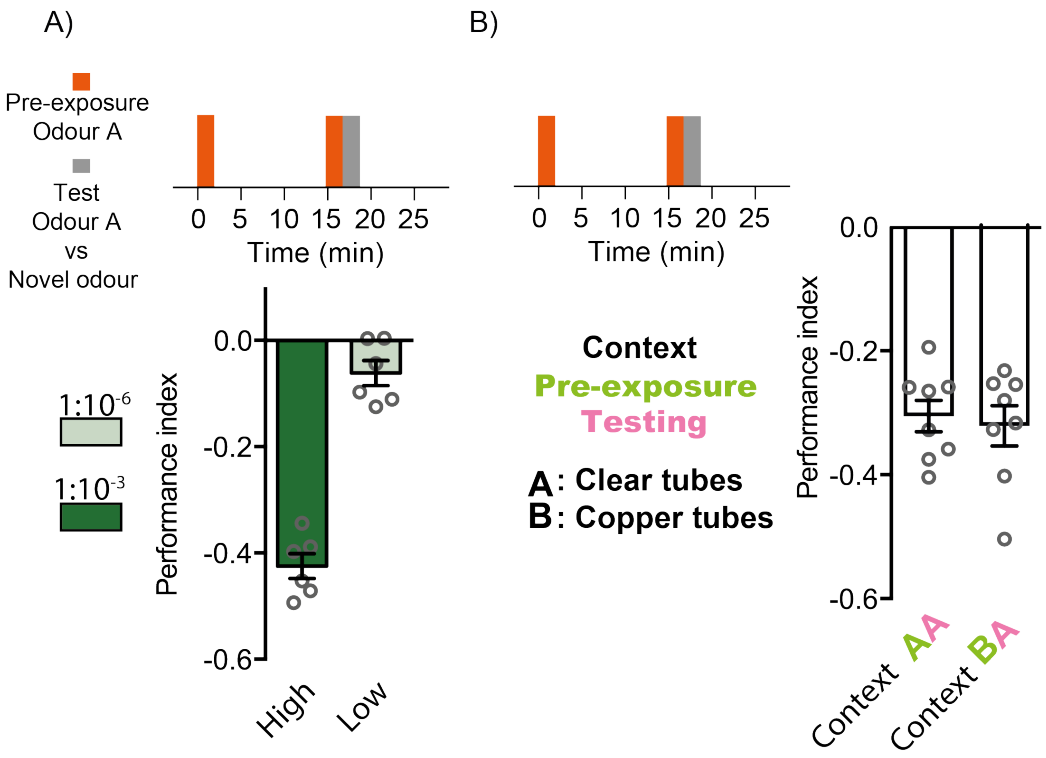


Figure 4.6: Pre-exposure memory depends on concentration but not context

A) Odour pre-exposure memory was only formed when flies were presented with high concentrations of odours ($1{:}10^{-3}$) and not low concentrations ($1{:}10^{-6}$, unpaired t-test; $t(10)=10.98$, $p<0.0001$, $n=6$ per group) B) High concentration pre-exposure memory does not get disrupted with context changes. Flies pre-exposed to the odour in clear tubes (context AA) did not differ from flies pre-exposed in copper tubes (context BA) when tested in clear tubes (unpaired t-test, $t(14)=0.3827$, $p=0.7077$, $n=8$ per group)

4.3.5 Neuronal Network Underlying Odour Pre-exposure Aversive Memory

4.3.5.1 Odour Pre-exposure Memory is Mushroom Body Dependent

The Mushroom Body (MB) is a centre for learning and memory in the fly, as olfactory memories actively depend on this region throughout acquisition, consolidation and retrieval [102]. We therefore tested whether the MB is required for pre-exposure memory. For this, we blocked Dynamin-dependent synaptic transmission in the α/β and ɣ KC subsets by expressing UAS-*Shibire*^{ts1} (see Methods 3.6 for more information on UAS-*Shibire*^{ts1}) [46, 49] using the MB247-GAL4 driver[6] (See Annex A for reference on patterns of expression for all driver lines). throughout the pre-exposure and testing. We found that when flies expressing both UAS and GAL4 transgenes placed at restrictive temperature (33°C), they did not show an aversive response to the odour following pre-exposure compared to both parental controls (Figure 4.7A). However, flies expressing these transgenes but kept at permissive temperature (23°C) showed an aversive memory, similar to the parental controls (data not shown). This control is to show that the lack of memory performance at (33°C) is not from a abnormal learning in these flies, but instead an effect of temperature-dependent neuronal inhibition. To determine if inhibition was required during a specific phase, we expressed the green light-activated anion Channelrhodopsin GtACR1 in these same neurons, which allowed us to more precisely temporally restrict neuronal inhibition to pre-exposure events (see 3.6). Once again flies expressing both transgenes had an impaired pre-exposure memory compared to flies carrying either the UAS or the GAL4 transgenes, demonstrating a requirement of these neurons for pre-exposure memory acquisition (Figure 4.7B).

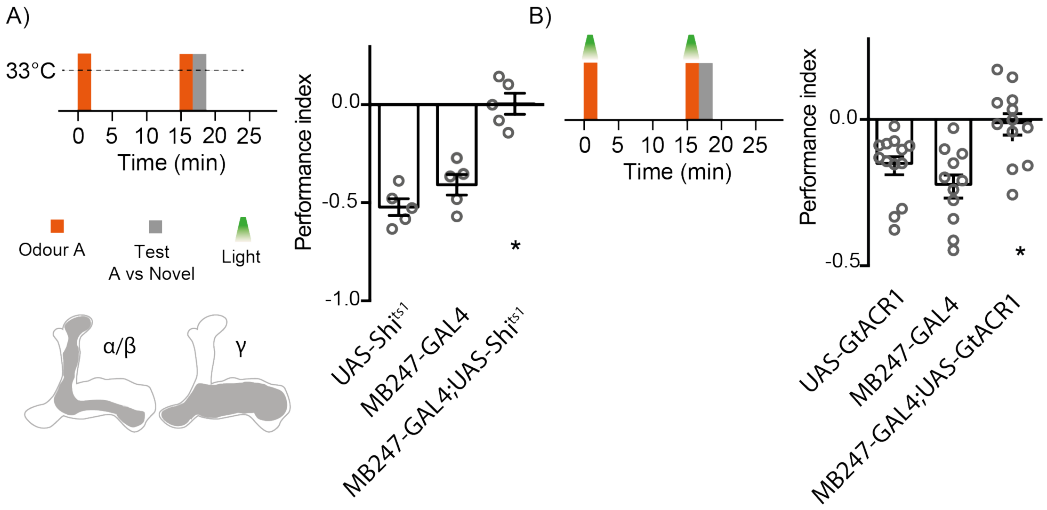


Figure 4.7: Formation of pre-exposure memory requires Kenyon Cell activity

A) Blocking synaptic transmission from KCs using the MB247-GAL4 driver throughout pre-exposure and testing disrupted pre-exposure memory compared to the parental controls (n=5 per group; one-way ANOVA F[2,12]=30.87, p<0.0001; Tukey's multiple comparisons test: UAS-*Shibire*^{ts1} vs. MB247-GAL4;*Shibire*^{ts1}, p<0.0001; MB247-GAL4 vs. MB247-GAL4;UAS-*Shibire*^{ts1}, p=0.0002). B) The MB was required for the acquisition of pre-exposure memory. Optogenetic inhibition of the same KC restricted to the pre-exposure phase disrupted the aversive memory compared to the parental controls (one-way ANOVA F[2,33]=9.088, p=0.0007; Tukey's multiple comparisons test: UAS-GtACR1 vs. MB247-GAL4;UAS-GtACR1, p=0.0183; MB247-GAL4 vs. MB247-GAL4;UAS-GtACR1, p=0.0006, UAS-GtACR1 n=13, MB247-GAL4 n=11, MB247>GtACR1 n=12).

4.3.5.2 Identification of Pre-exposure Reinforcing DANs

Dopaminergic neurons (DANs) innervating the MB provide aversive and appetitive reinforcement signals. DANs from the PPL1 cluster provide aversive reinforcement, whereas DANs from the PAM cluster provide reward reinforcement. Because KCs are required for acquisition of pre-exposure memory, we sought to determine whether DANs provide reinforcement for our repeated odour pre-exposure protocol. To inhibit synaptic transmission of PPL1 and PAM DANs, we

expressed UAS-*Shibire*^{ts1} using the TH-GAL4 and the R58E02-GAL4 drivers, respectively. We found that inhibition of aversive reinforcing PPL1 DANs was sufficient to flip the aversion to approach (Figure 4.8A). Flipping to approach suggests that a positive reinforcement signal may be unmasked when removing PPL1 signalling. Consistent with this, silencing PAM DANs led to an enhancement of aversive memory (Figure 4.8B), suggesting that by removing positive reinforcement behavioural inhibition of avoidance is eliminated, leading to a stronger avoidance. These results indicate that odour pre-exposure engages DANs from both the PPL1 and the PAM clusters which interact in an additive way.

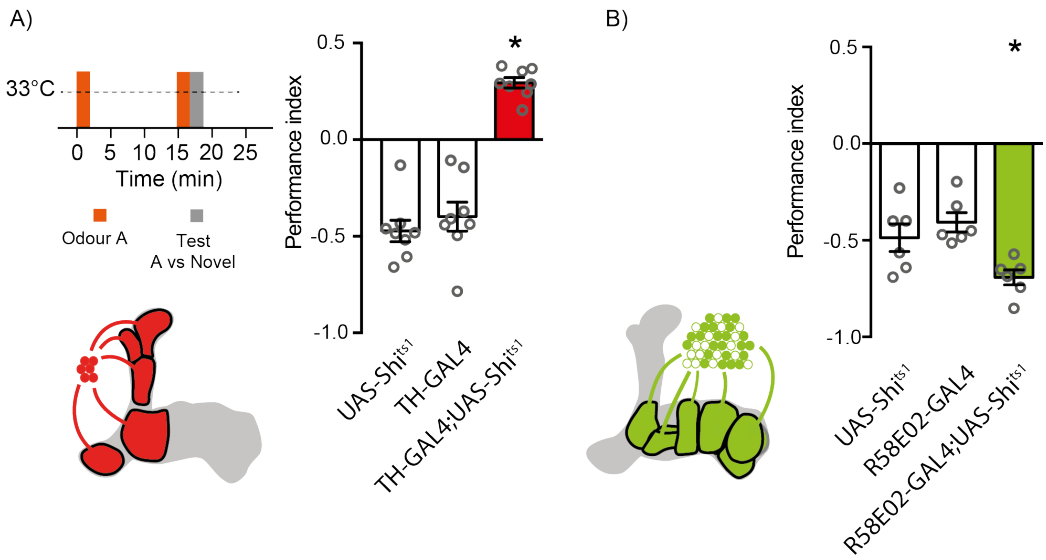


Figure 4.8: Reward and punishment reinforcing memories are involved in pre-exposure memory

A) Blocking punishment reinforcing PPL1 DANs labelled by the TH-GAL4 driver throughout pre-exposure and testing leads to a switch in the pre-exposure memory from aversion to approach, which was not seen in the parental controls (one-way ANOVA $F[2,21]=57.26$, $p<0.0001$; Tukey's multiple comparisons: UAS-*Shibire*^{ts1} vs. TH-GAL4;UAS-*Shibire*^{ts1}, $p<0.0001$; TH-GAL4 vs. TH-GAL4;UAS-*Shibire*^{ts1}, $p<0.0001$, n=8 per group). B) Blocking of reward-reinforcing DANs labelled by the R58E02-GAL4 driver led to an enhancement of pre-exposure memory compared to parental controls (one-way ANOVA $F[2,15]=7.126$, $p=0.0067$; Tukey's multiple comparisons test: UAS-*Shibire*^{ts1} versus R58E02-GAL4;UAS-*Shibire*^{ts1}, $p=0.0473$; R58E02-GAL4 vs. R58E02-GAL4;UAS-*Shibire*^{ts1}, $p=0.0062$, n=6 per group).

Given that the overall effect of pre-exposure results in avoidance, we focused on the PPL1 cluster as a reinforcer. Inhibition of a restricted subset of PPL1 labelled by the MB504B-GAL4 driver, led to a disruption of pre-exposure memory (Figure 4.9A). Lack of memory performance was evident when PPL1 synaptic transmission was blocked throughout pre-exposure and retrieval, but also when temporally restricting neuronal inhibition to the pre-exposure phase using GtACR1 (Figure 4.9B). Surprisingly, despite PPL1-γ1 DAN being shown to be involved

in aversive shock, bitter taste and heat reinforcement [32, 103], selective inhibition of γ1 using the c601-Gal4;MBGal80 driver [5] throughout pre-exposure and testing (Figure 4.10A) did not affect pre-exposure memory (Figure 4.10B). To identify the reinforcing DANs involved in pre-exposure memory, we inhibited individual subsets of PPL1 DANs targeting the following MB compartments (Figure 4.10A): α3 (MB630B-GAL4), α2α′2 (MB058B-GAL4) and γ2α′1 (MB296B-GAL4)(Figure 4.10C). Inhibition of either γ2α′1 or α3 disrupted pre-exposure memory; α3 demonstrated a much more robust switch from aversive to appetitive seeking of the pre-exposure odour (Figure 4.10C,D).

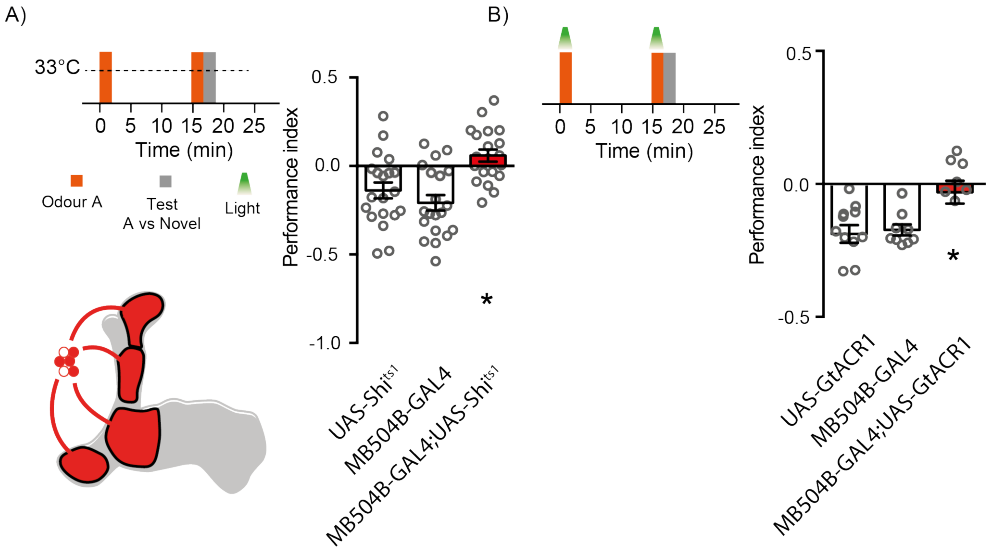


Figure 4.9: Pre-exposure memory requires PPL1 DANs during acquisition

A) Blocking synaptic transmission from neurons labelled by the PPL1 driver MB504B-GAL4 throughout the protocol, showed an impairment in pre-exposure memory performance (one-way ANOVA F[2,57]=11.30, p<0.0001; Tukey's test: UAS-*Shibire*^{ts1} vs. MB504B-GAL4;UAS-*Shibire*^{ts1}, p=0.0037; MB504B-GAL4 vs. MB504B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001, n=20 per group). B) Restricting optogenetic inhibition to the pre-exposure/acquisition phase of the protocol was enough to disrupt memory performance (one-way ANOVA F[2,24]=6.325, p=0.0062; Tukey's test: UAS-GtACR1 vs. MB504B-GAL4;UAS-GtACR1, p=0.0112; MB504B-GAL4 vs. MB504B-GAL4;UAS-GtACR1, p=0.0169, UAS-GtACR1 n=11, MB504B-GAL4 n=9, MB504B>GtACR1 n=7).

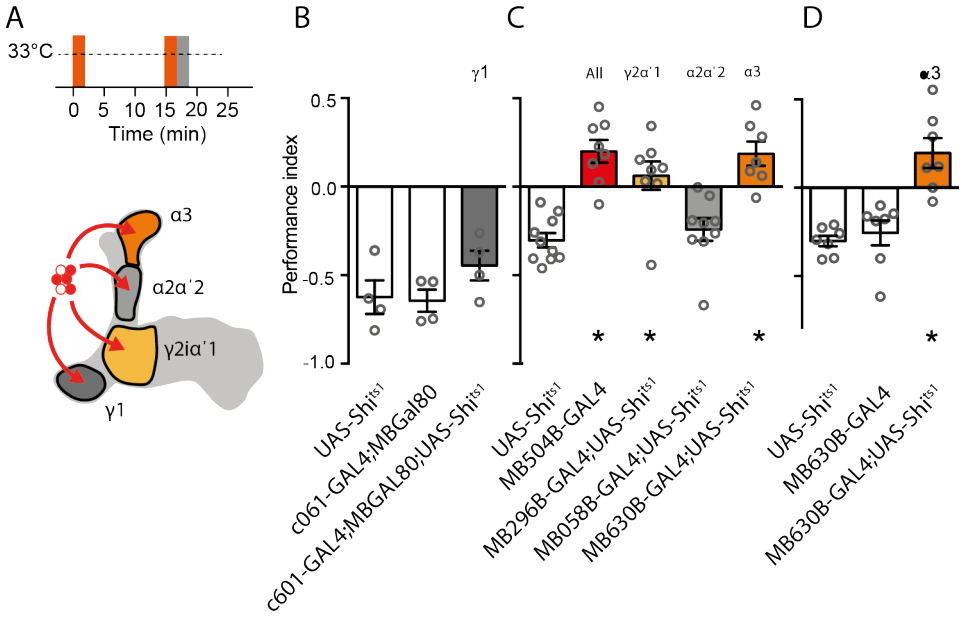


Figure 4.10: Pre-exposure memory requires α3 and γ2α′1 DANs

A) Top - Schematic of training protocol. Flies were kept at restrictive temperature (33°C) throughout stimuli exposure and testing. Bottom - Colour coded schematic of the mushroom body compartments labelled by each driver line used. B) Inhibition of PPL1-γ1-pedc did not show a different performance from the parental controls(One-way ANOVA F[2,9]=1.785, p=0.2223, n=4 per group). C) Blocking neurons labelled by MB296B-GAL4 (γ2α′1) or MB630B-GAL4 (α3), as well as the full PPL1 driver MB504B-GAL4 disrupted memory performance compared to the UAS-*Shibire*^{ts1} parental control (One-way ANOVA F[4,37]=14.80, p<0.0001; Dunnett's test: UAS-*Shibire*^{ts1} vs. MB504B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001; UAS-*Shibire*^{ts1} vs. MB296B-GAL4;UAS-*Shibire*^{ts1}, p=0.0006; UAS-*Shibire*^{ts1} vs. MB630B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001; *Shibire*^{ts1} n=10, MB504B>*Shibire*^{ts1} n=8, MB296B>*Shibire*^{ts1} n=8, MB059B>*Shibire*^{ts1} n=9, MB630B>*Shibire*^{ts1} n=7) D) PPL1-a3 inhibition was significantly different from both parental controls (one-way ANOVA F[2,29]=23.16, p<0.0001; Tukey's test: UAS-*Shibire*^{ts1} vs. MB630B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001; MB630B-GAL4 vs. MB630B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001, n=7 per group).

4.3.5.3 Role of PPL1-α3 in Aversive Reinforcement

Because inhibition of PPL1-α3 provided strong aversive reinforcement during odour pre-exposure, we investigated whether it also plays a role in other aversive conditioning paradigms, i.e. shock. To test this, we first blocked synaptic

transmission across PPL1 DANs (MB504B-GAL4;UAS-*Shibire*^{ts1}) during training and testing in a standard aversive electric shock conditioning paradigm. As expected, blocking synaptic transmission reduced aversive memory performance compared to genetic controls (Figure 4.11A). Surprisingly, blocking α3-PPL1 throughout shock conditioning did not impact memory performance (Figure 4.11A). Despite the fact that both shock and pre-exposure leads to conditioned aversive responses, odour pre-exposure memory recruits a distinct neuronal pathway to that of shock reinforcement. Furthermore, single odour-paired activation of PPL1 DANs labelled by MB504B-GAL4 induced a conditioned avoidance response to the CS^+ compared to parental control flies; whereas this was not the case for α3 activation (Figure 4.11B). This is consistent with previous reports demonstrating that paired α3-PPL1 requires repeated activation to form an aversive memory [78].

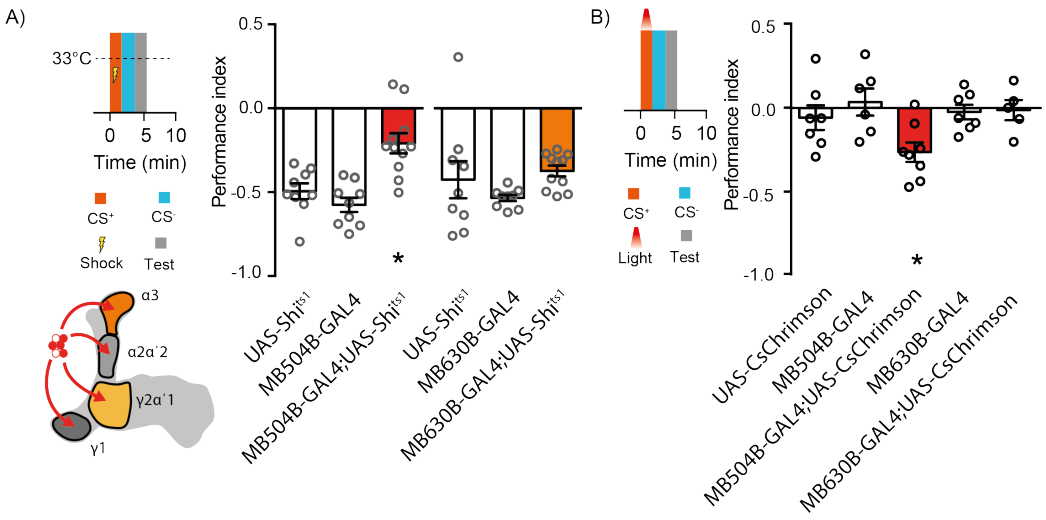


Figure 4.11: PPL1-α3 are dispensable for aversive shock conditioning
A) Inhibiting all PPL1 DANs during 90V shock aversive conditioning led to an impairment of memory performance compared to the parental controls (one-way ANOVA F[2,26]=14.43, p<0.0001; Tukey's test: UAS-*Shibire*^{ts1} (n=9) vs. MB504B-GAL4;UAS-*Shibire*^{ts1} (n=11) p=0.0016; MB504B-GAL4 (n=9) vs. MB504B-GAL4;UAS-*Shibire*^{ts1}, p<0.0001). However, restricting inhibition to PPL1-α3 (MB630-GAL4;UAS-*Shibire*^{ts1}, n=11) did not influence aversive shock memory performance compared to the controls (one-way ANOVA F[2,26]=1.692, p=0.2039, n=9 for parental controls). B) Paired odour presentation with optogenetic activation of PPL1-DANs in the MB504-GAL4 driver implants an artificial aversive memory. However, activation of only PPL1-α3 does not produce a memory performance different from the parental controls (UAS-Chrimson n=11, MB504B-GAL4 n=7, MB504B>CsChrimson n=10, MB630B-GAL4 n=10, MB630B>CsChrimson n=8; One-way ANOVA F[4,41]=5.665, p=0.0010; Sidak's test: UAS-CsChrimson vs. MB504B-GAL4;UAS-CsChrimson, p=0.0025; MB504B-GAL4 vs. MB504B-GAL4;UAS-CsChrimson, p=0.0028. All other comparisons p>0.9).

4.3.6 Neuronal Network Underlying Behavioural Output

Simultaneous activation of DANs and KCs leads to a depression of KC->MBON synapses, which tunes activity of selective MBONs ultimately skewing behavioural output between approach and avoidance (see 2.3.2). Because KCs and DANs are required for pre-exposure memory, we sought to examine whether correspond-

ing MBONs guide odour pre-exposure behaviour. For this, we blocked synaptic transmission in γ2α′1-MBON (G0239-GAL4;UAS-*Shibire*^{ts1}) or α3-MBON (MB077B-GAL4;UAS-*Shibire*^{ts1})(Figure 4.12A). Blocking synaptic output of either γ2α′1-MBON and α3-MBON throughout the pre-exposure protocol led to a disruption in aversive memory (Figure 4.12B and C).

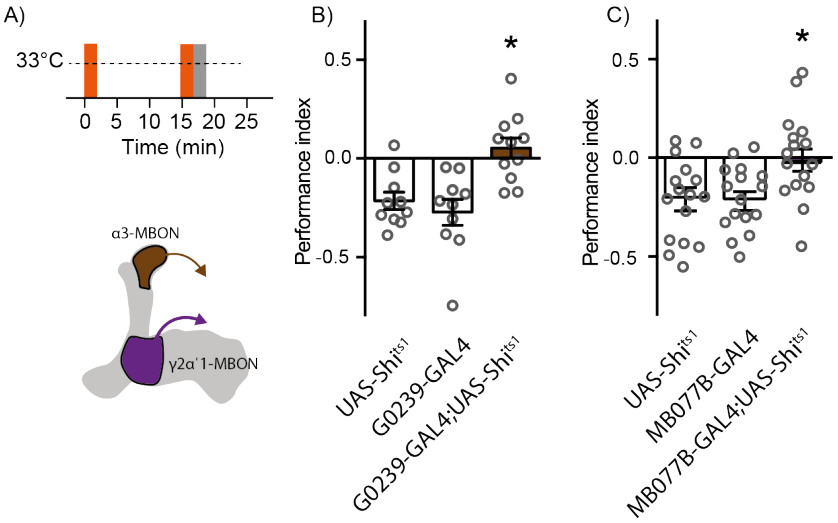


Figure 4.12: Blocking α3 and γ2α′1 MBONs disrupts pre-exposure memory

A) Top- Schematic of exposure protocol. Flies were kept at restrictive temperature (33°C) throughout pre-exposure and testing. Bottom- Schematic of neurons labelled by the drivers used. In brown α3-MBON. In purple γ2α′1-MBON. B) Inhibition of α3-MBON throughout the pre-exposure protocol led to a disruption of pre-exposure memory compared to the parental controls (one-way ANOVA F[2,28]=10.50, p=0.0004; Tukey's test: UAS-*Shibire*^{ts1} vs. G0239-GAL4;UAS-*Shibire*^{ts1}, p=0.0043; G0239-GAL4 vs. G0239-GAL4;UAS-*Shibire*^{ts1}, p = 0.0006, n=10 for parental controls, n=11 for G0239>*Shibire*^{ts1}). C) Ihibition of γ2α′1-MBON disrupts memory performance compared to parental controls (one-way ANOVA F[2,45]=5.063, p=0.0104; Tukey's test: UAS-*Shibire*^{ts1} vs. MB077B-GAL4;UAS-*Shibire*^{ts1}, p=0.0284; MB077B-GAL4 vs. MB077B-GAL4;UAS-*Shibire*^{ts1}, p=0.0181; All other comparisons p>0.5, n=16 for all groups).

Restricting inhibition of γ2α′1-MBON to pre-exposure memory testing suppressed memory expression, indicating a requirement of this neuron for retrieval (Figure 4.13A). Inhibiting α3-MBON exclusively during pre-exposure retrieval did not

significantly reduce memory performance compared to the parental controls (Figure 4.13A). However, inhibiting α3-MBON during retrieval in our LI protocol suppressed LI of appetitive memory, suggesting a requirement for α3-MBON for pre-exposure retrieval in the presence of a competing memory (Figure 4.13B).

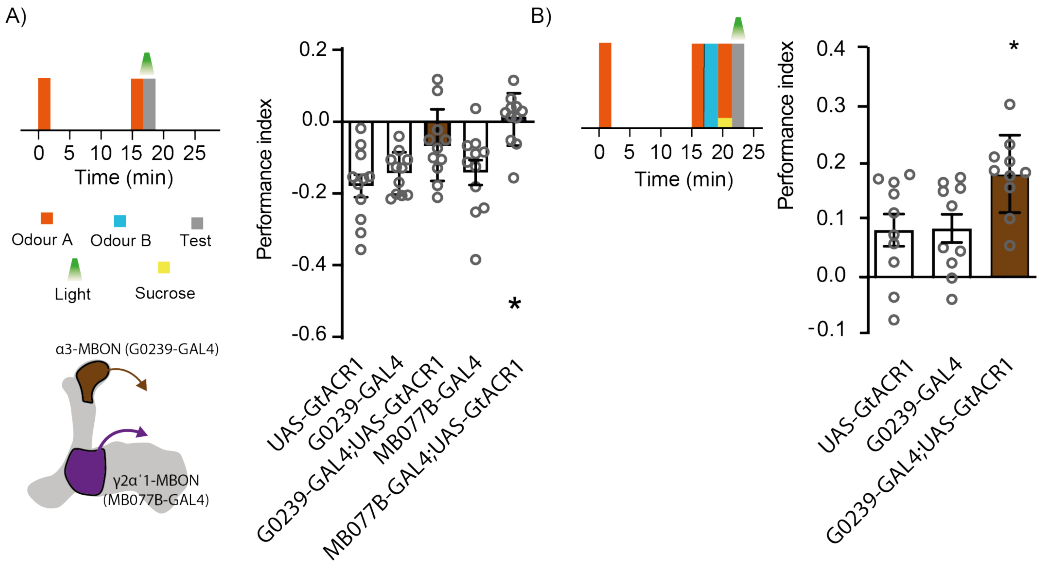


Figure 4.13: γ2α′1-MBON is required for retrieval of pre-exposure memory

A) Restricting optogenetic inhibition of γ2α′1-MBON but not of α3-MBON to the retrieval phase of the pre-exposure protocol disrupted pre-exposure memory when compared to the parental controls (n=11 for all groups. one-way ANOVA $F[4,50]=7.188$, $p=0.0001$; Sidak's test: UAS-GtACR1 vs. G0239-GAL4;UAS-GtACR1 (n=11) $p=0.0337$; G0239-GAL4 vs. G0239-GAL4;UAS-GtACR1, $p=0.2668$; UAS-GtACR1 vs. MB077B-GAL4;UAS-GtACR1, $p=0.0001$; MB077B-GAL4 vs. MB077B-GAL4;UAS-GtACR1, $p = 0.0027$). B) Inhibition of γ2α′1-MBON during the retrieval of the latent inhibition protocol was sufficient to recover appetitive memory performance compared to the parental controls (n=10 for each for all groups, one-way ANOVA $F[2,27]=5.063$, $p=0.0136$; Tukey's test: UAS-GtACR1 vs. G0239-GAL4;UAS-GtACR1, $p=0.0248$; G0239-GAL4 vs. G0239-GAL4;UAS-GtACR1, $p=0.0297$). For all other comparisons $p>0.5$.

4.4 Discussion

Two main hypothesis have been proposed to explain how latent inhibition arises. First, works in vertebrates suggested that latent inhibition arises through pre-exposure-induced reduction of attention impacting acquisition during training [98, 104]. We showed that spacing pre-exposure and training from testing allowed emergence of the conditioned memory, showing that latent inhibition does not impact learning. A second hypothesis is that latent inhibition occurs through inhibition of retrieval during testing. It has been suggested that during pre-exposure, the CS/No-US is associated to the context and only when the context matches in testing, this No-US prediction is retrieved, leading to latent inhibition of subsequent learning [100, 105]. The data presented here is in agreement with the requirement of context consistency between pre-exposure and testing for latent inhibition in *Drosophila*. However, we observed a shift in odour preference after pre-exposure. If a CS/no-US memory was to get retrieved when in a given context, it could explain why an animal would go towards a novel odour. However, we saw that changing the context after pre-exposure only did not inhibit aversive memory. The requirement of context consistency for latent inhibition suggests an association exists between the stimulus, its valence and the context. We suggest that context is only required to provide more information for resolution between conflicting memories. Therefore, pre-exposure memory will not be retrieved unless necessary, i.e. in the appropriate context. When presented with conflicting context across pre-exposure and testing, this information can aid in retrieval and allow expression of other more relevant memories.

In this study we demonstrated that LI in flies arises from a competing aversive memory generated during odour pre-exposure. This pre-exposure memory is integrated in an additive way with subsequently acquired memories, which lead to

a suppression of appetitive memories and the enhancement of aversive memories. We demonstrate that this learning is orchestrated by neurons innervating the α3 and the γ2α′1 compartments of the MB. PPL1-α3 and PPL1-γ2α′1 DANs provide reinforcing signals which result in synaptic depression of the corresponding MBONs. Recording calcium activity within these neurons during pre-exposure showed an increased Ca^{2+} activity of the α3 DANs during the second odour pre-exposure (See Annex B) [69]. Subsequently, recording activity in α3-MBON during odour exposure revealed a reduced calcium activity.

Interestingly, flies did not form pre-exposure memories with all stimuli, as concentration of the odour was an important regulator of the pre-exposure memory. High concentrations of the odour are aversive to flies, leading to an aversive memory performance post odour pre-exposure; whereas low concentrations were less aversive and did not elicit an aversive pre-exposure memory. Investigating the role of PAM-DANs in pre-exposure memory to aversive concentrations could help us understand why we did not see an appetitive pre-exposure to appetitive concentrations. However, we cannot reject the notion that lower appetitive concentrations could form an appetitive memory through odour pre-exposure, perhaps by increasing the amount of pre-exposure events or by using a different exposure context.

5 | Investigating the Emergence of Appetitive Long-term Memory

5.1 Chapter abstract

This second experimental chapter investigates the neural circuits involved in the dynamic retrieval of nutrient-dependent appetitive memories. Prior work has shown that activity of two distinct subsets of rewarding dopaminergic neurons are necessary for acquisition of sweet and nutrient-dependent memories, independently [30, 75]. These experiments proposed that paired activation of α1 during training leads to a delayed emergence of LTM in absence of STM; suggesting that α1 PAM-DANs are responsible for nutrient-dependent memory acquisition and consolidation. However, the lack of specificity with the drivers used for neuronal activation occlude the contribution that other DANs could have had in suppressing STM.

The data presented in this chapter addresses limitations of the previous studies and provides evidence for how α1 PAM-DANs are mechanistically involved in regulating both short- and long-term memories. We demonstrate a transient inhibition of sucrose-reinforced STM following activation of the broad neuronal subset used in the prior studies. We determined that the inhibitory effect arises from activation of dopaminergic neurons projecting to the γ5n compartment. This inhibition seems to be transient given that the memory recovers after 24h. Sucrose memory can be disrupted if unpaired activation of these neurons occurs before 24h memory retrieval, suggesting that γ5n-PAMs activation might disrupt motivational drive. Taken together, this chapter provides enough evidence to suggest the delayed emergence of nutrient-dependent memory is unlikely to be derived from

α1 PAMs activation. Moreover, this data suggests this delayed memory phenotype is created by inhibition of memory retrieval by co-activation of ɣ5n PAMs

5.2 Introduction

In nature, animals can remember environmental cues such as odours that are predictive of rewards such as food. Animals will approach these predictive cues when hungry to enhance their chances of finding food. When flies are exposed to an odour paired to a sucrose reward, they will preferentially approach the conditioned odour (CS^+) over a control odour that was presented without the reward (CS^-) [33, 55]. This memory appears immediately after training and persists for days [5, 106]. Interestingly, work from Burke and Waddell 2011 [31] showed that long-lasting approach to CS^+ is not present when the odour-paired sugar has no nutritional value, such as with D-Arabinose or D-Xylose. In this case, flies show a preference for the odour paired to the non-nutritious sugar immediately after training, but this memory performance is abolished 24h after training. Other studies have also demonstrated a requirement of nutrient intake for memory persistence [29, 107].

A sugar reward memory is thought to arise from two separate neuronal pathways within the mushroom body leading to two independent memories: a sweet-taste-reinforced appetitive memory that decays quickly after acquisition and a slowly emerging long-term memory that arises once the animal detects the nutrients post-ingestion [30, 75]. Consistent with this, Das et al. 2014 showed that octopamine synthesis is crucial for appetitive short-term memory but dispensable for appetitive long-term memory [32]. Octopamine deficient flies carrying a mutation in Tyramine β Hydroxylase, Tbh^{M18}, lack short-term memory but have intact long-term memory. Thus, octopamine is believed to exclusively encode the sweet, short-lived memory, allowing an isolated measurement of the emergent nutrient-reinforced memory in Tbh^{M18} mutants. However, it is not clear how nutrient detection is conveyed and how it triggers consolidation of long-term memory.

Moreover, this also suggests that nutrient information influences behaviour long after the CS^+ presentation has ended. Thus, a question arises: How can nutrient detection, which is temporally separated from the conditioning event, be attributed to the correct conditioned stimulus and not to the control stimulus? This type of retroactive association is also seen in paradigms such as taste aversion learning, suggesting that the delayed emergence of a nutrient-dependent memory is likely.

Yamagata et. al [30] showed that thermogenetic activation of a subgroup of DANs (labelled by the R15A04-GAL4 driver line) paired to CS^+ generates an apparent artificial long-term appetitive memory independent from any evident short-term memory. The R15A04-GAL4 driver labels reward-reinforcing DANs targeting 5 different regions within the mushroom body: $\alpha 1$, $\beta' 1$, $\beta 2sc$, and both sub-compartments of $\gamma 5$: $\gamma 5b$ and $\gamma 5n$ (Figure 5.1). Given that the $\alpha 1$ PAM-DANs receive inputs from nutrient sensing Gr43a neurons, Yamagata et. al attributed the emergence of memory to be a reflection of acquisition and consolidation of nutrient-dependent memory via paired activation of $\alpha 1$ DANs by Gr43a neurons.

In this model, the retrieval of the two independent memories at different time points allows the animals to approach the predictive cue throughout time without interruptions; initially seeking sweet reward and later nutritious reward. However, caveats in previous research need to be addressed in order to support the notion that the $\alpha 1$ compartment encodes this delayed nutrient consolidation. As mentioned throughout this thesis, multiple factors promote inhibition of memory expression and modulate behaviour across different time points. In this chapter, we investigated whether parallel neuronal activation of DANs within the R15A04-GAL4 could lead to a disruption of short-term memory retrieval simulating the emergence of memory following activation of these neurons.

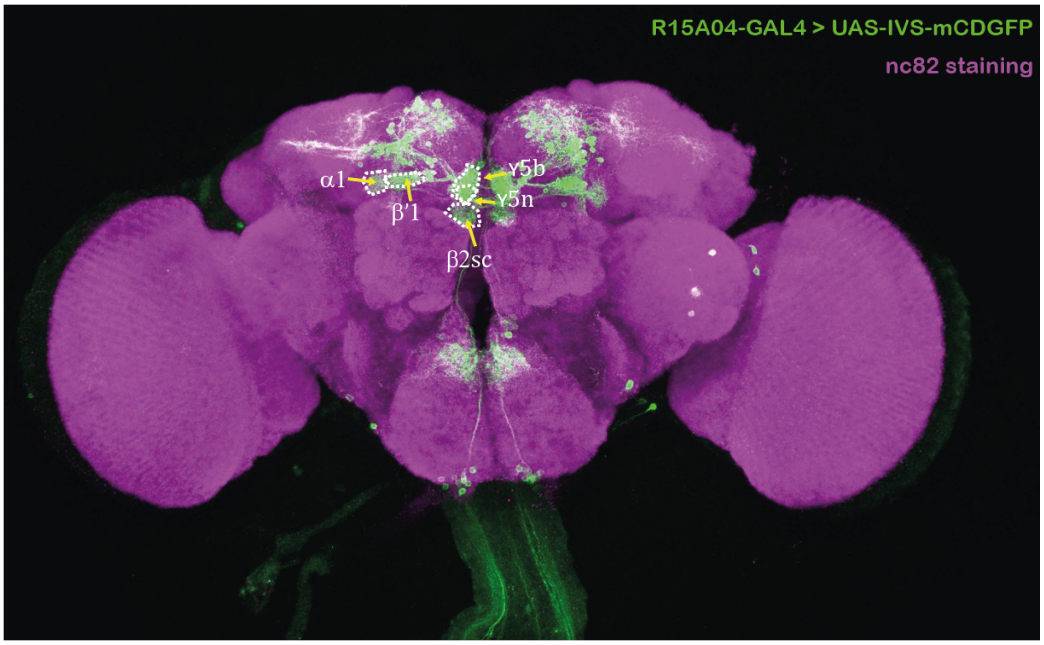


Figure 5.1: R15A04-GAL4 driven expression of UAS-GFP

GFP expression in green shows labelling of neurons targeting 5 different MB compartments. α1, β′1, β2sc, ɣ5b and ɣ5n, white dotted lines outline each compartment. Labelling was added to the expression image from the Fly Light GAL4 catalogue [43].

5.3 Results

5.3.1 Optogenetic Activation of R15A04-GAL4 DANs Produces Long-term but not Short-term Memory

Consistent with a previous study which used thermogenetic stimulation [30], pairing optogenetic activation of a subset of PAM-DANs (R15A04-GAL4) with an odour (Figure 5.2A) was enough to produce a memory performance observable at 24h after training (Figure 5.2B,E). This artificial memory was not seen 2min after training (Figure 5.2C). In contrast to the previous study, we did not observe a memory performance 3h after paired optogenetic activation (Figure 5.2D). This could be due to differences in the tools used for neuronal activation as well as differences in the calculation of the Performance Index (PI) (see Chapter 3).

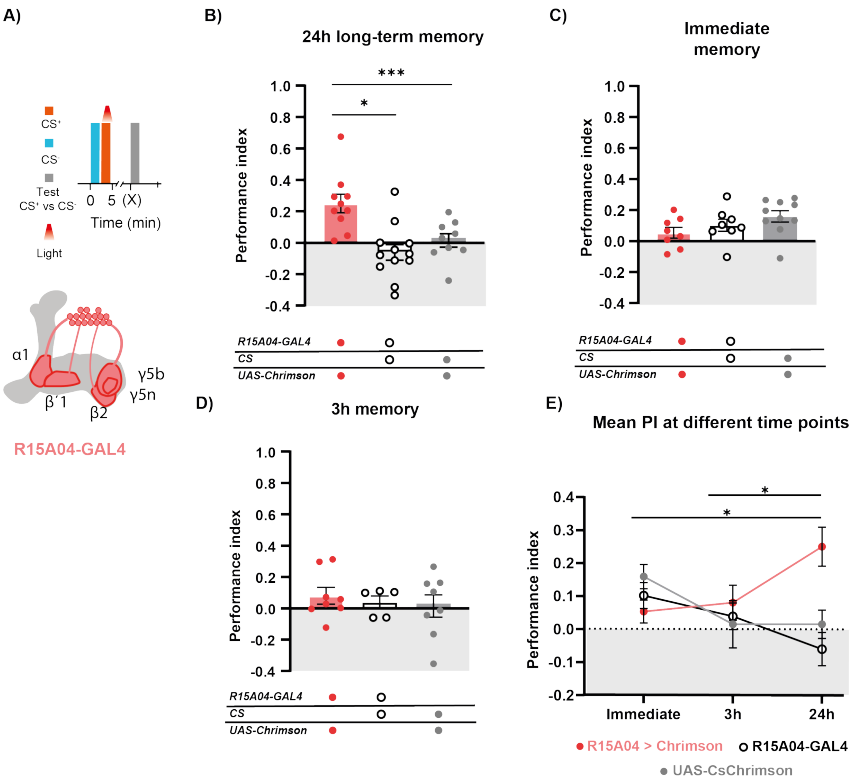


Figure 5.2: Activation of R15A04-GAL4 labelled PAM-DANs induce LTM

A) Upper: Training protocol used for (B)-(E). Time between training and testing was variable (X). Flies were presented with 2min CS^-, clean air for 30s and then 2min of CS^+ paired to light presentation. Lower: The PAM-DANs labelled by the R15A04-GAL4 driver. B) Flies trained to associate artificial neuronal activation with a CS^+ approached the CS^+ when tested 24h after training (R15A04-GAL4;UAS-CsChrimson one-sample t-test: n=10,df=9, t=4.249, p=0.0021). This was higher than the parental controls (one-way ANOVA F(2,28)=9.981, p=0.0005, Tukeys's group comparison R15A04-GAL4;UAS-CsChrimson vs. R15A04-GAL4 p= 0.0005 and R15A04-GAL4;UAS-CsChrimson vs. UAS-CsChrimson p= 0.0126). C) Flies trained to associate artificial neuronal activation with a CS^+ and tested immediately after training did not show any memory performance (One-sample t-test R15A04-GAL4;UAS-CsChrimson n=8, df=7, t=1.534, p=0.1690). This was indistinguishable from the parental controls (one-way ANOVA F(2,23)=2.108, p=0.1444). D) Flies trained to associate artificial neuronal activation with a CS^+ and tested 3h after training did not show any memory performance (one sample t-test R15A04-GAL4;UAS-CsChrimson n=8, df=7, t=1.506, p=0.1757). This was indistinguishable from the parental controls (R15A04-GAL4 Shapiro-Wilk p=0.0168; Kruskal-Wallis statistic =0.1929, p=0.9141). E) Mean performance across time shows the delayed emergence of the memory at 24h only in the experimental group (2-way ANOVA genotype/time interaction p=0.0023, F(4,69)=4.607, time factor F(2,69)=0.966 p=0.385, genotype factor (2,69)=2.86 p=0.063 Tukey's test: R15A04-GAL4;UAS-CsChrimson immediate vs. 24h p=0.0188 and 3h vs. 24h p= 0.0491.

5.3.2 Dissecting the Absence of Short-term Memory

The emergence of delayed long-term memory after neuronal stimulation suggests that activation of these neurons is sufficient to produce an appetitive long-term memory despite the absence of a short-term memory. The emergence of nutrient-dependent long-term memory could result from absence of short-term memory acquisition or from inhibition of immediate memory retrieval. To test whether memory retrieval is being impaired, we investigated whether testing conditions, parallel memories and motivational state could be inhibiting short-term memory performance via as the following possibilities:

1. Odour competition: memory performance could be occluded by an unexpected memory to the CS^-. Competing approach to CS^+ and CS^- would lead to an incorrect assessment of memory retrieval. Changing sequential presentation and removing CS^- form testing could reveal whether odour competition is inhibiting memory performance in our protocol.

2. Activation of PAM-DANs projecting to other compartments labelled by the R15A04-GAL4 driver could lead to a transient motivational switch, resulting in impaired memory retrieval. To investigate this, we assessed the effect of activation of compartment specific PAM-DANs in inhibition of short-term sucrose memory performance.

3. Fast-decaying parallel memories resulting from PAM-DAN activation could transiently inhibit short-term memory. Assessment of immediate artificial memory following activation of compartment specific PAM-DANs using restrictive drivers would help us determine whether a competing memory is created during activation of neurons labelled by R15A04-GAL4.

5.3.2.1 Odour Competition

In a differential associative learning paradigm, learning is assessed by examining the flies' change in preference to the conditioned odour (CS^+) with respect to the control odour (CS^-). Naïve flies have a balanced preference between the two odours. After appetitive conditioning, flies bias their approach towards the CS^+. After aversive training, flies will also break this balance and instead avoid the CS^+ (and therefore approach the CS^-). It has been shown that CS^- can acquire a valence opposite of that from the CS^+ when repeatedly presented after CS^+, signalling termination of the reinforcer [69]. Given that in our optogenetic activation protocol the CS^+ and CS^- are presented sequentially, there is a possibility that the CS^- could acquire a short-term competing appetitive valence similar to that of the CS^+. This could happen due to the CS^- acquiring predictability of the subsequent appetitive CS^+/light pairing. Consequently, measuring the CS^+ memory against the CS^- leads to a simultaneous retrieval of both competing memories, masking the memory to the conditioned odour. Therefore, changing sequential presentation of the odours and removing a potentially confounding odour should allow the expression of any CS^+ memory.

To determine whether short-term odour competition could explain the lack of performance in the short-term, we tested flies with CS^+ and a novel odour which was not presented during training. Moreover, we changed the sequential presentation of the odours to have CS^+ preceding CS^-. By removing the possibility of choosing the CS^- we could determine if there truly was short-term CS^+ memory. Novel vs. CS^+ odour discrimination testing was performed after the standard training protocol (CS^+ was presented second), as well as an inverted training (CS^+ was presented first). In both the standard training and the inverted training, flies did not approach the CS^+ over the novel odour immediately after training (standard

training: Figure 5.3A, inverted training: Figure 5.3B). Taken together, these data reject the possibility that the unconditioned odour masks the expression of any conditioned odour memory during immediate testing.

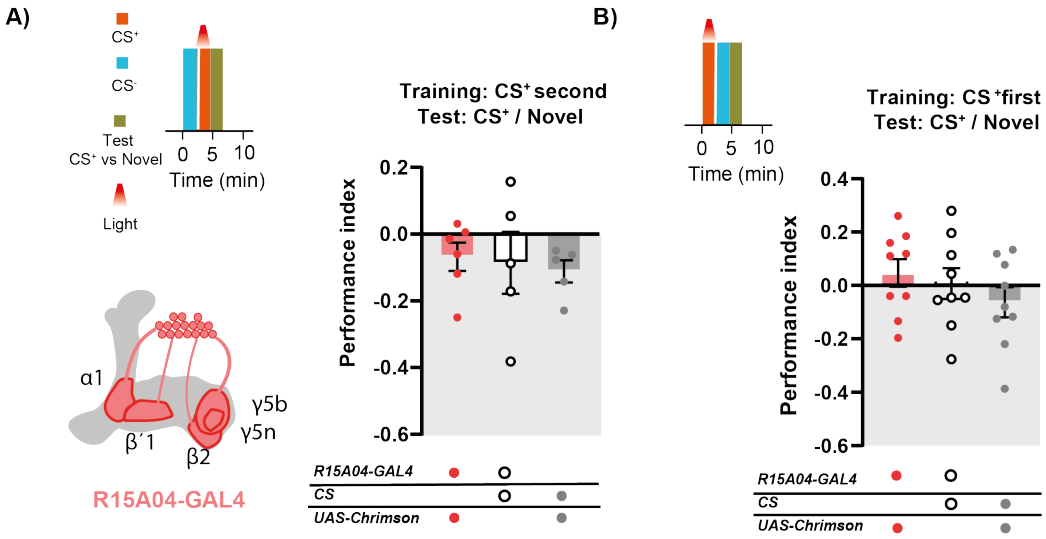


Figure 5.3: Inverted sequential odour presentation and testing CS^+ vs novel odour do not reveal CS^+-specific immediate valence

A) Flies presented with a CS^- followed by CS^+ paired to light presentation did not show any immediate memory when testing CS^+ vs. a novel odour (one-sample t-test R15A04-GAL4;UAS-CsChrimson n=6, df=5,t=1.601, p=0.1703). This was indistinguishable from the parental controls (one-way ANOVA (2,12)=0.1381 p=0.8723). B) When flies were trained in an inverted protocol, where CS^+ was presented before CS^-, no memory was seen immediately after training (R15A04-GAL4;UAS-CsChrimson one-sample t-test n=9, df=8 t=0.9109, p=0.3890). This was indistinguishable from the controls (one-way ANOVA (2,24)=1.015, p=0.3774.

5.3.2.2 Exclusive Activation of α1 PAM-DANs

Yamagata et al. [30] proposed that despite R15A04-GAL4 labelling a broad subset of PAM-DANs it was likely the α1 DANs produce this emergent memory. However, they did not test whether activation of exclusively the α1 DANs leads to the same phenotype. The R15A04-GAL4 driver labels neurons projecting to 4 other compartments: β′1, β2sc, ɣ5b and ɣ5n.

To test whether emergent memory arises from α1 DAN stimulation, we used a more restrictive split-GAL4 driver MB043C-GAL4 to achieve exclusive activation of α1-DANs and measured short-term memory performance in the T-maze. For this, UAS-CsChrimson expression was driven by the MB043C-GAL4 driver. Neuronal activation was paired to the CS^+ presentation (Figure 5.4A). Surprisingly, flies approached the odour that was paired with artificial activation immediately after training (Figure 5.5A,B). This memory was also present 24h after training (Figure 5.5A,C).

Consistent with this, a study using a quadrant arena assay to measure memory performance upon activation of different DANs, showed that immediately after odour paired activation of α1, flies spend more time in the quadrant containing the conditioned odour compared to the control odour which was also presented during training. Taken together, these results suggest that α1 activation leads to both short- and long-term memory performance.

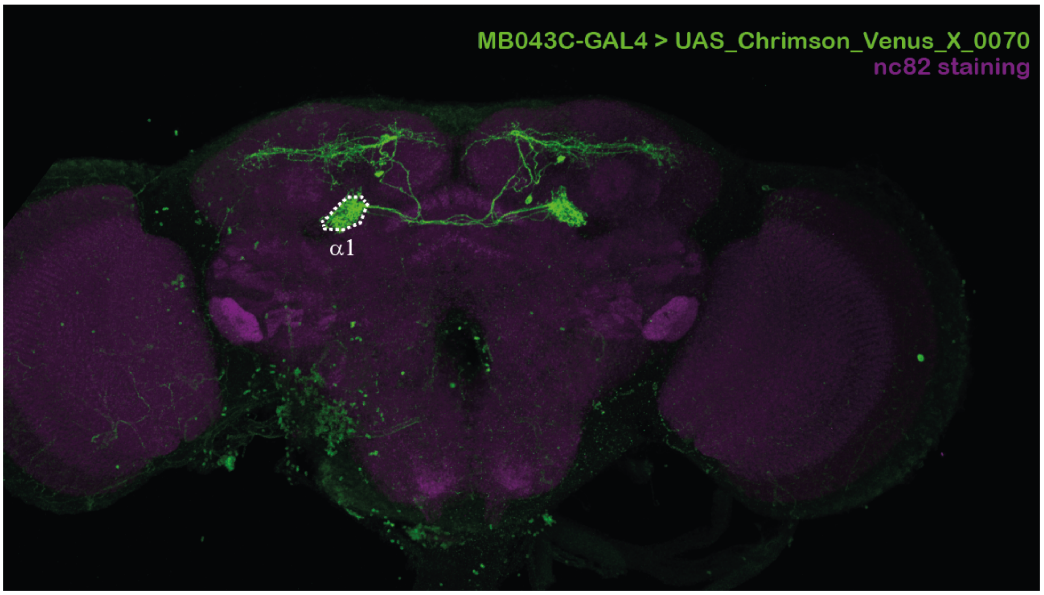


Figure 5.4: PAM α1 labelling by the MB043C-GAL4 driver

MB043C-GAL4 driven GFP expression in green shows labelling of DANs targeting the α1 compartment of the MB - white dotted line. Labelling was added to the expression image from the Fly Light GAL4 catalogue [43]

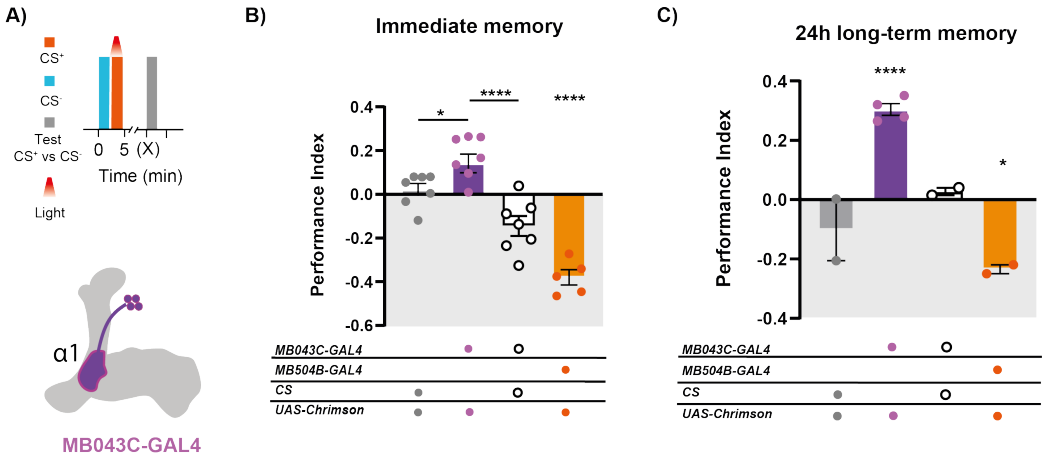


Figure 5.5: Activation of α1 leads to both short- and long-term memory performance

A) Protocol schematic for artificial training. Flies were presented a control odour CS^- (blue bar) followed by a second odour (CS^+) paired to light activation of α1 using the MB043C-GAL4 driver. Memory was measured at different intervals (X) after training. B) Flies tested immediately after optogenetic training showed a preference for the CS^+ (MB043C-GAL4;UAS-CsChrimson one sample t-test n=7, df=6, t=4.632, p=0.0036) compared to the parental controls (One-way ANOVA, F(2,18)=17.44, p<0.0001. Dunnett's test MB043C-GAL4;UAS-CsChrimson vs. UAS-CsChrimson mean diff=0.1486, p=0.0225; MB043C-GAL4;UAS-CsChrimson vs. MB043C-GAL4 mean diff=-0.3146, p<0.0001). C) Flies tested 24h after optogenetic activation approached the CS^+ (MB043C-GAL4;UAS-CsChrimson one sample t-test n=4, df=3,t=15.47 p=0.0006). B) and C) Activation of aversive DANs using MB504B-GAL4 (orange) was used as a control for UAS-CsChrimson activation (one sample t-test n=5, df=4 t=10.78, p=0.0004 for immediate, n=2, df=1, t=15.40, p=0.0413 for 24h memory). This group was exclusively as activation control and excluded from the group analysis.

5.3.2.3 Sucrose Memory is Inhibited via γ5n Activation

Activation of α1, β′1, β2sc, γ5b and γ5n using the R15A04-GAL4 driver leads exclusively to a long-term appetitive memory but α1 PAM-DAN activation produces both short- and long-term memory. We investigated whether a short-term inhibitory force resulting from the parallel activation of one of the other compartments labelled in the R15A04-GAL4 driver was responsible for the lack of

short-term memory performance.

To examine this, we used 6 drivers that contain different subsets of neurons labelled within the R15A04-GAL4 driver, we tested whether partial pairing of neuronal activation during the sucrose presentation phase of a standard appetitive conditioning paradigm was capable of inhibiting short-term memory performance, but not long-term memory (Figure 5.6). As in a standard appetitive training, CS^- was presented for 2 minutes followed by 30 seconds of clean air and finally CS^+ was presented with sucrose for 2 minutes. Neuronal activation was performed for 1 minute starting half-way through sucrose presentation (Figure 5.6A). Flies were then tested for preference between the CS^+ and CS^-. Partial pairing of neuronal activation was performed instead of full pairing to avoid potential confounding with activation that could interfere with feeding behaviour and thus prevent flies from learning the initial odour/sucrose association. Therefore, any effect in the immediate memory would be attributable to a retrieval or consolidation deficit rather than a disruption in acquisition.

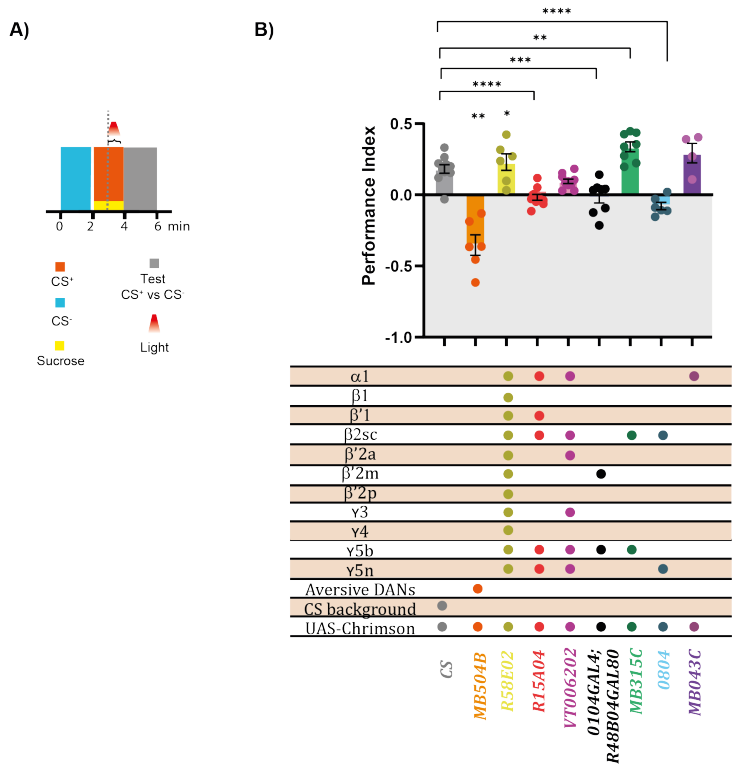


Figure 5.6: Partially paired activation of ɣ5n suppresses immediate memory performance

A) Schematic of the training protocol used for partial paired activation: CS^- was presented alone followed by CS^+ paired to sucrose. Optogenetic neuronal activation was induced during the second minute of CS^+ presentation. Flies were tested for CS^+ vs. CS^- preference immediately after training. B) Short-term memory retrieval was impaired following neuronal activation in two different drivers which included labelling of ɣ5n DANs (R15A05>CsChrimson one-sample t-test n=10, df=9, t=0.8586, p=0.419, and 0804>CsChrimson one-sample t-test n=6, df=5, t=3.032, p=0.0290, mean=-0.07), and in 0104-GAL4;R48B04-GAL80 (n=8, df=7, t=0.3847, p=.7119). These performances were statistically different from the UAS-CsChrimson control (One-way ANOVA (6,49)=23.7, p<0.0001, Dunnett's post-hoc test R15A04>CsChrimson vs. UAS-CsChrimson p=<0.0001; VT006202>CsChrimson vs. UAS-Chrimson p=0.1488; 0104-GAL4;R48B04-GAL80>CsChrimson vs. UAS-CsChrimson p=0.0001; MB315C>CsChrimson vs. UAS-CsChrimson p=0.0029; 0804>CsChrimson vs. UAS-CsChrimson p<0.0001). For each group, the labelled DANs are represented in the table. Positive controls MB504B-GAL4 (orange) and R58E02-GAL4 (yellow) were crossed to UAS-CsChrimson as activation control for artificial aversive and appetitive memory and excluded from the group analysis. One-sample t-tests: MB504B>CsChrimson n=6, df=5 t=4.885 p=0.0045; R58E02>CsChrimson n=6, df=5,t=3.921, p=0.0112; VT006202>CsChrimson n=10, df=9 t=6.108, p=0.0002; MB315C>CsChrimson t(7)=9.751, p<0.0001; MB043C>CsChrimson t(3)=4.279, p=0.0235.

Interestingly, we found that activation of DANs projecting to the γ5n compartment, had impaired immediate memory performance compared to the wild-type CS control (Figure 5.6B). Moreover, when memory performance was measured 24h after this same training protocol (Figure 5.7A), the γ5n activated flies approached the odour paired to the sucrose and stimulation (Figure 5.7B). These results clearly demonstrate that activation of γ5n neurons leads to an inhibition of immediate sucrose memory. However, is not yet clear whether the recovery of 24h memory performance corresponds to a recovery of the sucrose memory or to an artificial memory generated through neuronal activation.

Similarly to how γ5n activation suppresses immediate memory performance, flies expressing UAS-CsChrimson in the β′2m and γ5b compartments (0104-GAL4;R48B04-GAL80) also do not show immediate memory performance. However, because activation of these neurons did not recover the memory 24h after training, we conclude that the lack of immediate memory performance in these flies is likely due to a disruption of the sucrose memory rather than a transient inhibition.

Partial activation of α1, β2sc, β′2a, γ3, γ5b and γ5n DANs labelled by VT006202-GAL4 did not show reduced STM performance despite labelling of γ5n (Figure 5.6). However, when these neurons were activated throughout the full duration of sucrose presentation (Figure 5.8A), memory performance was impaired. This was significantly different from the UAS-CsChrimson parental control, but not from the GAL4 parental control. Memory performance in fully paired activation of VT006202-GAL4 flies was not seen at 24h after training. However, the GAL4 control did not show memory performance different from 0 either (Figure 5.8B) making these results difficult to interpret.

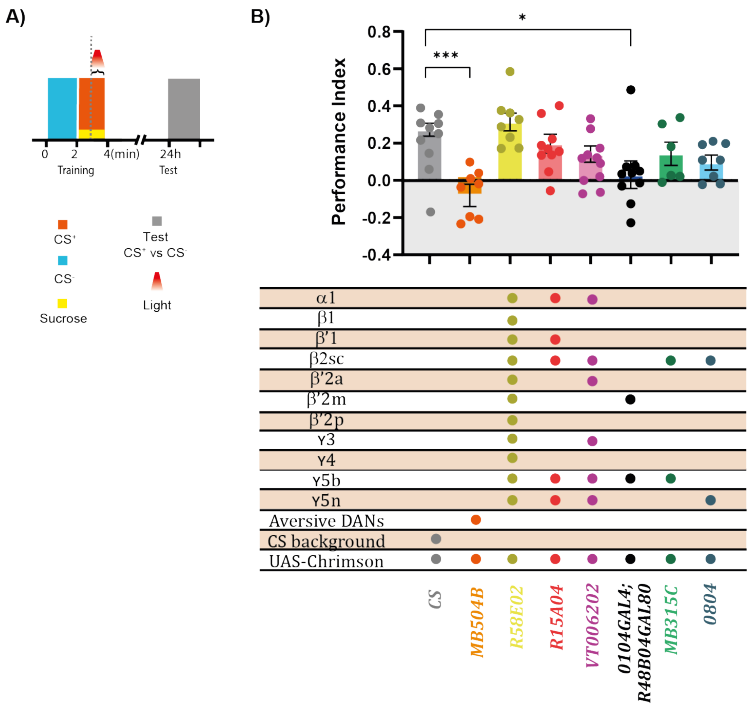


Figure 5.7: Memory is recovered 24h after γ5n neuronal activation

A) Memory performance was measured 24 hours after the modified appetitive protocol where neuronal activation was partially paired to the sucrose/CS^+ presentation during the second minute of exposure. B) Memory performance was recovered at 24h for the two drivers labelling γ5n (one-sample t-test R15A04>CsChrimson n=10, df=9, t=4.205, p=0.0023, and 0804>CsChrimson n=8, df=7, t=2.938, p=0.0218). Memory was not recovered for 0104-GAL4;R48B04-GAL80>CsChrimson (one-sample t-test n=10, df=9, t=.6262 p=0.5467). UAS-CsChrimson transgene was crossed to all experimental drivers and to the wild-type CS as control. For each group, the driver and the corresponding labelled DANs are represented in the table below. Parental group UAS-CsChrimson crossed to CS wild-type (grey) was used as a performance control and compared to all genotypes (one-way ANOVA F(5,50)=1.874 p=0.1151; Dunnett's post-hoc test: UAS-CsChrimson vs. R15A04>CsChrimson p=0.9576; UAS-CsChrimson vs. VT006202>CsChrimson p=0.3286; UAS-CsChrimson vs. 0104-GAL4;R48B04-GAL80>CsChrimson p=0.0312; UAS-Chrimson vs. MB315C>CsChrimson p=0.7861; UAS-CsChrimson vs. 0804>CsChrimson p=0.3306). Positive controls MB504 (orange) and R58E02-GAL4 (yellow) were used as neuronal activation controls for artificial aversive and appetitive memory and excluded from the group analysis (one sample t-tests: MB504B>CsChrimson n=8, df=7, t=1.418, p=0.1990; R58E02>CsChrimson n=8, df=7, t=6.605, p=0.0003; VT006202>CsChrimson n=12, df=11, t=3.269, p=0.0075; MB315C>CsChrimson n=6, df=5, t=2.296, p=0.0701).

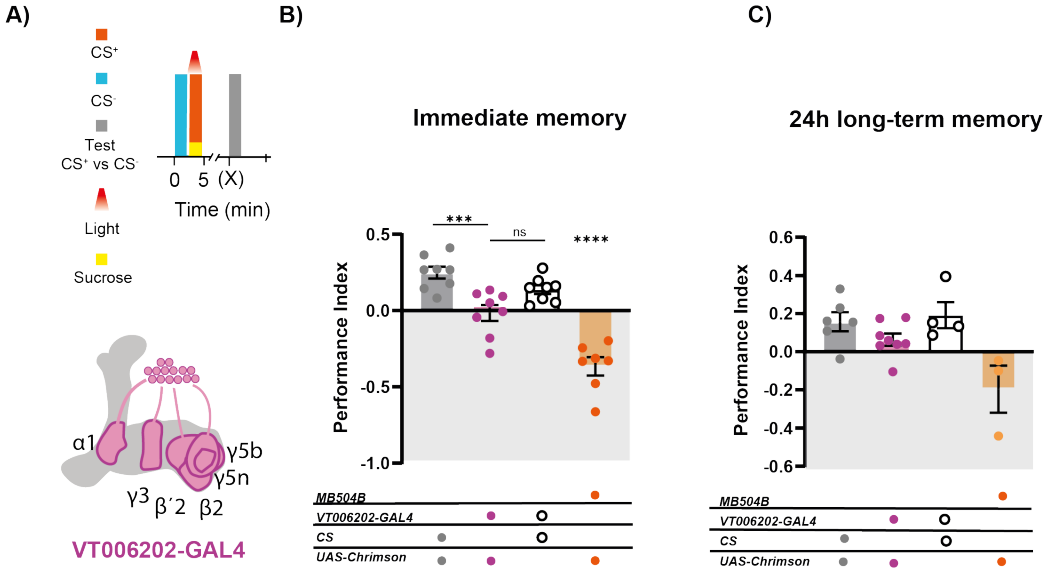


Figure 5.8: Paired activation of VT006202-GAL4 throughout sucrose and odour presentation

A) Training protocol used (top) and schematic of labelled neurons by the VT006202-GAL4 driver (bottom). Light-induced neuronal activation was performed throughout the 2 minutes of CS^{+}/sucrose presentation B) Activation of neurons labelled by VT006202-GAL4 inhibited performance immediately after training (one-sample t-test: VT006202>CsChrimson n=8, df=7, t=0.3002, p=0.7728; UAS-CsChrimson n=8, df=7, t=6.371, p=0.0004; VT006202-GAL4 n=8, df=7, t=4.799, p=0.0020). This memory was different from the UAS-CsChrimson parental control but not from the GAL4 parental control (one-way ANOVA: F(2,21)=10.39, p=0.0007, Dunnett's post-hoc test: VT006202>CsChrimson vs. UAS-CsChrimson p=0.0004; VT006202>CsChrimson vs. VT006202-GAL4 p=0.1242) C) No memory was observed 24h after optogenetic activation in the VT006202>CsChrimson group or in the VT006202-GAL4 parental control (One-sample t-test: VT006202>CsChrimson n=8, df=7, t=2.003, p=0.0853; VT006202-GAL4 n=4, df=3, t=2.790, p=0.0684). No difference was seen between the groups (one-way ANOVA (2,15)=2.167, p=0.1490. B) and C) MB504B>CsChrimson was used as an activation control for aversive performance and excluded from group analysis (one-sample t-test in B n=7, df=6, t=6.136, p=0.0009; in A n=3, df=2, t=1.581, p= 0.2546).

5.3.3 Long-term Memory Retrieval Inhibition via γ5n DANs Activation

The ability of γ5n to inhibit short-term memory could arise through the creation of a parallel short-lived aversive memory to the CS^+, which would compete with the appetitive sucrose memory to counteract approach. Alternatively, these neurons could represent a motivational state that negatively regulates approach, such as satiety that would ultimately prevent flies from seeking reward in the short-term.

To test these possibilities, we investigated whether unpaired activation of γ5n prior to 24h memory retrieval inhibits long-term memory retrieval. By activating γ5n following sugar training in the absence of paired odour presentation, we can decouple γ5n activation with formation of an aversive memory. If γ5n encodes satiety state, post-training uncoupled activation prior to testing will suppress expression of appetitive memory. We found that by activating neurons labelled by R15A04-GAL4, VT006202-GAL4 and 0804-GAL4 drivers, all of which contain γ5n, two minutes prior to 24h memory retrieval, memory performance was disrupted (Figure 5.9). These results demonstrate that γ5n activation is suppressing memory retrieval by acting as a potential motivational switch.

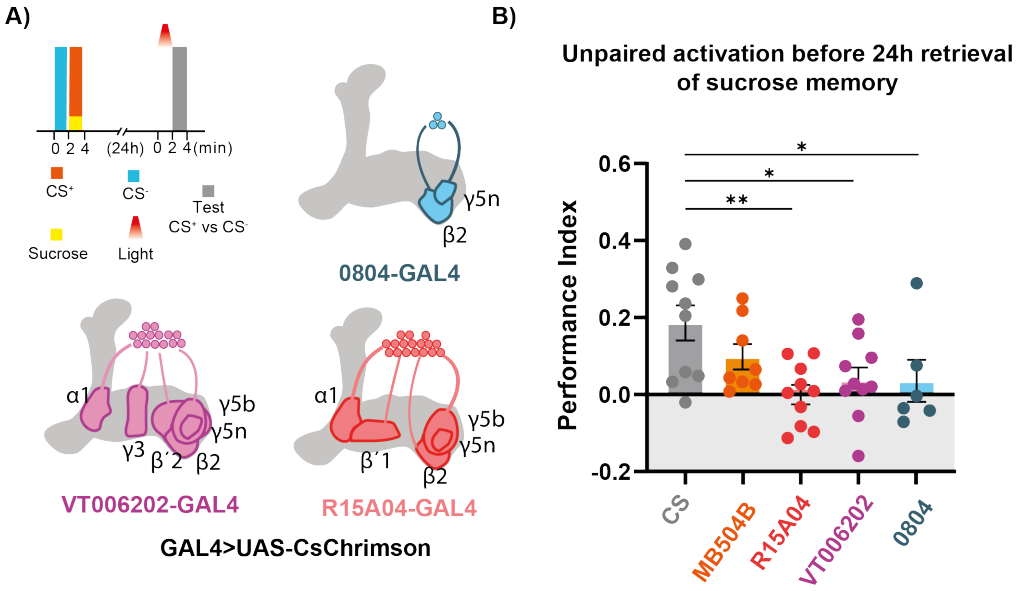


Figure 5.9: Unpaired activation of γ5n inhibits long-term memory retrieval

A) Schematic of the protocol and driver lines used. Flies carrying a UAS-CsChrimson transgene and different GAL4 drivers were trained using a standard appetitive learning paradigm. Neuronal activation without odour pairing was performed for 2 minutes immediately prior to memory retrieval test. B) Optogenetic activation prior to 24h memory retrieval suppressed performance in flies expressing UAS-CsChrimson in three drivers labelling γ5n (R15A04>CsChrimson n=10, df=9, t=0.01872, p=0.9855; VT006202>CsChrimson n=10, df=9, t=1.177, p=0.2694; 0804>CsChrimson n=6, df=5, t=0.6490, p=0.5450). This was significantly different from the wild-type CS control (one-way ANOVA $F(4,39)=4.125$, $p=0.007$. Dunnett's post-hoc test: MB504B>CsChrimson vs. UAS-CsChrimson p=0.3072; R15A04>CsChrimson vs. UAS-CsChrimson p= 0.0024; VT006202>CsChrimson vs. UAS-CsChrimson p= 0.0190; 0804>CsChrimson vs. UAS-CsChrimson p= 0.0453. Memory performance was not suppressed when activating aversive DANs labelled by MB504B-GAL4 prior to retrieval. (One-sample t-test MB504B>CsChrimson n=8, df=7, t=2.976, p=0.0206).

We then addressed whether a competing aversive memory created through paired activation could contribute to the transient inhibition of performance in addition to the motivational shift. For this, neuronal activation was paired to CS^+ presentation, in absence of sucrose. Memory performance was measured immediately and 3 hours after training. We did not find evidence of an artificial memory implanted by optogenetic activation of the γ5n DAN-labelling drivers (Figure 5.10A,B).

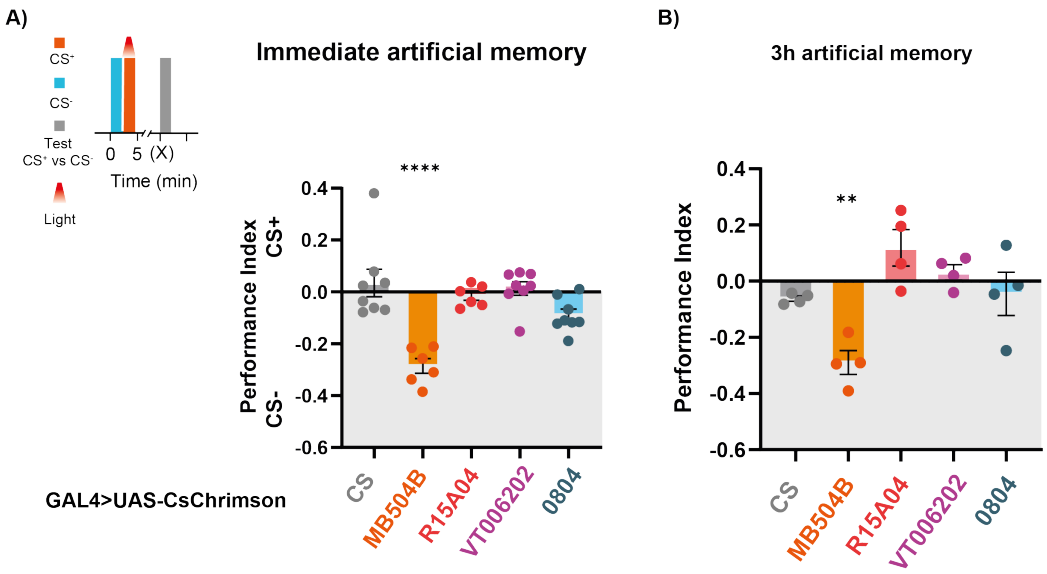


Figure 5.10: Paired activation of 0804-GAL4 does not lead to competing short-term memory

A) CS^{+}-paired optogenetic activation of drivers labelling of γ5n did not lead to any immediate artificial memory (R15A04>CsChrimson n=6, df=5, t=0.8958, p=0.4114; VT006202>CsChrimson n=8, df=7, t=0.5182, p=0.6203) except in 0804>CsChrimson (n=8, df=7, t=3.886, p=0.006. However, performance of all experimental groups, including 0804>CsChrimson was indistinguishable from the CS parental control (Kruskal-Wallis test: p=0.0266, Dunn's post-hoc test: 0804>CsChrimson vs. CS>CsChrimson p=0.06, all other comparisons p>0.2). B) No memory was observed 3h after training in groups labelling γ5n different from the UAS-Chrimson parental control (one-way ANOVA: F(3,12)=2.489, p=0.1101). A) and B) MB504B-GAL4 was used as an activation control and excluded from group analysis (one sample t-test n=6, df=5, t=10.01, p=0.0002 in A, and n=4, df=3, t=6.800, p=0.0065 in B).

5.4 Summary and Discussion

The work presented here elucidates results from a previous study reporting the presence of an emergent LTM, that arose from α1 activation [30]. However, because the GAL4 driver used in the previous study labelled several other compartments, we used more specific drivers to demonstrate that exclusive α1 activation leads to both STM and LTM. We showed that previous reports of an apparent emergence of only the long-term memory from the broad driver can be explained by concomitant activation of a non-α1 compartment that resulted in behavioural inhibition of the appetitive short-term memory. This behavioural inhibition arises through simultaneous activation of γ5n DANs within the broad R15A04-GAL4 driver.

I propose that γ5n DANs encode satiety and activation of these neurons and can engage a motivational switch to toggle memory expression, as activation of these neurons lead to suppression of short- and long-term sucrose memory retrieval. Activation of γ5n DANs during the second minute of sucrose presentation disrupted immediate memory retrieval. In addition, activating γ5n DANs prior to 24h sucrose memory testing impaired memory performance, suggesting that γ5n activation mainly impairs retrieval by artificially inducing a satiated state to suppress memory expression. However, it is also possible that inhibition of sucrose STM via partial pairing of neuronal activation is confounded by a reduction in sucrose consumption derived from satiety in the second minute of training.

To determine whether these neurons are necessary for state-dependent inhibition of memory performance, clear future experiments can be done. Measuring food consumption [108] in starved flies following γ5n activation would allow to determine the impact these manipulations have on state regulation. Although we tested

γ5n activation for immediate and 3h memory, it is unclear whether this can generate a 24h memory. Assessing whether 24h artificial memory can be implanted via activation of γ5n using restrictive drivers such as 0804-GAL4 could show any potential artificial memory contributing towards 24h memory performance. Another experiment involves γ5n-DAN inhibition in sugar trained flies that are re-fed before testing. In normal flies that are re-fed before testing, re-feeding suppresses memory expression [55]. However, we expect that γ5n inhibition would lead to inappropriate expression of sucrose memory in satiated flies. Inhibition experiments provide the advantage over activation studies since they allow the study of an ongoing process by disrupting its course. In the contrary, activation studies could lead to noise introduced into a different ongoing process, not specifically the one of interest. Moreover, stimulation under inappropriate conditions might lead to stimulation-induced inhibition[109]. Therefore, although they're a good proxy for studying the effect of the activity of a neuron and the computation that it might lead to, this is artificial stimulation might not always reflect how this neuron is activated in physiological circumstances.

Perhaps the most intriguing phenotype is that of the Tbh^{M18} mutant. Disruption of octopamine synthesis in these mutants leads exclusively to a long-term appetitive memory performance after sucrose training [32]. The authors hypothesise that this occurs because octopaminergic signalling conveys sweet-taste reinforcement, mitigating acquisition of sweet-reinforced memory. However, it is unclear how loss in octopamine signalling is selectively affecting short term memory.

One possibility is that Tbh^{M18} mutants have impaired short-term memory retrieval rather than impaired acquisition. This could result from a fast octopamine-induced inhibition of satiety-signalling neurons, which would normally underlie enhancement in performance for immediate memory, whereas loss of octopaminergic signalling results in failure to inhibit satiety-signalling neurons that would suppress

immediate performance. In this case, octopamine would be released when flies experience sweet-taste to promote retrieval of short-term memory and food seeking, by opposing satiety signals induced by the recently consumed meal. Further signalling from satiety-signalling molecules, such as DSK, Allatostatin-A or Insulin-like peptides, could terminate feeding [110–112]. Consistent with this, a study showed that consuming non-nutritive sweeteners increases subsequent feeding in wild-type flies but not in Tbh^{M18} mutants [113].

Calcium imaging of state modulating neurons, such as γ5n DANs or γ1-pedc [5], in Tbh^{M18} mutants and wild-type flies is crucial for understanding how octopaminergic signalling modulates activity of state-encoding neurons to ultimately modulate retrieval. Additional experiments knocking down expression of *Octβ1R*, an inhibitory octopamine receptor [114], in γ5n neurons could reveal a direct role for octopamine in immediate satiety suppression. However, more work is needed to test these hypotheses and to shed light on whether two independent memories become integrated to promote seeking of food-predictive odours across time.

6 | Discussion

Throughout this thesis we have demonstrated how different factors such as outcome evaluation, memory integration, state, and stimulus-outcome time contingency regulate behavioural output. In this final chapter we will discuss the implications of this work and the contributions it offers for the broader understanding of how these factors flexibly modulate behaviour.

Outcome Evaluation

Animals are able to evaluate stimuli valence and integrate multiple events leading to different outcomes [78]. Correct evaluation and integration of stimulus predicted outcomes allows animals to rely on previously formed associations to appropriately guide behaviour. Presentations of unreinforced stimuli are thought to lead to habituation and sensitisation that induce non-associative changes in behaviour. When presented after training, such as during memory extinction, animals can evaluate the absence of an explicit outcome using the expected outcome valence as reference, leading to a formation of an opposing valence memory which competes with the retrieval of the original memory [61, 88, 89]. In Chapter 4 we showed that flies can evaluate stimuli valence in the absence of explicit or expected outcomes. We showed that presentation of a stimulus without a reinforcer or expectation results in the inherent valence of the stimulus serving as its own reinforcer. We demonstrated that repeated pre-exposure to an aversive odour can further reinforce conditioned aversion. However, we did not see appetitive memories following pre-exposure to attractive concentrations of the odour. Interestingly, bidirectional changes in self-reported stimulus preference occur in humans through mere stimulus exposure, depending on stimulus category [115–117]. It has been proposed that preference changes to previously exposed stimuli arises from a context-dependent associative learning process [118, 119]; similar to what

we describe in Chapter 4.

Memory Integration

One strategy to optimise behavioural flexibility in a system is to maintain parallel memories of a stimulus that can be integrated to guide behaviour. We determined that a fast-decaying pre-exposure memory competes with subsequent learning to the pre-exposed stimulus, leading to latent inhibition of memory retrieval. Previously, it has been suggested that latent inhibition arises from a transient impairment of memory acquisition. However, single-trial appetitive conditioning in the fly following stimulus pre-exposure demonstrated that this is not the case. Instead, we find that a retrieval deficit underlies latent inhibition in the fly. In contrast to canonical aversive training paradigms such as in shock, heat and bitter training, pre-exposure memory occurs independently of γ1-pedc DANs.

By comparing our pre-exposure protocol to other exposure protocols with equal exposure time in a single event, we show that changes in stimulus preference do not occur via habituation. Moreover, familiarity leading to habituation in the fly is thought to occur via the α′3 compartment [92]. We showed that pre-exposure memory requires the α3 compartment of the mushroom body. Taken together, these data demonstrate that unreinforced stimulus presentations can acquire conditioned valence that gets integrated with subsequent memories to guide behaviour independently from a reference valence or habituation.

Behavioural Guidance

Dynamic retrieval of multiple associations is crucial for animals to be able to change behaviour across time and meet physiological and hedonic needs. Deprivation state can bias behaviour to satisfy these needs [120, 121]. Neuropeptide F (NPF), which signals hunger in flies, is known to inhibit γ1-pedc DANs, allowing the modulation of memory expression by state dependent signals [5]. In Chapter 5 we demonstrated that activation of γ5n DANs suppresses memory retrieval. We

propose this occurs through a motivational switch, which potentially signals satiation state.

Inhibition of γ5n in fed flies could lead to unexpected memory expression, showing a role for these neurons in state modulation. Investigating the role of γ5n neurons in suppressing memory retrieval in different physiological states could help in the understanding of whether this neuronal activation leads to a hedonic or a physiological motivational switch.

In addition to internal state, context can provide crucial information on environmental availability of rewards, allowing animals to guide their behaviour accordingly. In Chapter 4, we demonstrate that by changing contexts between pre-exposure and testing, latent inhibition is impaired, biasing expression of subsequent associations within similar contexts. Interestingly, pre-exposure memory alone is not context dependent, suggesting that when memories are in conflict, context provides an extra layer of information to influence memory retrieval and to prioritise expression of context-relevant memories.

Stimulus-Outcome Contingency

Temporal contingency between stimulus and outcome is critical for robust memory formation [14]. Nutrient-dependent memory is thought to exclusively contribute to the long-term memory performance of sucrose appetitive memory and is proposed to operate independently from any short-term contributions. However, it is unclear how a robust long-term memory is formed despite the delay between the stimulus and the onset of nutrient signal. Stimulus-nutrient specificity has been suggested to occur via delayed memory consolidation triggered by stimulus paired activation of α1 PAM-DANs [30]. However, previous work used broader drivers for α1 that included expression within other compartments, confounding the interpretation of these results.

By using restrictive drivers, the data presented through Chapter 5 demonstrated

that activation of PAM-α1 DANs results in robust immediate memory performance that lasts up to 24h. This opposes the idea that activation of α1 DANs leads to the emergence of a long-term memory in the absence of short-term memory. Moreover, we showed that the previously reported LTM-exclusive memory phenotype [30] arises from short-term inhibition of memory performance from simultaneous α1 and γ5n DAN activation.
Interestingly, inhibition of α1 DANs during short-term memory retrieval failed to disrupt memory performance, suggesting α1 DANs are not required in the short term [30]. Emerging LTM could arise from delayed activation of α1 DANs. Stimulus specificity could be maintained through some innate link between ingestion and nutrition, similar to an ingestion-illness association occurring in test aversion learning [24]. However, this could also be explained by the robustness of the network and the role of other compartments in short-term memory. Calcium imaging of α1 DANs before and immediately after training should indicate whether there is a delayed activation of α1 DANs.

Alternatively, and most likely, nutrient-dependent memory does not occur independently from short-term memory. One possibility is that nutrient detection occurs immediately upon consumption or within a very short period, allowing stimulus specificity to be maintained and triggering the formation of a persistent α1 DAN-dependent memory. Consistent with this idea, Burke et al. [31] showed that flies preferentially seek an odour predictive of nutritious sugars over an odour predictive of a non-nutritious sugar, even immediately after training, despite a similar proboscis extension reflex (PER) to both sugars. Association and consumption of both sugars within a short period of time would make retroactive nutrient-reinforcement difficult to allocate to either of the odour/sugar pairs, suggesting that nutrient-stimulus specificity is maintained at the moment of consumption or that flies can differentiate between the sugars despite similar PER.

Moreover, studies have shown that fly larvae are able to immediately detect different nutrients, such as amino acids, to guide food intake [122]. Similarly, adult female flies assess nutrient quality of the environment for oviposition decision-making and to guide food consumption according to deprivation state [123].
It has been suggested that feeding after training triggers sleep-dependent memory consolidation, which will recruit a different neuronal network than starved/sleep-independent memory consolidation [124]. Persisting nutrients in this case would not be creating an emerging memory, but instead promoting the consolidation of short-term memory into long-term memory, potentially by triggering sleep-dependent consolidation.

6.1 Conclusions

Learning from experience is crucial for predicting outcomes and engaging the right behavioural response. But its only through the combination of learned memories with retrieval factors that animals are allowed to dynamically act upon the relevant memories to guide behaviour in the most appropriate way at the given circumstances. This thesis focused on two cases, latent inhibition and nutrient-dependent memory, in which animals do not show a memory performance immediately after training but performance appears later in time. While these phenomenon had been suggested to occur through the lack of acquisition of memory traces (via lack of attention or delayed memory formation); the later appearance of memory performance allowed us to look into ways in which memory retrieval can be suppressed to allow a dynamic changes in behaviour, masking the existence of the memory.

In Chapter 4, we demonstrated that the exposure to a stimulus without explicit outcome can acquire a conditioned response through repetition. In this case the

stimulus valence serves as its own reinforcement signal. In the right context, this memory can be retrieved and get integrated to compete with other memories leading to latent inhibition. We showed that LI emerged as a consequence of interference and suppression of appetitive memory retrieval. Upon decay of pre-exposure memory, other memories can be expressed. Moreover, this data shows that that pre-exposure underlying latent inhibition in *Drosophila* does not result from an impact of pre-exposure in learning, but rather acts as a performance suppressor.

The role of nutrients in memory consolidation has been demonstrated across species [125–127]. Research in humans shows that caloric consumption positively affects memory processing, enhancing memory performance [126, 128]. In the contrary, hypoglycemic state in type I diabetic patients negatively impact consolidation of declarative memory [127]. In *Drosophila*, training paradigms that lead to LTM have shown to be more energetically costly compared to those leading to only STM [125]. It is clear that nutrition plays a critical role for memory consolidation. It is therefore not surprising that nutrient reinforced memories last longer[31]. This long-lasting memory was thought to arise through the delayed processing of a nutrient signal, creating a delayed memory trace[30, 75]. However, through the experiments performed in Chapter 5, we showed that nutrient-dependent memory is unlikely to occur through delayed emergence of a memory trace reinforced by α1-DANs. Interestingly, nutrient consumption following an odour/non-nutritious association allows consolidation of the association, despite lack of pairing between the nutrient and the odour [29]. Taken together, this suggests that nutrients are more likely to promote persistence or consolidation of a short term memory than to arise independently.

This work shows that factors such as integration of parallel memories, context changes, and internal state shift, are capable of inhibiting behaviour despite intact

memory acquisition. Uncovering the molecular and neural correlates of these factors will not only further our understanding of how animals adapt their behaviour to a constantly changing environment, but also will allow us to evaluate these factors as temporal inhibitors of performance in paradigms where memories are thought to not be longer present, such as forgetting.

Bibliography

[1] Amrita Mukherjee and Darren W. Williams. "More Alive than Dead: Non-Apoptotic Roles for Caspases in Neuronal Development, Plasticity and Disease". In: *Cell Death and Differentiation* 24.8 (Aug. 2017), pp. 1411–1421.

[2] Zheng Li et al. "Caspase-3 Activation via Mitochondria Is Required for Long-Term Depression and AMPA Receptor Internalization". In: *Cell* 141.5 (May 2010), pp. 859–871.

[3] Julien Gibon et al. "The X-linked Inhibitor of Apoptosis Regulates Long-Term Depression and Learning Rate". In: *FASEB journal: official publication of the Federation of American Societies for Experimental Biology* 30.9 (Sept. 2016), pp. 3083–3090.

[4] T. Tully et al. "Genetic Dissection of Consolidated Memory in Drosophila". In: *Cell* 79.1 (Oct. 1994), pp. 35–47.

[5] Michael J. Krashes et al. "A Neural Circuit Mechanism Integrating Motivational State with Memory Expression in Drosophila". In: *Cell* 139.2 (Oct. 2009), pp. 416–427.

[6] Emmanuel Perisse et al. "Aversive Learning and Appetitive Motivation Toggle Feed-Forward Inhibition in the Drosophila Mushroom Body". In: *Neuron* 90.5 (June 2016), pp. 1086–1099.

[7] Feng Li et al. "The Connectome of the Adult Drosophila Mushroom Body Provides Insights into Function". In: *eLife* 9 (Dec. 2020). Ed. by Leslie C Griffith et al., e62576.

[8] Ladan Shams and Aaron R. Seitz. "Benefits of Multisensory Learning". In: *Trends in Cognitive Sciences* 12.11 (Nov. 2008), pp. 411–417.

[9] Christopher R. Fetsch, Gregory C. DeAngelis, and Dora E. Angelaki. "Bridging the Gap between Theories of Sensory Cue Integration and the Physiology of Multisensory Neurons". In: *Nature Reviews. Neuroscience* 14.6 (June 2013), pp. 429–442.

[10] S. Benzer. "BEHAVIORAL MUTANTS OF Drosophila ISOLATED BY COUNTERCURRENT DISTRIBUTION". In: *Proceedings of the National Academy of Sciences of the United States of America* 58.3 (Sept. 1967), pp. 1112–1119.

[11] Zeynep Okray et al. *Multisensory Learning Binds Modality-Specific Neurons into a Cross-Modal Memory Engram*. July 2022.

[12] P Ivan Pavlov. "Conditioned Reflexes: An Investigation of the Physiological Activity of the Cerebral Cortex". In: *Annals of Neurosciences* 17.3 (1927), pp. 136–141.

[13] E. L. Thorndike. "Animal Intelligence: An Experimental Study of the Associative Processes in Animals". In: *Psychological review* 5.5 (1898), pp. 551–553.

[14] Mark E. Bouton. *Learning and Behaviour. A Contemporary Synthesis*. Sinauer Associates Inc., 2007.

[15] John H. Byrne. "Learning and Memory: Basic Mechanisms". In: *Fundamental Neuroscience (Fourth Edition)*. Ed. by Larry R. Squire et al. San Diego: Academic Press, Jan. 2013, pp. 1009–1027.

[16] Charles Tayloe. *The Explanation of Behaviour*. London: Routledge & Kegan Paul Ltd, 1964.

[17] Donald M. Thomson and Endel Tulving. "Associative Encoding and Retrieval: Weak and Strong Cues." In: *Journal of Experimental Psychology* 86.2 (1971), p. 255.

[18] Mark E. Bouton and Douglas C. Brooks. “Time and Context Effects on Performance in a Pavlovian Discrimination Reversal.” In: *Journal of Experimental Psychology: Animal Behavior Processes* 19.2 (19930701), p. 165.

[19] Pepe J. Hernandez and Ted Abel. “The Role of Protein Synthesis in Memory Consolidation: Progress amid Decades of Debate”. In: *Neurobiology of learning and memory* 89.3 (Mar. 2008), pp. 293–311.

[20] Edward A. Wasserman and Ralph R. Miller. “What’s Elementary About Associative Learning?” In: *Annual Review of Psychology* 48.1 (1997), pp. 573–607.

[21] Eric R. Kandel. “The Biology of Memory: A Forty-Year Perspective”. In: *Journal of Neuroscience* 29.41 (Oct. 2009), pp. 12748–12756.

[22] Robert A. Rescorla. “Pavlovian Conditioning: It’s Not What You Think It Is”. In: *American Psychologist* 43.3 (1988), pp. 151–160.

[23] J. Garcia, W. G. Hankins, and K. W. Rusiniak. “Behavioral Regulation of the Milieu Interne in Man and Rat”. In: *Science (New York, N.Y.)* 185.4154 (Sept. 1974), pp. 824–831.

[24] John Garcia and Robert A. Koelling. “Relation of Cue to Consequence in Avoidance Learning”. In: *Psychonomic Science* 4.1 (Jan. 1966), pp. 123–124.

[25] Geraldine A. Wright et al. “Parallel Reinforcement Pathways for Conditioned Food Aversions in the Honeybee”. In: *Current Biology* 20.24 (Dec. 2010), pp. 2234–2240.

[26] Aurélie Babin et al. “Fruit Flies Learn to Avoid Odours Associated with Virulent Infection”. In: *Biology Letters* 10.3 (Mar. 2014), p. 20140048.

[27] Andrés Molero-Chamizo. “Taste Aversions”. In: June 2018.

[28] Jan Bures, Federico Bermudez-Rattoni, and Takashi Yamamoto. *Conditioned Taste Aversion: Memory of a Special Kind*. Oxford: Oxford Univ. Press, 2002.

[29] Pierre-Yves Musso, Paul Tchenio, and Thomas Preat. "Delayed Dopamine Signaling of Energy Level Builds Appetitive Long-Term Memory in Drosophila". In: *Cell Reports* 10.7 (Feb. 2015), pp. 1023–1031.

[30] Nobuhiro Yamagata et al. "Distinct Dopamine Neurons Mediate Reward Signals for Short- and Long-Term Memories". In: *Proceedings of the National Academy of Sciences* 112.2 (Jan. 2015), pp. 578–583.

[31] Christopher J. Burke and Scott Waddell. "Remembering Nutrient Quality of Sugar in Drosophila". In: *Current biology: CB* 21.9 (May 2011), pp. 746–750.

[32] Gaurav Das et al. "Drosophila Learn Opposing Components of a Compound Food Stimulus". In: *Current Biology* 24.15 (Aug. 2014), pp. 1723–1730.

[33] Bruce L. Tempel et al. "Reward Learning in Normal and Mutant Drosophila". In: *Proceedings of the National Academy of Sciences of the United States of America* 80.5 (Mar. 1983), pp. 1482–1486.

[34] Kent C. Berridge and Terry E. Robinson. "Liking, Wanting and the Incentive-Sensitization Theory of Addiction". In: *The American psychologist* 71.8 (Nov. 2016), pp. 670–679.

[35] Mark E. Bouton. "Context, Time, and Memory Retrieval in the Interference Paradigms of Pavlovian Learning". In: *Psychological Bulletin* 114.1 (1993), pp. 80–99.

[36] Karl-Heinz T. Bäuml. "Context Retrieval as a Critical Component in Selective Memory Retrieval". In: *Current Directions in Psychological Science* 28.2 (Apr. 2019), pp. 177–182.

[37] Karen G. Hales et al. “Genetics on the Fly: A Primer on the Drosophila Model System”. In: *Genetics* 201.3 (Nov. 2015), pp. 815–842.

[38] A. H. Brand and N. Perrimon. “Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes”. In: *Development (Cambridge, England)* 118.2 (June 1993), pp. 401–415.

[39] Sean E. McGuire, Zhengmei Mao, and Ronald L. Davis. “Spatiotemporal Gene Expression Targeting with the TARGET and Gene-Switch Systems in Drosophila”. In: *Science’s STKE: signal transduction knowledge environment* 2004.220 (Feb. 2004), pl6.

[40] T. Osterwalder et al. “A Conditional Tissue-Specific Transgene Expression System Using Inducible GAL4”. In: *Proceedings of the National Academy of Sciences of the United States of America* 98.22 (Oct. 2001), pp. 12596–12601.

[41] Haojiang Luan et al. “Refined Spatial Manipulation of Neuronal Function by Combinatorial Restriction of Transgene Expression”. In: *Neuron* 52.3 (Nov. 2006), pp. 425–436.

[42] Tony D. Southall, David A. Elliott, and Andrea H. Brand. “The GAL4 System: A Versatile Toolkit for Gene Expression in Drosophila”. In: *Cold Spring Harbor Protocols* 2008.7 (July 2008), pdb.top49.

[43] Arnim Jenett et al. “A GAL4-Driver Line Resource for Drosophila Neurobiology”. In: *Cell Reports* 2.4 (Oct. 2012), pp. 991–1001.

[44] Barret D Pfeiffer et al. “Refinement of Tools for Targeted Gene Expression in Drosophila”. In: *Genetics* 186.2 (Oct. 2010), pp. 735–755.

[45] Yoshinori Aso et al. “The Neuronal Architecture of the Mushroom Body Provides a Logic for Associative Learning”. In: *eLife* 3 (Dec. 2014), e04577.

[46] Junko Kasuya, Hiroshi Ishimoto, and Toshihiro Kitamoto. “Neuronal Mechanisms of Learning and Memory Revealed by Spatial and Temporal Sup-

pression of Neurotransmission Using Shibirets1, a Temperature-Sensitive Dynamin Mutant Gene in Drosophila Melanogaster". In: *Frontiers in Molecular Neuroscience* 2 (2009).

[47] Adam Claridge-Chang et al. "Writing Memories with Light-Addressable Reinforcement Circuitry". In: *Cell* 139.2 (Oct. 2009), pp. 405–415.

[48] Nathan C. Klapoetke et al. "Independent Optical Excitation of Distinct Neural Populations". In: *Nature Methods* 11.3 (Mar. 2014), pp. 338–346.

[49] T. Kitamoto. "Conditional Modification of Behavior in Drosophila by Targeted Expression of a Temperature-Sensitive Shibire Allele in Defined Neurons". In: *Journal of Neurobiology* 47.2 (May 2001), pp. 81–92.

[50] J. Hirsch and J. C. Boudreau. "Studies in Experimental Behavior Genetics. I. The Heritability of Phototaxis in a Population of Drosophila Melanogaster". In: *Journal of Comparative and Physiological Psychology* 51.6 (Dec. 1958), pp. 647–651.

[51] Tim Tully. "Discovery of Genes Involved with Learning and Memory: An Experimental Synthesis of Hirschian and Benzerian Perspectives". In: *Proceedings of the National Academy of Sciences of the United States of America* 93.24 (Nov. 1996), pp. 13460–13467.

[52] S. Benzer. "From the Gene to Behavior". In: *JAMA* 218.7 (Nov. 1971), pp. 1015–1022.

[53] Tim Tully and William G. Quinn. "Classical Conditioning and Retention in Normal and mutantDrosophila Melanogaster". In: *Journal of Comparative Physiology A* 157.2 (Mar. 1985), pp. 263–277.

[54] E Folkers, P Drain, and W G Quinn. "Radish, a Drosophila Mutant Deficient in Consolidated Memory." In: *Proceedings of the National Academy of Sciences of the United States of America* 90.17 (Sept. 1993), pp. 8123–8127.

[55] Michael J. Krashes and Scott Waddell. “Rapid Consolidation to a Radish and Protein Synthesis-Dependent Long-Term Memory after Single-Session Appetitive Olfactory Conditioning in Drosophila”. In: *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 28.12 (Mar. 2008), pp. 3103–3113.

[56] Yoshinori Aso et al. “Mushroom Body Output Neurons Encode Valence and Guide Memory-Based Action Selection in Drosophila”. In: *eLife* 3 (Dec. 2014). Ed. by Liqun Luo, e04580.

[57] David Owald and Scott Waddell. “Olfactory Learning Skews Mushroom Body Output Pathways to Steer Behavioral Choice in Drosophila”. In: *Current Opinion in Neurobiology* 35 (Dec. 2015), pp. 178–184.

[58] Efrain O. Aceves-Piña and William G. Quinn. “Learning in Normal and Mutant Drosophila Larvae”. In: *Science* 206.4414 (Oct. 1979), pp. 93–96.

[59] W. G. Quinn, W. A. Harris, and S. Benzer. “Conditioned Behavior in Drosophila Melanogaster”. In: *Proceedings of the National Academy of Sciences of the United States of America* 71.3 (Mar. 1974), pp. 708–712.

[60] W. G. Quinn and Y. Dudai. “Memory Phases in Drosophila”. In: *Nature* 262.5569 (Aug. 1976), pp. 576–577.

[61] Johannes Felsenberg et al. “Integration of Parallel Opposing Memories Underlies Memory Extinction”. In: *Cell* 175.3 (Oct. 2018), 709–722.e15.

[62] Martin Heisenberg et al. “Drosophila Mushroom Body Mutants Are Deficient in Olfactory Learning”. In: *Journal of Neurogenetics* 2.1 (Jan. 1985), pp. 1–30.

[63] Jill R. Crittenden et al. “Tripartite Mushroom Body Architecture Revealed by Antigenic Markers”. In: *Learning & Memory* 5.1 (May 1998), pp. 38–51.

[64] Yoshinori Aso et al. “Three Dopamine Pathways Induce Aversive Odor Memories with Different Stability”. In: *PLoS Genetics* 8.7 (July 2012).

[65] Oliver Barnstedt et al. “Memory-Relevant Mushroom Body Output Synapses Are Cholinergic”. In: *Neuron* 89.6 (Mar. 2016), pp. 1237–1247.

[66] Shin-ya Takemura et al. “A Connectome of a Learning and Memory Center in the Adult Drosophila Brain”. In: *eLife* 6 (July 2017). Ed. by Ronald L Calabrese, e26975.

[67] Tamara Boto et al. “Independent Contributions of Discrete Dopaminergic Circuits to Cellular Plasticity, Memory Strength, and Valence in Drosophila”. In: *Cell Reports* 27.7 (May 2019), 2014–2021.e2.

[68] Zhengmei Mao and Ronald L. Davis. “Eight Different Types of Dopaminergic Neurons Innervate the Drosophila Mushroom Body Neuropil: Anatomical and Physiological Heterogeneity”. In: *Frontiers in Neural Circuits* 3 (July 2009).

[69] Pedro F. Jacob et al. “Prior Experience Conditionally Inhibits the Expression of New Learning in Drosophila”. In: *Current Biology* (June 2021).

[70] Robert A. A. Campbell et al. “Imaging a Population Code for Odor Identity in the Drosophila Mushroom Body”. In: *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 33.25 (June 2013), pp. 10568–10581.

[71] Andrew C. Lin et al. “Sparse, Decorrelated Odor Coding in the Mushroom Body Enhances Learned Odor Discrimination”. In: *Nature Neuroscience* 17.4 (Apr. 2014), pp. 559–568.

[72] Glenn C. Turner, Maxim Bazhenov, and Gilles Laurent. “Olfactory Representations by Drosophila Mushroom Body Neurons”. In: *Journal of Neurophysiology* 99.2 (Feb. 2008), pp. 734–746.

[73] Chang Liu et al. “A Subset of Dopamine Neurons Signals Reward for Odour Memory in Drosophila”. In: *Nature* 488.7412 (Aug. 2012), pp. 512–516.

[74] Yoshinori Aso et al. “Specific Dopaminergic Neurons for the Formation of Labile Aversive Memory”. In: *Current Biology* 20.16 (Aug. 2010), pp. 1445–1451.

[75] Wolf Huetteroth et al. “Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in Drosophila”. In: *Current Biology* 25.6 (Mar. 2015), pp. 751–758.

[76] Nils Otto et al. “Input Connectivity Reveals Additional Heterogeneity of Dopaminergic Reinforcement in Drosophila”. In: *Current Biology* 30.16 (Aug. 2020), 3200–3211.e8.

[77] Laszlo Tirian and Barry J. Dickson. *The VT GAL4, LexA, and Split-GAL4 Driver Line Collections for Targeted Expression in the Drosophila Nervous System*. Oct. 2017.

[78] Yoshinori Aso and Gerald M Rubin. “Dopaminergic Neurons Write and Update Memories with Cell-Type-Specific Rules”. In: *eLife* 5 (July 2016). Ed. by Liqun Luo, e16135.

[79] Thomas A Grigliatti et al. “Temperature-Sensitive Mutations in Drosophil-amelanogaster”. In: (), p. 8.

[80] J H Koenig, K Saito, and K Ikeda. “Reversible Control of Synaptic Transmission in a Single Gene Mutant of Drosophila Melanogaster.” In: *Journal of Cell Biology* 96.6 (June 1983), pp. 1517–1522.

[81] Pedro F. Jacob and Scott Waddell. “Spaced Training Forms Complementary Long-Term Memories of Opposite Valence in Drosophila”. In: *bioRxiv* (Sept. 2019), p. 785618.

[82] Christopher J. Burke et al. “Layered Reward Signalling through Octopamine and Dopamine in Drosophila”. In: *Nature* 492.7429 (Dec. 2012), pp. 433–437.

[83] Farhan Mohammad et al. “Optogenetic Inhibition of Behavior with Anion Channelrhodopsins”. In: *Nature Methods* 14.3 (Mar. 2017), pp. 271–274.

[84] Alexander R. Ochs et al. “Optogenetic Stimulation Using Anion Channelrhodopsin (GtACR1) Facilitates Termination of Reentrant Arrhythmias With Low Light Energy Requirements: A Computational Study”. In: *Frontiers in Physiology* 12 (2021).

[85] Alex S. Mauss, Christian Busch, and Alexander Borst. “Optogenetic Neuronal Silencing in Drosophila during Visual Processing”. In: *Scientific Reports* 7.1 (Oct. 2017), p. 13823.

[86] Martín Klappenbach, Ayelén Nally, and Fernando Federico Locatelli. “Parallel Memory Traces Are Built after an Experience Containing Aversive and Appetitive Components in the Crab Neohelice”. In: *Proceedings of the National Academy of Sciences of the United States of America* 114.23 (June 2017), E4666–E4675.

[87] Martín Klappenbach, Agustín E. Lara, and Fernando F. Locatelli. “Honey Bees Can Store and Retrieve Independent Memory Traces after Complex Experiences That Combine Appetitive and Aversive Associations”. In: *Journal of Experimental Biology* 225.11 (June 2022), jeb244229.

[88] Xiangyu Zhang, Joshua Kim, and Susumu Tonegawa. “Amygdala Reward Neurons Form and Store Fear Extinction Memory”. In: *Neuron* 105.6 (Mar. 2020), 1077–1093.e7.

[89] Susana S Correia et al. “Amygdala-Ventral Striatum Circuit Activation Decreases Long-Term Fear”. In: *eLife* 5 (Sept. 2016), e12669.

[90] Jean-Christophe Sandoz and Minh-Hà Pham-Delègue. “Spontaneous Recovery After Extinction of the Conditioned Proboscis Extension Response in the Honeybee”. In: *Learning & Memory* 11.5 (Sept. 2004), pp. 586–597.

[91] Isabell Twick, John Anthony Lee, and Mani Ramaswami. “Olfactory Habituation in Drosophila-odor Encoding and Its Plasticity in the Antennal Lobe”. In: *Progress in Brain Research* 208 (2014), pp. 3–38.

[92] Daisuke Hattori et al. “Representations of Novelty and Familiarity in a Mushroom Body Compartment”. In: *Cell* 169.5 (May 2017), 956–969.e17.

[93] N. J. Mackintosh. “Stimulus Selection: Learning to Ignore Stimuli That Predict No Change in Reinforcement”. In: *Constraints on Learning: Limitations and Predispositions*. Oxford, England: Academic Press, 1973, pp. xv, 488–xv, 488.

[94] Steven Reiss and Allan R. Wagner. “CS Habituation Produces a “Latent Inhibition Effect” but No Active “Conditioned Inhibition””. In: *Learning and Motivation* 3.3 (Aug. 1972), pp. 237–245.

[95] Ourania Semelidou, Summer F Acevedo, and Efthimios MC Skoulakis. “Temporally Specific Engagement of Distinct Neuronal Circuits Regulating Olfactory Habituation in Drosophila”. In: *eLife* 7 (Dec. 2018), e39569.

[96] E. C. Tolman and C. H. Honzik. “Introduction and Removal of Reward, and Maze Performance in Rats”. In: *University of California Publications in Psychology* 4 (1930), pp. 257–275.

[97] H. C. Blodgett. “The Effect of the Introduction of Reward upon the Maze Performance of Rats”. In: *University of California Publications in Psychology* 4 (1929), pp. 113–134.

[98] R. E. Lubow and A. U. Moore. “Latent Inhibition: The Effect of Nonreinforced Pre-Exposure to the Conditional Stimulus”. In: *Journal of Comparative and Physiological Psychology* 52 (Aug. 1959), pp. 415–419.

[99] K. R. Weiss and B. L. Brown. “Latent Inhibition: A Review and a New Hypothesis”. In: *Acta Neurobiologiae Experimentalis* 34.2 (1974), pp. 301–316.

[100] Allan R. Wagner. “Context-Sensitive Elemental Theory”. In: *The Quarterly Journal of Experimental Psychology. B, Comparative and Physiological Psychology* 56.1 (Feb. 2003), pp. 7–29.

[101] C. D. Beck, B. Schroeder, and R. L. Davis. “Learning Performance of Normal and Mutant Drosophila after Repeated Conditioning Trials with Discrete Stimuli”. In: *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 20.8 (Apr. 2000), pp. 2944–2953.

[102] Michael J. Krashes et al. “Sequential Use of Mushroom Body Neuron Subsets during Drosophila Odor Memory Processing”. In: *Neuron* 53.1 (Jan. 2007), pp. 103–115.

[103] Dana Shani Galili et al. “Converging Circuits Mediate Temperature and Shock Aversive Olfactory Conditioning in Drosophila”. In: *Current Biology* 24.15 (Aug. 2014), pp. 1712–1722.

[104] R. E. Lubow, Paul Schnur, and Bathsheva Rifkin. “Latent Inhibition and Conditioned Attention Theory”. In: *Journal of Experimental Psychology: Animal Behavior Processes* 2 (1976), pp. 163–174.

[105] R. Frederick Westbrook and Mark E. Bouton. “Latent Inhibition and Extinction: Their Signature Phenomena and the Role of Prediction Error”. In: *Latent Inhibition: Cognition, Neuroscience and Applications to Schizophrenia*. Ed. by Ina Weiner and Robert Lubow. Cambridge: Cambridge University Press, 2010, pp. 23–39.

[106] J. Colomb et al. “Parametric and Genetic Analysis of Drosophila Appetitive Long-Term Memory and Sugar Motivation”. In: *Genes, Brain and Behavior* 8.4 (2009), pp. 407–415.

[107] Pierre-Yves Plaçais et al. “Upregulated Energy Metabolism in the Drosophila Mushroom Body Is the Trigger for Long-Term Memory”. In: *Nature Communications* 8.1 (June 2017), p. 15510.

[108] William W. Ja et al. “Prandiology of Drosophila and the CAFE Assay”. In: *Proceedings of the National Academy of Sciences of the United States of America* 104.20 (May 2007), pp. 8253–8256.

[109] Mohammed Filali et al. “Stimulation-Induced Inhibition of Neuronal Firing in Human Subthalamic Nucleus”. In: *Experimental Brain Research* 156.3 (June 2004), pp. 274–281.

[110] Allan-Hermann Pool and Kristin Scott. “Feeding Regulation in Drosophila”. In: *Current opinion in neurobiology* 0 (Dec. 2014), pp. 57–63.

[111] Jeannette A. E. Söderberg, Mikael A. Carlsson, and Dick R. Nässel. “Insulin-Producing Cells in the Drosophila Brain Also Express Satiety-Inducing Cholecystokinin-Like Peptide, Drosulfakinin”. In: *Frontiers in Endocrinology* 3 (2012), p. 109.

[112] Anne Christina Hergarden, Timothy D. Tayler, and David J. Anderson. “Allatostatin-A Neurons Inhibit Feeding Behavior in Adult Drosophila”. In: *Proceedings of the National Academy of Sciences of the United States of America* 109.10 (Mar. 2012), pp. 3967–3972.

[113] Qiao-Ping Wang et al. “Sucralose Promotes Food Intake through NPY and a Neuronal Fasting Response”. In: *Cell Metabolism* 24.1 (July 2016), pp. 75–90.

[114] Alex C. Koon and Vivian Budnik. “Inhibitory Control of Synaptic and Behavioral Plasticity by Octopaminergic Signaling”. In: *The Journal of Neuroscience* 32.18 (May 2012), pp. 6312–6322.

[115] Robert B. Zajonc. “Attitudinal Effects of Mere Exposure”. In: *Journal of Personality and Social Psychology* 9.2, Pt.2 (1968), pp. 1–27.

[116] Junghyun Park, Eiko Shimojo, and Shinsuke Shimojo. “Roles of Familiarity and Novelty in Visual Preference Judgments Are Segregated across Object Categories”. In: *Proceedings of the National Academy of Sciences* 107.33 (Aug. 2010), pp. 14552–14555.

[117] Hsin-I Liao, Su-Ling Yeh, and Shinsuke Shimojo. “Novelty vs. Familiarity Principles in Preference Decisions: Task-Context of Past Experience Matters”. In: *Frontiers in Psychology* 2 (Mar. 2011), p. 43.

[118] R.B. Zajonc. “Mere Exposure: A Gateway to the Subliminal”. In: *Current Directions in Psychological Science* 10.6 (Dec. 2001), pp. 224–228.

[119] Marieke de Vries et al. “Happiness Cools the Warm Glow of Familiarity: Psychophysiological Evidence That Mood Modulates the Familiarity-Affect Link”. In: *Psychological Science* 21.3 (Mar. 2010), pp. 321–328.

[120] Sherry J. Cheriyamkunnel et al. “A Neuronal Mechanism Controlling the Choice between Feeding and Sexual Behaviors in Drosophila”. In: *Current Biology* 31.19 (Oct. 2021), 4231–4245.e4.

[121] Verónica María Corrales-Carvajal, Aldo A Faisal, and Carlos Ribeiro. “Internal States Drive Nutrient Homeostasis by Modulating Exploration-Exploitation Trade-Off”. In: *eLife* 5 (Oct. 2016). Ed. by Iain D Couzin, e19920.

[122] Marianne Bjordal et al. “Sensing of Amino Acids in a Dopaminergic Circuitry Promotes Rejection of an Incomplete Diet in Drosophila”. In: *Cell* 156.3 (Jan. 2014), pp. 510–521.

[123] Mathieu Lihoreau et al. “Drosophila Females Trade off Good Nutrition with High-Quality Oviposition Sites When Choosing Foods”. In: *The Journal of Experimental Biology* 219.Pt 16 (Aug. 2016), pp. 2514–2524.

[124] Nitin S. Chouhan et al. “Availability of Food Determines the Need for Sleep in Memory Consolidation”. In: *Nature* 589.7843 (Jan. 2021), pp. 582–585.

[125] Frederic Mery and Tadeusz J. Kawecki. “A Cost of Long-Term Memory in Drosophila”. In: *Science (New York, N.Y.)* 308.5725 (May 2005), p. 1148.

[126] Sandra I. Sünram-Lea et al. “The Effect of Retrograde and Anterograde Glucose Administration on Memory Performance in Healthy Young Adults”. In: *Behavioural Brain Research* 134.1 (Aug. 2002), pp. 505–516.

[127] Kamila Jauch-Chara et al. “Hypoglycemia During Sleep Impairs Consolidation of Declarative Memory in Type 1 Diabetic and Healthy Humans”. In: *Diabetes Care* 30.8 (Aug. 2007), pp. 2040–2045.

[128] Nina Herzog et al. “Effects of Daytime Food Intake on Memory Consolidation during Sleep or Sleep Deprivation”. In: *PLOS ONE* 7.6 (June 2012), e40298.

[129] Yoshinori Aso et al. “The Mushroom Body of Adult Drosophila Characterized by GAL4 Drivers”. In: *Journal of Neurogenetics* 23.1-2 (Jan. 2009), pp. 156–172.

[130] Pierre-Yves Plaçais et al. “Two Pairs of Mushroom Body Efferent Neurons Are Required for Appetitive Long-Term Memory Retrieval in Drosophila”. In: *Cell Reports* 5.3 (Nov. 2013), pp. 769–780.

A | Expression patterns of drivers used

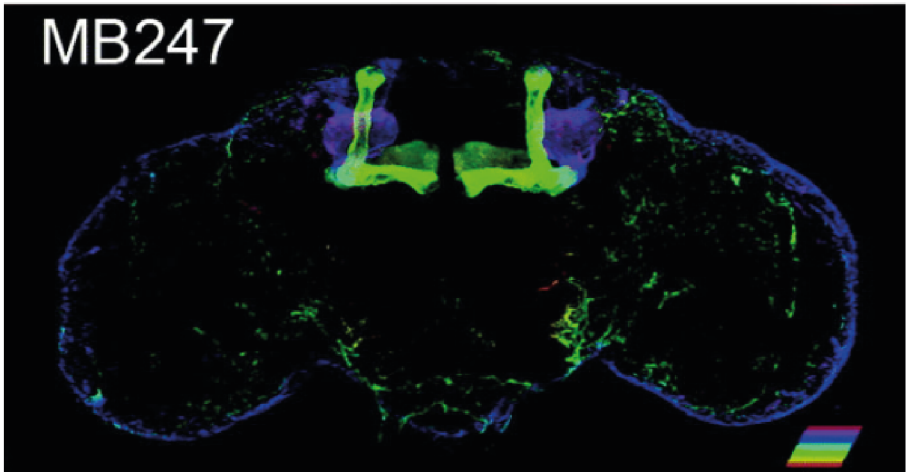


Figure A.1: MB247-GAL4 expression pattern

MB247-GAL4 driving the expression of CD8::GFP shows expression in the α/βand γKCs (in green). Image from Aso et al 2009 [129]

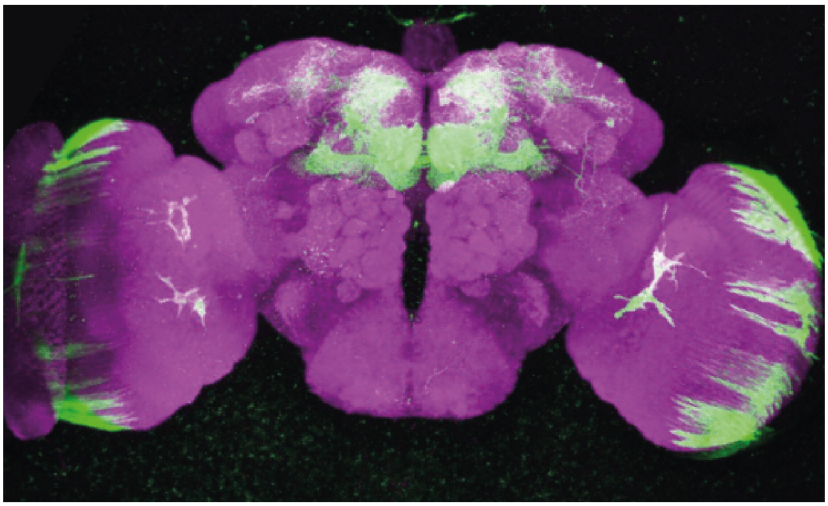


Figure A.2: R58E02-GAL4 driver expression pattern

R58E02-GAL4 driving expression of CD8::GFP (in green) shows labelling of PAM DANs innervating the horizontal compartments of the mushroom body[45]. Image from the Fly Light collection. [43]

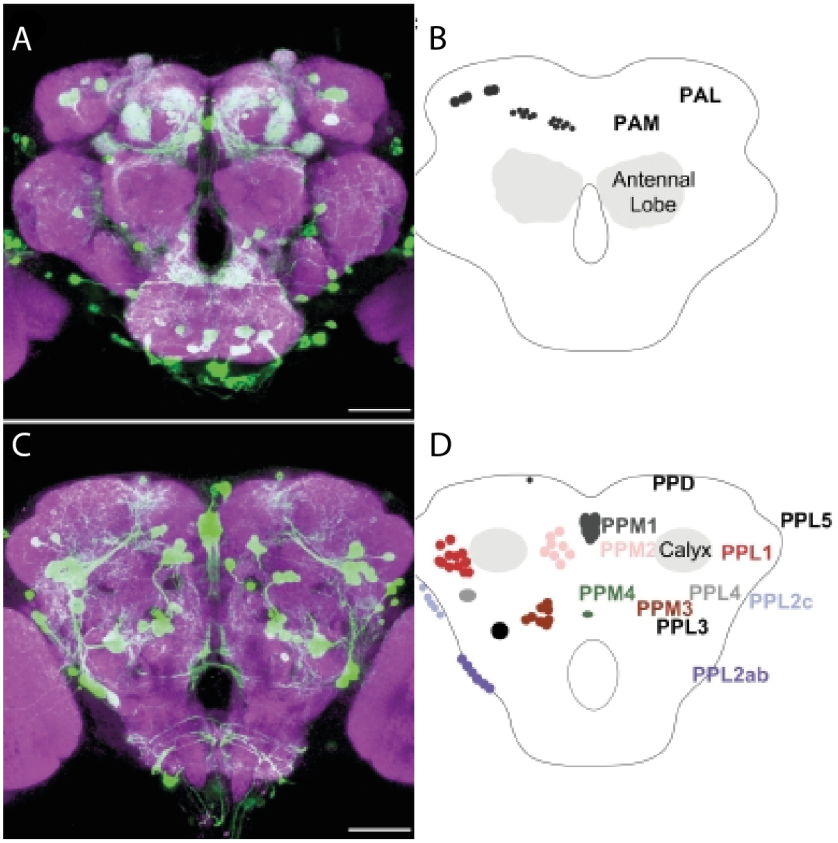


Figure A.3: TH-GAL4 labels multiple subsets of Dopaminergic cells in the fly brain

Image modified from from Mao et al 2009 [68]. A-B) Anterior view. C-D) Posterior view A,C) Brain stained with magenta with anti-nc82. In green, TH-GAL4 expressing GFP. B,E) Cell body clusters labelled. TH-Gal4 is a broad labeling driver which includes labelling of most DANs in the fly brain, including aversive reinforcing PPL1 cluster. For PAM DANS, TH-GAL4 only labels 12 out of 100 PAM DANS. For details see [68]

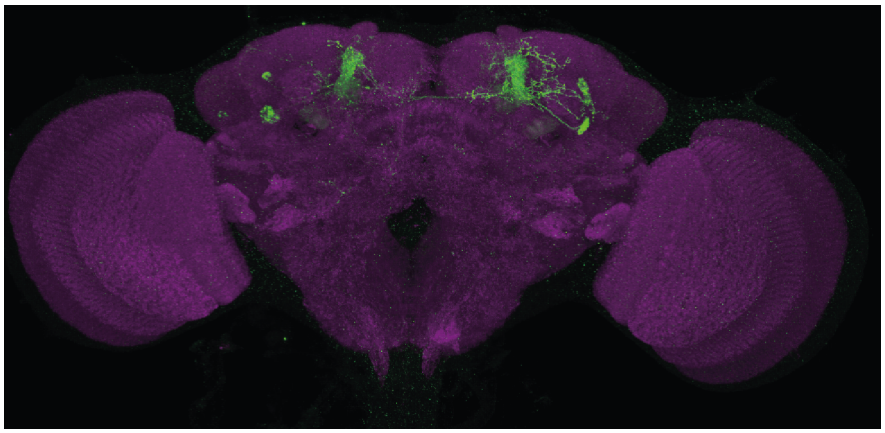


Figure A.4: MB504B-GAL4 labels PPL1 DANS

MB504B-GAL4 driving expression of GFP shows PPL1 DANs innervating the vertical lobe of the mushroom body. Figure taken from Fly Light catalogue [43]

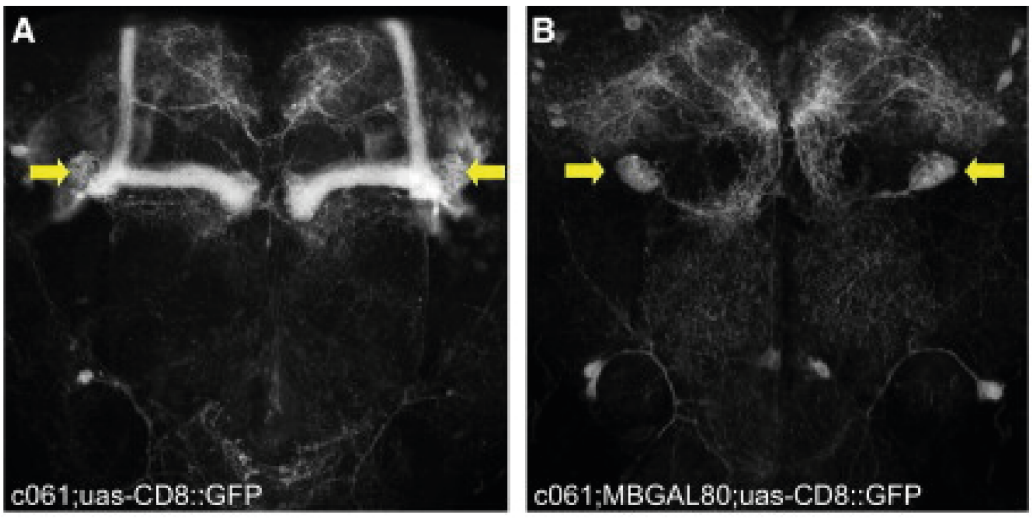


Figure A.5: C601GAL4;MBGAL80 driver

A) c601-GAL4 labels Kenyon Cells in addition to DANs innervating the heel of the mushoom body. B) c601GAL4 can be combined with transcriptional repressor MBGAL80 to achieve labelling of ɣ1-pedc DANs (yellow arrows) without expression in KCs. Image from Krashes et al 2009 [5].

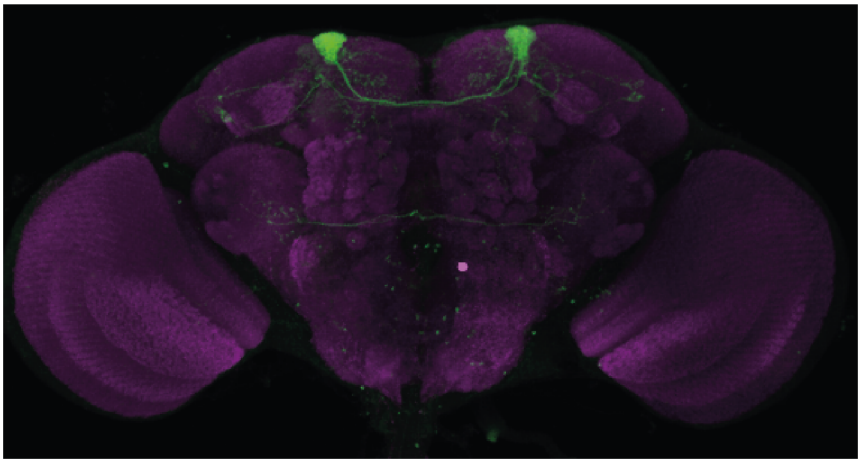


Figure A.6: MB630B-GAL4 driver

MB530B-GAL4 driving expression of GFP shows PPL1-α3 DANs innervating the tip of the vertical lobe of the mushroom body[45]. Figure taken from Fly Light catalogue [43]

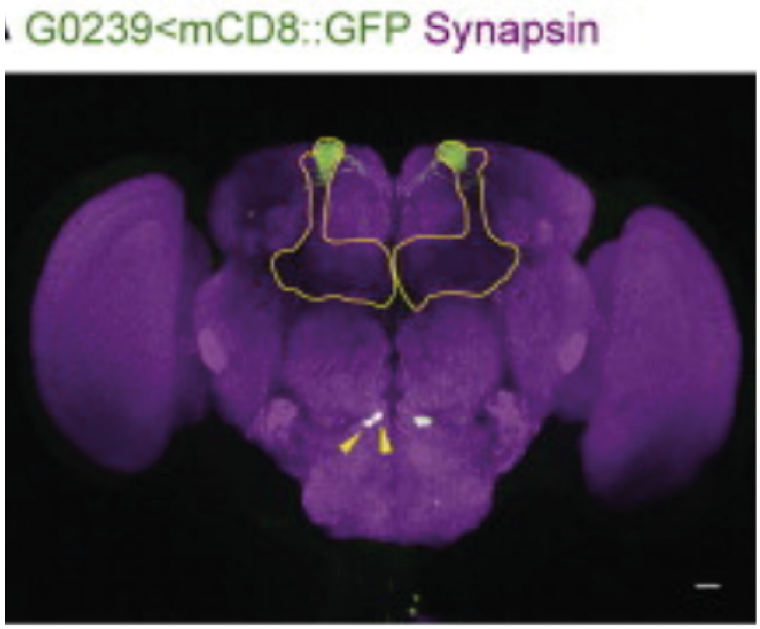


Figure A.7: G0239-GAL4

G0239-GAL4 driving expression of GFP shows labelling of MBONs innervating the α3 compartment of the mushroom body. Figure taken from Placais et al 2013 [130] [43]

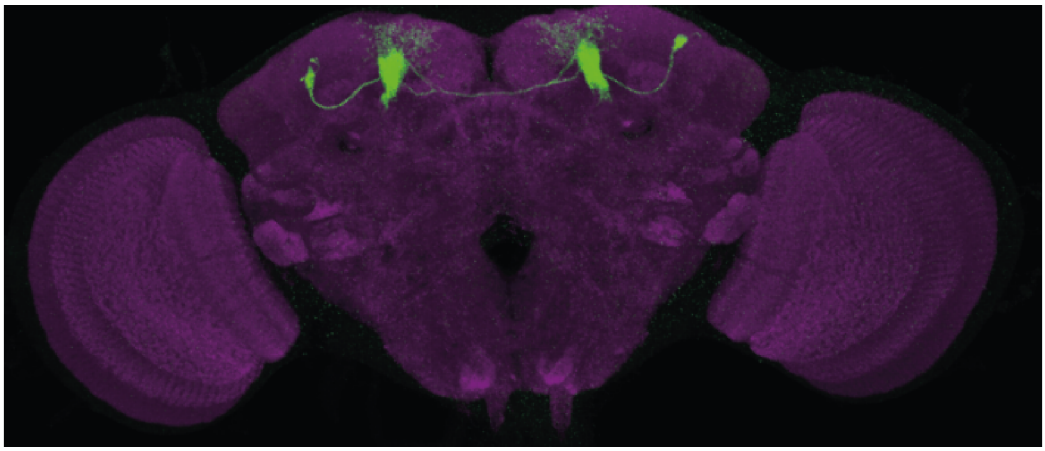


Figure A.8: MB058B-GAL4 driver

MB058B-GAL4 driving expression of GFP shows labelling of PPL1 DANs projecting to the $\alpha 2\alpha' 2$ compartments of the mushroom body[45]. Figure taken from Fly Light catalogue [43]

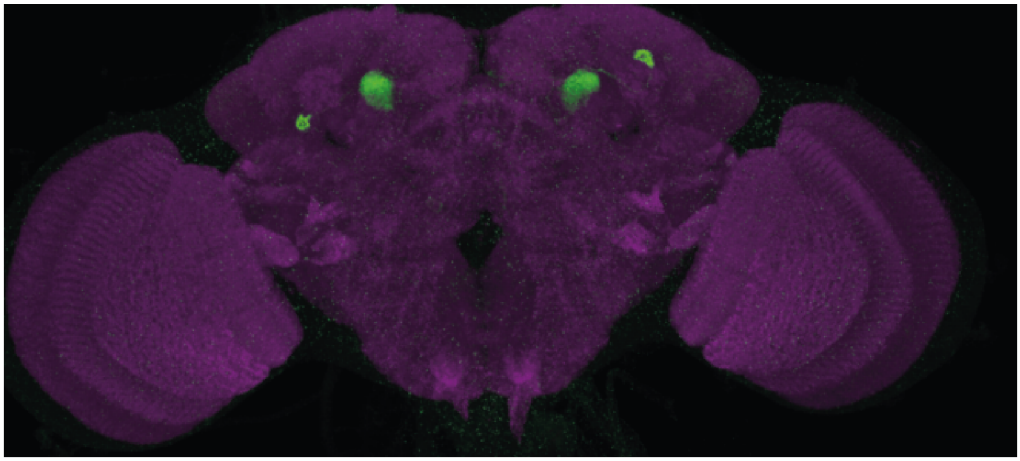


Figure A.9: MB296-GAL4 driver

MB296B-GAL4 driving expression of GFP shows labelling of PPL1 DANs innervating the $\gamma 2\alpha' 1$ compartment of the mushroom body[45]. Figure taken from Fly Light catalogue [43]

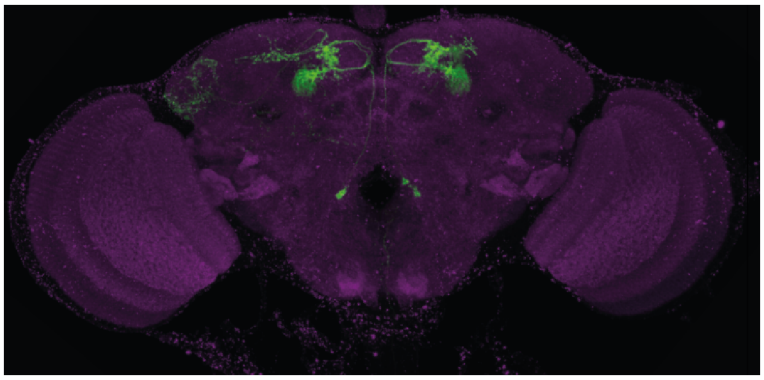


Figure A.10: MB077B-GAL4 driver

MB077B-GAL4 driving expression of GFP shows labelling of MBONs innervating the γ2α′1 compartment of the mushroom body[45]. Figure taken from Fly Light catalogue [43]

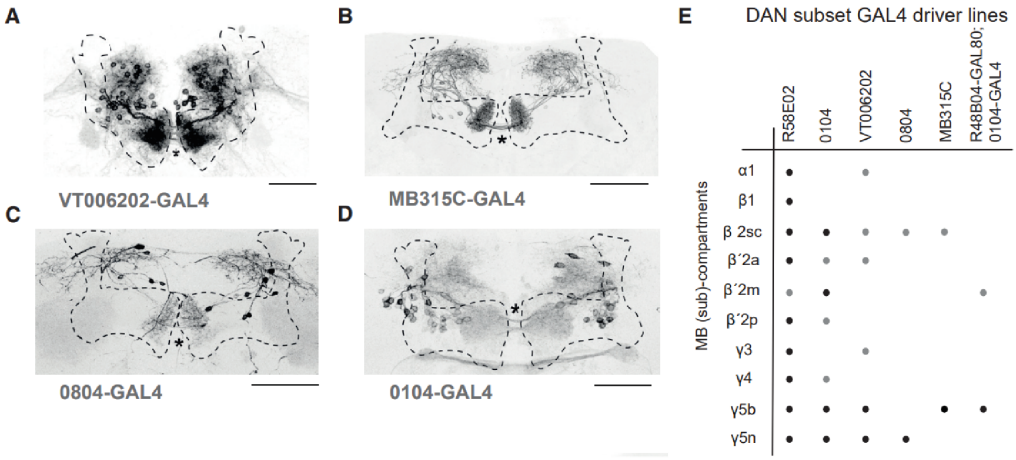


Figure A.11: Drivers for PAMs labelling γ5 compartment

Figure taken from Otto et al 2020 [76]. A) shows labelling by VT006202-GAL4. This driver labels 20 PAM γ5. Which include γ5n and γ5b, in addition to DANs projecting to other compartments, detailed in E. B)MB315C-GAL4 labels 8 PAM DANs targetting the γ5b plus the β2sc DANs. C) 0804-GAL4 labels 5 PAMs targetting the γ5n compartment. D) 0104-GAL4 labels PAM DANs projecting to γ5b plus other compartments that can be eliminated by expression of transcriptional repressor GAL80 as shown in E). E) Summary table of expression pattern of DANs in each driver line. For further reference see [76]

B | Publication - Prior experience conditionally inhibits the expression of new learning in Drosophila

Article

Current Biology

Prior experience conditionally inhibits the expression of new learning in *Drosophila*

Highlights

- Odor pre-exposure alters the expression of a learned association of that odor
- Pre-exposure memory only affects subsequent retrieval if context is consistent
- Pre-exposure memory can complement or compete with a learned association
- Odor pre-exposure forms a labile mushroom body-dependent aversive memory

Authors

Pedro F. Jacob, Paola Vargas-Gutierrez, Zeynep Okray, Stefania Vietti-Michelina, Johannes Felsenberg, Scott Waddell

Correspondence

scott.waddell@cncb.ox.ac.uk

In brief

Jacob et al. show that prior experience of an odor can inhibit the retrieval of a newly formed memory for that same odor. Odor pre-exposure forms a labile mushroom body-dependent aversive memory that competes with expression of new learning if the context is appropriate. This study explains how a retrieval defect can underlie latent inhibition.

Jacob et al., 2021, Current Biology *31*, 3490–3503
August 23, 2021 © 2021 The Author(s). Published by Elsevier Inc.
https://doi.org/10.1016/j.cub.2021.05.056

CellPress

CellPress
OPEN ACCESS

Current Biology

Article

Prior experience conditionally inhibits the expression of new learning in *Drosophila*

Pedro F. Jacob,[1] Paola Vargas-Gutierrez,[1] Zeynep Okray,[1] Stefania Vietti-Michelina,[1,3] Johannes Felsenberg,[1,2] and Scott Waddell[1,4,5,*]

[1]Centre for Neural Circuits and Behaviour, University of Oxford, Oxford OX1 3TA, UK
[2]Present address: Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland
[3]Present address: Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford OX1 3SR, UK
[4]Twitter: @scottishwaddell
[5]Lead contact
*Correspondence: scott.waddell@cncb.ox.ac.uk
https://doi.org/10.1016/j.cub.2021.05.056

SUMMARY

Prior experience of a stimulus can inhibit subsequent acquisition or expression of a learned association of that stimulus. However, the neuronal manifestations of this learning effect, named latent inhibition (LI), are poorly understood. Here, we show that prior odor exposure can produce context-dependent LI of later appetitive olfactory memory performance in *Drosophila*. Odor pre-exposure forms a short-lived aversive memory whose lone expression lacks context-dependence. Acquisition of odor pre-exposure memory requires aversively reinforcing dopaminergic neurons that innervate two mushroom body compartments—one group of which exhibits increasing activity with successive odor experience. Odor-specific responses of the corresponding mushroom body output neurons are suppressed, and their output is necessary for expression of both pre-exposure memory and LI of appetitive memory. Therefore, odor pre-exposure attaches negative valence to the odor itself, and LI of appetitive memory results from a temporary and context-dependent retrieval deficit imposed by competition with the parallel short-lived aversive memory.

INTRODUCTION

Keeping track of life experience allows animals to benefit from all of their prior knowledge when learning new information and using their memory to direct behavior. Although the subject of great early debate among learning theorists, it is now accepted that learning occurs even without explicit rewards or punishment. Classic experiments showed that rats given the prior opportunity to roam in an empty maze performed better when they were later trained with rewards presented in specific locations.[1] Becoming familiar with the maze without explicit reinforcement and an obvious initial change in the animal's behavior was called "latent learning."[2]

Attempts to replicate a facilitating effect of latent learning using classical conditioning led to an unexpected observation. Pre-exposing animals to a stimulus instead often inhibited the ability of the animal to learn using that stimulus—a phenomenon given the name "latent inhibition" (LI).[3,4] LI has been heavily studied for the last 50 years, and two alternative theories have been proposed to account for the inhibitory effect of stimulus pre-exposure. In the acquisition (A) model, subsequent learning is considered to be impaired because pre-exposure alters the capacity for the stimulus to enter into new associations.[5–7] In contrast, in the retrieval (R) model, learning is still believed to occur but memory expression is impaired.[8–11] A strong argument in favor of the R model is the observation that LI often appears to be limited in time, leading to expression of the subsequent learning undergoing "spontaneous recovery." Importantly, both theories of latent inhibition assume that something is learned during pre-exposure such as primitive properties of the stimulus including its specific identity, intensity (e.g., concentration), and salience. In addition, LI is often sensitive to the consistency of the context within which the animal is pre-exposed, taught, and tested for memory expression. This led to the proposal that first learning an association between the stimulus and its context makes it difficult for the animal to subsequently associate the stimulus with reinforcement during training.[12,13]

Studying olfactory learning in the relatively small brain of *Drosophila* has potential to define how LI can operate and reveal an underlying neuronal circuit mechanism. Several earlier studies in both adult flies and larvae demonstrated that repeated exposure to an odor can alter its apparent valence to the fly, either making the fly avoid it more or become unresponsive to it.[14–18] Although an A model for LI has been reported with appetitive conditioning in the honeybee,[19–21] a prior study in adult *Drosophila* did not observe any effect on aversive conditioning following a single odor pre-exposure.[22]

Associative olfactory learning in *Drosophila* relies on the neuronal circuitry of the mushroom body (MB). Individual odors are represented as activity in sparse and largely non-overlapping subpopulations of the ~4,000 intrinsic neurons called Kenyon cells (KCs). Positive or negative valence can be assigned to these odor representations by anatomically discrete dopaminergic neurons (DANs) which, via dopamine receptor-directed

 © 2021 The Author(s). Published by Elsevier Inc.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

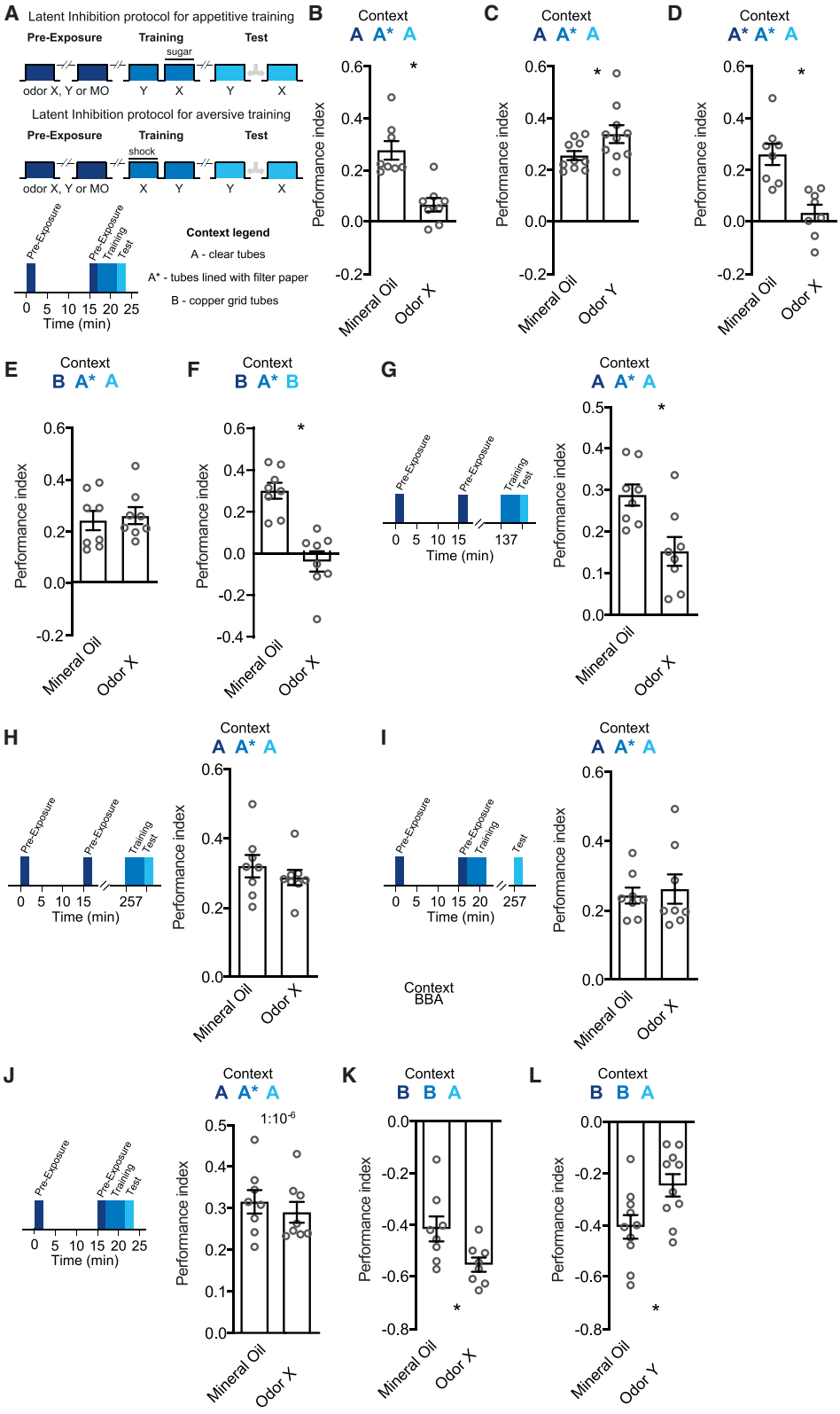


Figure 1. Odor pre-exposure induces context-dependent latent inhibition of appetitive learning

(A) Schematic for behavioral paradigms in this study. Flies were twice pre-exposed to either an odor X, Y, or mineral oil (MO), with a 15-min inter-trial interval (dark blue phase; pre-exposure). Flies were then either trained appetitively (with sugar) or aversively (with electric shock) (mid blue phase, training) and tested (light blue

(legend continued on next page)

cyclic AMP (cAMP)-dependent plasticity,[23–29] modulate the efficacy of KC output synapses onto different downstream mushroom body output neurons (MBONs), whose dendrites occupy the same MB compartment. Aversive learning depresses KC synapses onto MBONs whose activation favors approach, whereas appetitive learning reduces odor-drive to MBONs favoring avoidance. By establishing a skew in the valence of the odor-driven MBON network, learned information subsequently directs either odor avoidance or attraction behavior.[30]

A number of studies indicate that discrete experience is represented as plasticity of different combinations of KC-MBON connections, directed by the engagement of unique combinations of DANs. For example, different types of DANs have been implicated in coding memories for specific rewards (e.g., water, the sweet taste and nutrient value of sugars, the absence of expected shock, and the delayed recognition of safety).[31–36] In contrast, the same PPL1 DANs appear to be required to code aversive memories for electric shock, bitter taste, and heat,[37–39] although imaging suggests they are activated by temperature decreases and to noxious heat.[39,40] By forming and storing conflicting and complementary memories in different places, the fly can more effectively direct its behavior to reflect a history of experience.

Here, we show that prior odor exposure can temporarily inhibit memory performance after subsequent appetitive learning in *Drosophila*. This inhibitory effect is sensitive to a change of context across the pre-exposure, training, and testing periods, consistent with it being a form of LI. Odor pre-exposure forms a short-lived odor-specific aversive memory, whose acquisition requires the $\gamma2\alpha'1$ and $\alpha3$ DANs, the latter of which become sensitized to consecutive odor presentation. As a consequence, aversive memory is apparent as a decrease in the odor-evoked response of the corresponding approach-directing $\gamma2\alpha'1$ and $\alpha3$ MBONs. Blocking the $\alpha3$ MBONs impairs the expression of the aversive odor pre-exposure memory and abolishes LI of appetitive memory. The short-lived presence of a parallel and differently located odor-specific aversive memory therefore temporarily inhibits the retrieval of a subsequently formed appetitive memory for that same odor. These data provide evidence for a context-dependent R model of latent inhibition in *Drosophila*.

RESULTS

Odor pre-exposure can produce temporary latent inhibition of appetitive memory

Drosophila can be appetitively conditioned by pairing odor presentation with sugar reward.[15,41] We therefore tested whether prior exposure to the to be sugar-paired odor (CS+) altered subsequent learned behavior. Starved flies were given two 2-min odor X presentations with a 15-min inter-trial interval (ITI) before being trained by presenting odor Y for 2 min then 30 s later presenting odor X with sucrose reward (two odors were reciprocally used as X and Y, and performance indices represent an average). Control flies were twice pre-exposed to the odor diluent of mineral oil (MO) before training (Figure 1A). When immediately tested after training, MO-exposed flies exhibited appetitive memory for odor X. However, the performance of flies pre-exposed to the conditioned odor X was significantly impaired (Figure 1B), a phenotype resembling LI. In contrast, flies pre-exposed to odor Y (the to be CS−) exhibited appetitive memory performance that was significantly enhanced to that of flies exposed to MO (Figure 1C). Because LI can typically be reduced by changing the context between pre-exposure, training, and testing, we next altered the

phase, test) for their preference between odors X and Y. To evaluate the effect of context, three different contexts were used: clear tubes (context A), tubes lines with filter paper (context A*), and copper grid tubes (context B). In subsequent figures, the changes in context between the pre-exposure, training, and test phases are marked using this nomenclature and are color-coded accordingly. For example, dark blue A, mid blue A*, and light blue A corresponds to clear tubes in the pre-exposure and test phases and tubes lined with filter paper in the training phase.

(B) Flies were twice pre-exposed to odor X or MO in clear tubes (context A), with a 15-min inter-trial interval. Immediately following the last exposure, they were trained by presenting odor Y for 2 min, then odor X for 2 min with sugar (CS+) in context A*. Flies pre-exposed to odor X exhibited appetitive memory performance to odor X (one-sample t test: $t(7) = 2.419$, $p = 0.0461$) that was significantly reduced (LI) in comparison to flies exposed to MO ($t(14) = 4.719$, $p = 0.0003$).

(C) When pre-exposed to odor Y (the to be CS−; context A), flies exhibited appetitive memory that was significantly increased in comparison to flies exposed to MO ($t(18) = 2.127$, $p = 0.0475$).

(D) When pre-exposure and training context were more closely matched, using filter paper in the pre-exposure tube (context A*A*A), flies pre-exposed to odor X did not exhibit appetitive memory performance (LI) (one-sample t test: $t(7) = 0.9866$, $p = 0.3567$). Performance was also significantly reduced compared to flies pre-exposed to MO ($t(14) = 4.376$, $p = 0.0006$).

(E) Pre-exposing flies to odor X in a different context of a copper grid tube (context BA*A) abolished the inhibitory effect (no LI). Appetitive memory performance of flies pre-exposed to odor X was similar to MO pre-exposed flies ($t(14) = 0.3902$, $p = 0.7023$).

(F) Matching context between pre-exposure and testing (both in copper grid tube; context BA*B) restored LI. Performance of flies pre-exposed to odor X was significantly impaired compared to flies pre-exposed to MO ($t(14) = 5.449$, $p < 0.0001$).

(G) The inhibitory effect on appetitive learning is evident 2 h after odor pre-exposure. Flies were pre-exposed to odor X or MO. 2 h later, they were appetitively trained and tested immediately for memory. Performance of flies pre-exposed to odor X was significantly reduced compared to flies pre-exposed to MO ($t(14) = 3.155$, $p = 0.007$).

(H) The inhibitory effect on learning was not evident 4 h after odor pre-exposure. Memory performance of flies pre-exposed to odor X or MO was similar when trained 4 h after the last pre-exposure ($t(14) = 0.8368$, $p = 0.4167$).

(I) The inhibitory effect of pre-exposure on learning was not evident 4 h after training. Performance was similar if flies were pre-exposed to odor X or MO, then immediately trained but tested for memory 4 h later ($t(14) = 0.3877$, $p = 0.7041$), demonstrating time-dependent loss of LI and recovery of appetitive memory.

(J) Pre-exposure to lower odor concentration did not induce LI. Flies were pre-exposed with $1{:}10^{-6}$ odor X or mineral oil (MO), with 15-min inter-trial interval, in clear tubes, immediately trained with $1{:}10^{-6}$ odor X paired with sugar (CS+) and tested for memory. Flies pre-exposed to $1{:}10^{-6}$ odor X showed similar memory performance to those pre-exposed to MO ($t(14) = 0.6741$, $p = 0.5112$).

(K) Odor pre-exposure facilitates memory performance after aversive conditioning. Flies were pre-exposed to odor X or mineral oil (MO) in a copper grid-lined tube (context B), then immediately trained by pairing odor X for 1 min with twelve 30 V electric shocks (CS+). Flies pre-exposed to odor X exhibited increased aversive memory performance in comparison to MO-exposed flies ($t(14) = 2.485$, $p = 0.0262$).

(L) When pre-exposed to odor Y (the to be CS; context B), flies exhibited aversive memory that was significantly decreased in comparison to flies exposed to MO ($t(18) = 2.560$, $p = 0.0197$).

CellPress
OPEN ACCESS

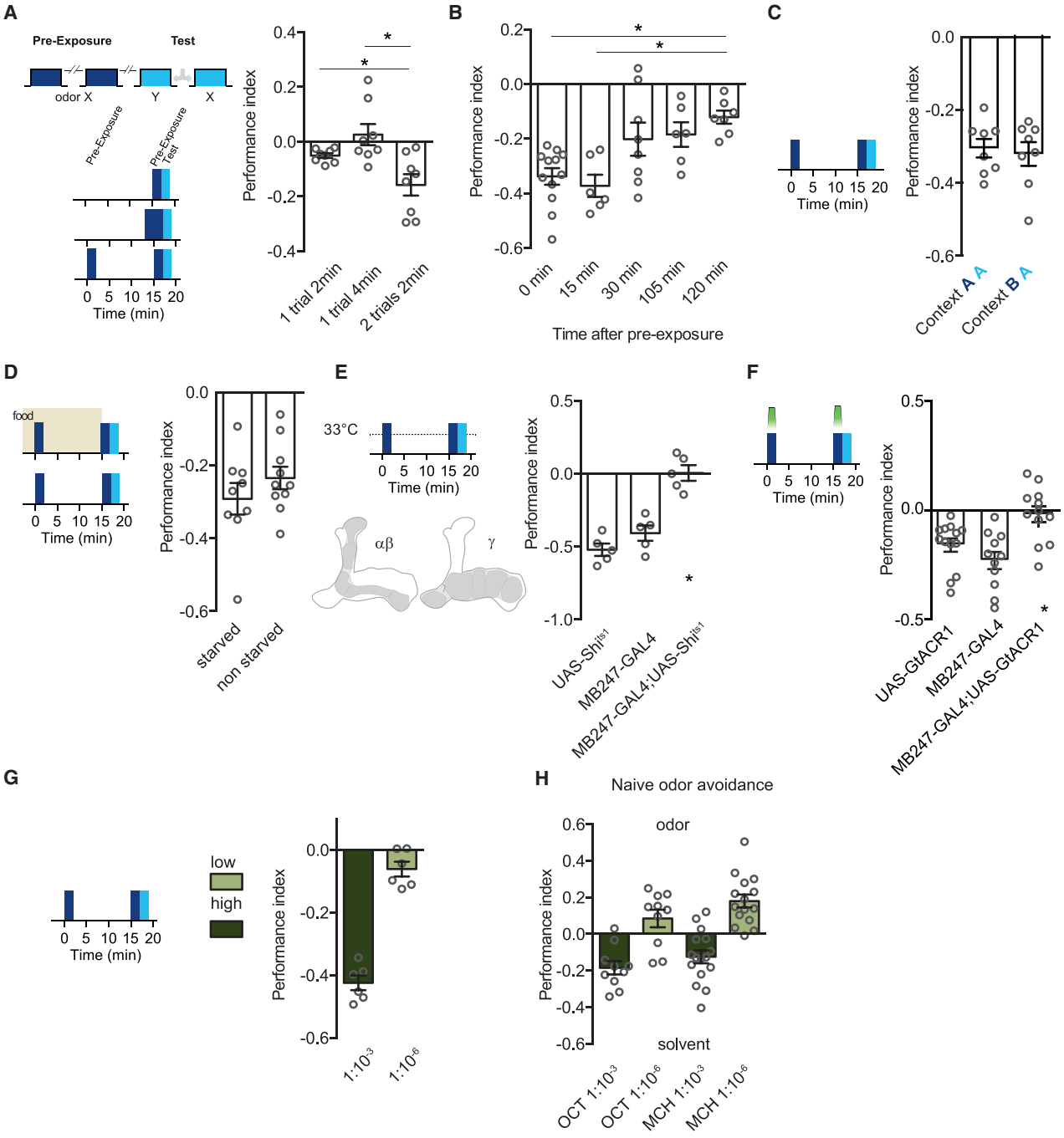


Figure 2. Odor pre-exposure forms a labile mushroom body-dependent aversive memory

(A) Repeated odor exposure forms aversive memory for that odor. Two 2-min odor exposures with a 15-min interval induced odor aversion ($F[2,21] = 8.365$, $p = 0.0023$, $n = 8$) whereas a single 2-min or 4-min exposure did not (Tukey's multiple comparisons test: 1 × 2 min versus 1 × 4 min, $p = 0.07$; 1 × 2 min versus 2 × 2 min, $p = 0.0182$; 1 × 4 min versus 2 × 2 min, $p = 0.0048$).

(B) Pre-exposure induced aversive memory is labile (one-sample t test $p < 0.05$ for all comparisons; $F[4,34] = 6.295$, $p = 0.007$, $n = 6–12$; Tukey's multiple comparisons test: 0 min versus 120 min, $p = 0.0036$ and 15 min versus 120 min, $p = 0.0040$, for all other comparisons $p > 0.05$).

(C) Expression of pre-exposure memory is not sensitive to changing context. Performance of flies pre-exposed in copper-lined and tested in clear tubes (context BA) was indistinguishable from that of flies pre-exposed and tested in clear tubes (context AA) ($t(14) = 0.3827$, $p = 0.7077$).

(D) Formation of odor pre-exposure memory does not depend on hunger ($t(17) = 1.090$, $p = 0.2911$).

(E) Blocking synaptic output from the MB KCs abolishes odor pre-exposure memory ($F[2,12] = 30.87$, $p < 0.0001$, $n = 5$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus MB247-GAL4, $p = 0.2773$; UAS-*Shi*^{ts1} versus MB247-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$; MB247-GAL4 versus MB247-GAL4;UAS-*Shi*^{ts1}, $p = 0.0002$).

(legend continued on next page)

context between these experimental stages. In our initial experiments (Figures 1B and 1C), the flies were pre-exposed in clear tubes, trained in tubes lined with filter paper, and then tested in clear tubes. We therefore pre-exposed and trained the flies in tubes lined with filter paper to make the context consistent during these stages. This constancy of pre-exposure and training context produced a particularly strong inhibitory effect of pre-exposure (Figure 1D). More importantly, these first two experiments suggest that flies may consider the context of clear (A) and paper (A*) tubes to be similar. We therefore next explicitly changed the context by pre-exposing flies to the to be CS+ in a copper grid-lined tube (B) before training them in tubes lined with filter paper (A*), and testing them in clear tubes (A) (Figure 1E). Strikingly, the robust change in context between copper grid tubes and paper/clear (BA*A) abolished the inhibitory effect of odor pre-exposure. A failure to observe LI across changing context could result from the inability of the flies to form or retrieve a pre-exposure memory. However, robust LI was recovered when the flies were trained on paper but were pre-exposed and tested in copper grid tubes (BA*B) (Figure 1F), showing that pre-exposure memory is formed but not retrieved in the BA*A scenario. Together, these context-shifting experiments demonstrate that the inhibitory effect of odor pre-exposure on subsequent appetitive memory is a form of LI whose expression depends on the context at testing approximating that experienced during odor pre-exposure. These experiments also suggest that this form of LI results from a retrieval deficit—an R model.

We next tested the persistence of LI. Whereas pre-exposure to the CS+ impaired memory performance when flies were trained 2 h after the last pre-exposure (Figure 1G), performance was unimpaired if training was delayed for 4 h after the last pre-exposure (Figure 1H). In addition, appetitive memory performance was unimpaired if flies were trained immediately after the second pre-exposure but tested 4 h later (Figure 1I). These experiments, therefore, suggest that the inhibitory effect of LI decays between 2 and 4 h after odor pre-exposure. In addition, the recovery of appetitive memory performance is also consistent with LI resulting from a temporary retrieval deficit, which provides further evidence that an R model is in operation here.

Many studies have shown that learning and LI is sensitive to properties (frequency, amount, and duration) of the conditioned stimulus.[42–44] We therefore also tested whether two exposures of a lower odor concentration (10^{-6} rather than 10^{-3}) with 15 min ITI induced LI of appetitive memory. Although training with this odor concentration produced robust appetitive memory, no LI effect was observed following odor pre-exposure (Figure 1J).

Odor pre-exposure can produce facilitation of aversive memory

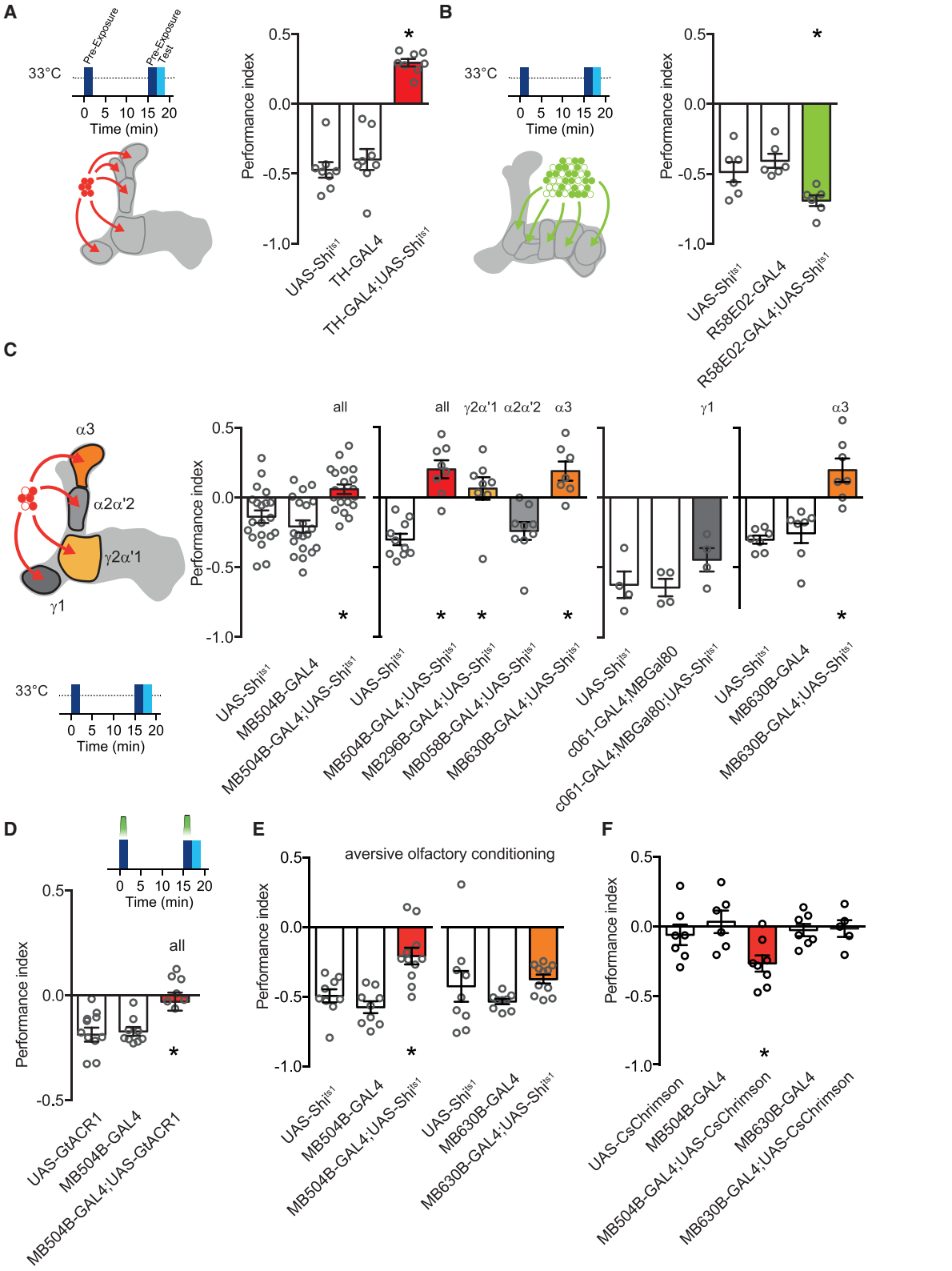
We also tested whether odor pre-exposure altered performance measured after aversive training, pairing odor with electric shock. Starved flies were given two 2-min odor X presentations with a 15 min ITI before being trained by presenting odor X for 1 min, paired with electric shocks, then 45 s later presenting odor Y (Figure 1A). Surprisingly, flies pre-exposed to the CS+ exhibited enhanced aversive memory performance as compared to flies pre-exposed to MO (Figure 1K). This enhancement may represent a form of "latent faciliation."[45,46] Finding that the same odor pre-exposure schedule can inhibit appetitive memory but facilitate aversive memory performance led us to hypothesize that pre-exposure might form an avoidance memory for the CS+; in the former case, this is competing, and in the latter case, this is complementary. In further support of this notion, flies pre-exposed to odor Y (the to be CS−) exhibited aversive memory performance that was significantly impaired to that of MO pre-exposed flies (Figure 1L)—a prediction of the flies acquiring an aversive pre-exposure memory for the CS− and a shock-reinforced aversive memory for the CS+. Importantly, finding that CS+ pre-exposure does not inhibit aversive learning argues against an A model accounting for the observed LI, because both appetitive and aversive learning should be reduced if pre-exposure prevented the CS+ entering into a learned association.

Pre-exposure forms a short-lived MB-dependent aversive odor memory

Although repeated odor presentation frequently leads to rapid sensory adaptation of insect olfactory sensory neurons and/or behavioral habituation, these effects often require many trials, and the response decrement decays within a few minutes.[17,18,47–51] Prior work has also shown that a few odor pre-exposures can enhance subsequent odor avoidance behavior in *Drosophila*,[14,15,34] consistent with our hypothesized aversive learning model. We therefore tested whether our LI odor exposure regimen altered odor avoidance behavior. As in the prior experiments, starved flies were exposed twice to 2 min of odor X (10^{-3} dilution in MO) with a 15 min ITI. They were then immediately tested for preference between the pre-exposed odor X and another odor Y without any training. Flies pre-exposed twice to odor X showed a selective avoidance of odor X, consistent with pre-exposure forming an aversive odor X memory (Figure 2A). In contrast, a single odor pre-exposure, of either 2 or 4 min, did not alter odor preference, suggesting that repeated exposure is required to form the avoidance memory (Figure 2A). Measuring odor preference at different times after pre-exposure revealed that the avoidance memory is labile and slowly decays between 15 min and 2 h (Figure 2B). We also tested whether expression of pre-exposure memory was sensitive to context by pre-exposing flies to odor in copper grid tubes and testing them for odor preference in clear tubes. Performance was unaffected by this change of context (Figure 2C), demonstrating that context is uniquely important for the LI effect of pre-exposure memory. We last tested whether the explicit absence of food acts as

(F) Forming pre-exposure memory requires KC activity during odor pre-exposure. Restricting KC block to the period of odor pre-exposure, using GtACR1 and green light, abolishes aversion to odor X ($F_{[2,33]} = 9.088$, $p = 0.0007$, n = 11–13; Tukey's multiple comparisons test: UAS-*GtACR1* versus MB247-GAL4, $p = 0.3423$; UAS-*GtACR1* versus MB247-GAL4;UAS-*GtACR1*, $p = 0.0183$; MB247-GAL4 versus MB247-GAL4;UAS-*GtACR1*, $p = 0.0006$).
(G) Formation of pre-exposure memory depends on odor concentration. Pre-exposure to high (10^{-3}) but not low (10^{-6}) concentration of odor induces aversive odor memory ($t(10) = 10.98$, $p < 0.0001$).
(H) Reducing odor concentration by 3 orders of magnitude can switch naive valence from avoidance to attraction. All pre-exposure experiments were performed in the context A (clear tubes) for pre-exposure and testing phases.

CellPress
OPEN ACCESS



(legend on next page)

aversive reinforcement for hungry flies by pre-exposing satiated flies. However, pre-exposure induced similar odor avoidance in satiated flies as compared to starved flies (Figure 2D). Because our LI effect was assayed in hungry flies, all subsequent experiments in this study were performed in hungry flies.

Olfactory memories typically depend on the neuronal circuitry of the MB.[41,52–56] We therefore tested the consequence of blocking output from αβ and γ subsets of MB KCs on the effect of odor pre-exposure. We expressed the dominant temperature-sensitive UAS-*Shibire*^{ts1} (UAS-*Shi*^{ts1}) transgene[57] with MB247-GAL4 and blocked KC output throughout the experiment by raising the temperature to restrictive 33°C. This manipulation abolished the development of odor avoidance performance (Figure 2E). Moreover, restricting inhibition of KC activity to only the periods of odor pre-exposure using expression of the green light-sensitive anion-selective GtACR1 channel[58] revealed that KC activity is necessary during pre-exposure (Figure 2F); this suggests that it is a MB-dependent process.

Because we observed LI when flies were twice pre-exposed to 10^{-3} odor concentration but not to 10^{-6}, we also tested whether this lower odor concentration produced an aversive pre-exposure memory. Consistent with the lack of LI, no enhanced avoidance was observed following 10^{-6} odor exposures (Figure 2G). In addition, when naive flies were given the choice between a 10^{-3} odor stream and air, they exhibited avoidance of the odor (Figure 2H). In contrast, flies either showed no preference, or odor approach when tested with a 10^{-6} odor stream and air. Therefore, we reasoned that lower odor concentrations, which are less repellent,[59] do not act as an aversive reinforcer during odor pre-exposure.

Pre-exposure memory requires γ2α′1 and α3 DANs

Aversive olfactory learning reinforced by electric shock, bitter taste, or heat depends on punishment coding DANs from the PPL1 cluster.[37,38,60–63] In contrast, DANs in the PAM cluster mostly code for reward.[31,35,64] We therefore first used TH-GAL4 and R58E02-GAL4 to express UAS-*Shi*^{ts1} and test the respective roles of PPL1 and PAM DANs in pre-exposure learning. Blocking TH-GAL4 neurons switched the effect of pre-exposure from generating aversion to approach (Figure 3A). This reversal of valence implies that removing aversive signaling may either release (mutually exclusive) or unmask (in parallel) positive reinforcement induced by odor pre-exposure. Blocking the rewarding DANs with R58E02-GAL4; UAS-*Shi*^{ts1} increased the aversive effect of pre-exposure (Figure 3B), consistent with odor exposure engaging negative and positive reinforcing DAN populations in parallel.

More specifically, blocking the four types of PPL1-DANs implicated in shock-reinforced olfactory learning,[63] either throughout the experiment (Figure 3C) or only during odor pre-exposure (Figure 3D), abolished the learned aversion. Blocking the individual types revealed that the PPL1-γ1pedc (MB-MP1) and PPL1-α′2α2 DANs are dispensable for pre-exposure learning. In contrast, blocking PPL1-γ2α′1 DANs (MB-MV1) abolished pre-exposure learning, whereas blocking the PPL1-α3 DANs converted pre-exposure-induced aversion to approach (Figure 3C).

Because PPL1-DANs are thought to provide an aversive teaching signal, common to electric shock, heat, and bitter taste,[37,38] we also tested for a role of PPL1-α3 DANs in shock-reinforced aversive memory. Although blocking the four PPL1-DANs impaired shock learning, blocking only the PPL1-α3 DANs left immediate shock memory intact (Figure 3E). In addition, replacing shock with optogenetic stimulation of the four PPL1-DANs could artificially implant an aversive memory, whereas a single pairing of odor presentation with PPL1-α3 DAN activation did not form aversive memory (Figure 3F). These data are consistent with a prior study that showed learning requires multiple trials of PPL1-α3 DAN activation.[65] These results demonstrate that two-trial pre-exposure learning and single-trial electric shock learning involve different PPL1-DANs and emphasize the importance of PPL1-α3 DANs for pre-exposure learning.

Figure 3. Forming pre-exposure memory requires γ2α′1 and α3 dopaminergic neurons

(A) Blocking punishment coding DANs switches the valence of odor pre-exposure learning. Exposure induced aversion becomes approach ($F[2,21] = 57.26$, $p < 0.0001$, $n = 8$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus TH-GAL4, $p = 0.6528$;UAS-*Shi*^{ts1} versus TH-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$; TH-GAL4 versus TH-GAL4; UAS-*Shi*^{ts1}, $p < 0.0001$).

(B) Blocking reward coding DANs increases the aversive effect of odor pre-exposure ($F[2,15] = 7.126$, $p = 0.0067$, $n = 6$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus R58E02-GAL4, $p = 0.5676$; UAS-*Shi*^{ts1} versus R58E02-GAL4/UAS-*Shi*^{ts1}, $p = 0.0473$; R58E02-GAL4 versus R58E02-GAL4/UAS-*Shi*^{ts1}, $p = 0.0062$).

(C) Pre-exposure-evoked aversive learning requires PPL1 DANs ($F[2,57] = 11.30$, $p < 0.0001$, $n = 20$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus MB504B-GAL4, $p = 0.4573$; UAS-*Shi*^{ts1} versus MB504B-GAL4;UAS-*Shi*^{ts1}, $p = 0.0037$; MB504B-GAL4 versus MB504B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$). A specific requirement was observed for PPL1-γ2α′1 (MV1) and PPL1-α3 DANs (for PPL1 screen: $F[4,37] = 14.80$, $p < 0.0001$, $n = 7–10$; Dunnett's multiple comparisons test: UAS-*Shi*^{ts1} versus MB504B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$; UAS-*Shi*^{ts1} versus MB296B-GAL4;UAS-*Shi*^{ts1}, $p = 0.0006$; UAS-*Shi*^{ts1} versus MB058B-GAL4;UAS-*Shi*^{ts1}, $p = 0.8902$; UAS-*Shi*^{ts1} versus MB630B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$; for PPL1-γ1: $F[2,9] = 1.785$, $p = 0.2223$, $n = 4$; for PPL1-α3: $F[2,29] = 23.16$, $p < 0.0001$, $n = 10–11$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus MB630B-GAL4, $p = 0.5575$; UAS-*Shi*^{ts1} versus MB630B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$; MB630B-GAL4 versus MB630B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$).

(D) Optogenetic silencing of PPL1 DANs during odor exposures blocks exposure-evoked aversive learning ($F[2,24] = 6.325$, $p = 0.0062$, $n = 9$; Tukey's multiple comparisons test: UAS-*GtACR1* versus MB504B-GAL4, $p = 0.9832$;UAS-*GtACR1* versus MB504B-GAL4;UAS-*GtACR1*, $p = 0.0112$; MB504B-GAL4 versus MB504B-GAL4;UAS-*GtACR1*, $p = 0.0169$).

(E) Blocking PPL1 DANs impairs immediate aversive memory following conditioning pairing odor with 90V electric shocks ($F[2,26] = 14.43$, $p < 0.0001$, $n = 9–11$; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus MB504B-GAL4, $p = 0.5452$; UAS-*Shi*^{ts1} versus MB504B-GAL4;UAS-*Shi*^{ts1}, $p = 0.0016$; MB504B-GAL4 versus MB504B-GAL4;UAS-*Shi*^{ts1}, $p < 0.0001$). However, only blocking the PPL1-α3 DANs has no effect ($F[2,26] = 1.692$, $p = 0.2039$, $n = 9–11$).

(F) Pairing odor exposure with optogenetic activation of PPL1 DANs induces aversive memory to the previously paired odor, whereas activation of only PPL1-α3 DANs is ineffective ($F[4,41] = 5.665$, $p = 0.0010$, $n = 7–11$; Bonferroni's multiple comparisons test: UAS-CsChrimson versus MB504B-GAL4, $p > 0.9999$; UAS-CsChrimson versus MB504B-GAL4;UAS-CsChrimson, $p = 0.0025$; MB504B-GAL4 versus MB504B-GAL4;UAS-CsChrimson, $p = 0.0028$; UAS-CsChrimson versus MB630B-GAL4, $p > 0.9999$; UAS-CsChrimson versus MB630B-GAL4;UAS-CsChrimson, $p > 0.9999$; MB630B-GAL4 versus MB504B-GAL4;UAS-CsChrimson, $p > 0.9999$). All pre-exposure experiments were performed in the context A (clear tubes) for pre-exposure and testing phases.

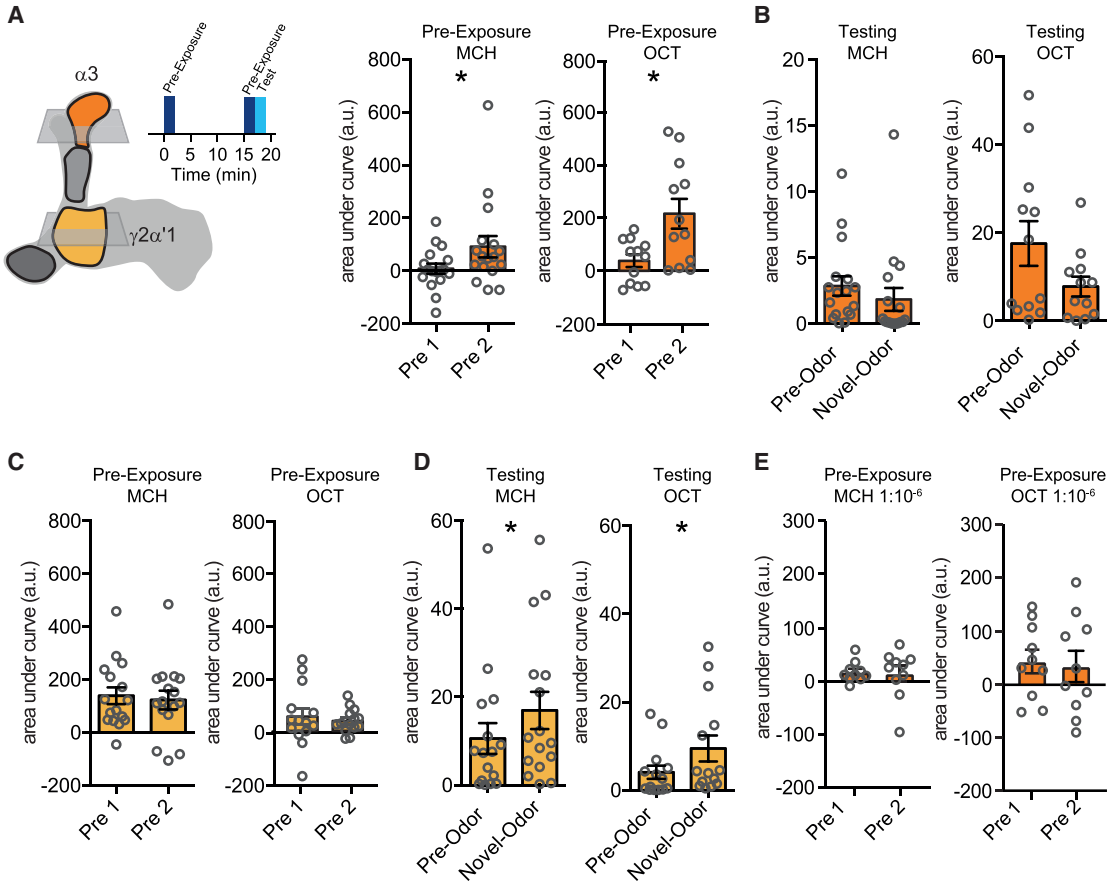


Figure 4. PPL1-γ2α′1 and PPL1-α3 DAN activity is altered following odor exposure

(A) Odor-evoked responses of PPL1-α3 DANs increase from the first to the second odor presentation (for MCH: W(18) = 91, p = 0.0438; for OCT: t(11) = 2.859, p = 0.0156).

(B) In the test responses of PPL1-α3 DANs to the pre-exposed odor were not different to those for the novel odor (for MCH: W(18) = −33, p = 0.4951; for OCT: W(12) = −40, p = 0.1294).

(C) Odor-evoked responses of PPL1-γ2α′1 DANs did not change between the first and second odor presentations (for MCH: t(17) = 0.4792, p = 0.6379; for OCT: t(13) = 0.5678, p = 0.5798).

(D) In the test, responses of PPL1-γ2α′1 DANs to the pre-exposed odor were reduced compared to those for the novel odor (for MCH: W(18) = 107, p = 0.0182; for OCT: W(14) = 71, p = 0.0245).

(E) Responses of PPL1-α3 DANs did not change when flies were twice exposed to lower concentration of odor (for MCH: t(9) = 0.1930, p = 0.8513; for OCT: t(9) = 0.2901, p = 0.7783). Imaging traces are presented in Figure S1.

The activity of PPL1-γ2α′1 and PPL1-α3 DANs is altered following odor presentation

Because pre-exposure learning depends on repeated trials and requires the PPL1-γ2α′1 and PPL1-α3 DANs, we imaged DAN activity during and after odor presentations under the microscope (Figures 4 and S1). Flies were constructed that expressed the fluorescent UAS-GCaMP6m calcium sensor in PPL1-γ2α′1 or PPL1-α3 DANs. As before in the behavioral experiments, flies were given two odor presentations with 15 min ITI. They were then immediately given another 5-s exposure of the trained odor followed by 5 s of a novel odor to mimic the behavioral test situation under the microscope. Odor-evoked responses of PPL1-α3 DANs increased from the first to the second trial (Figures 4A and S1A), whereas between trial PPL1-γ2α′1 DAN responses did not evidently change (Figures 4C and S1B). However, PPL1-γ2α′1 DANs exhibited a reduced response to the pre-exposed compared to the novel odor in the 5-s test, whereas PPL1-α3 DANs showed no difference between novel and test odor responses (Figures 4B, 4D, S1A, and S1B).

In addition, consistent with a lack of LI (Figure 1H) and pre-exposure memory (Figure 2F), lower 10^{-6} odor concentration did not increase the odor-evoked responses of PPL1-α3 DANs (Figures 4E and S1C). Together, these results suggest that the increased odor-driven activity of the PPL1-α3 DANs, specifically in the 2nd pre-exposure, is crucial for the formation of an aversive pre-exposure memory. The relative change at test of the pre-exposed odor responses of PPL1-γ2α′1 DANs also implies an exposure-dependent plasticity of their activity, perhaps occurring earlier than that of the α3 DANs.

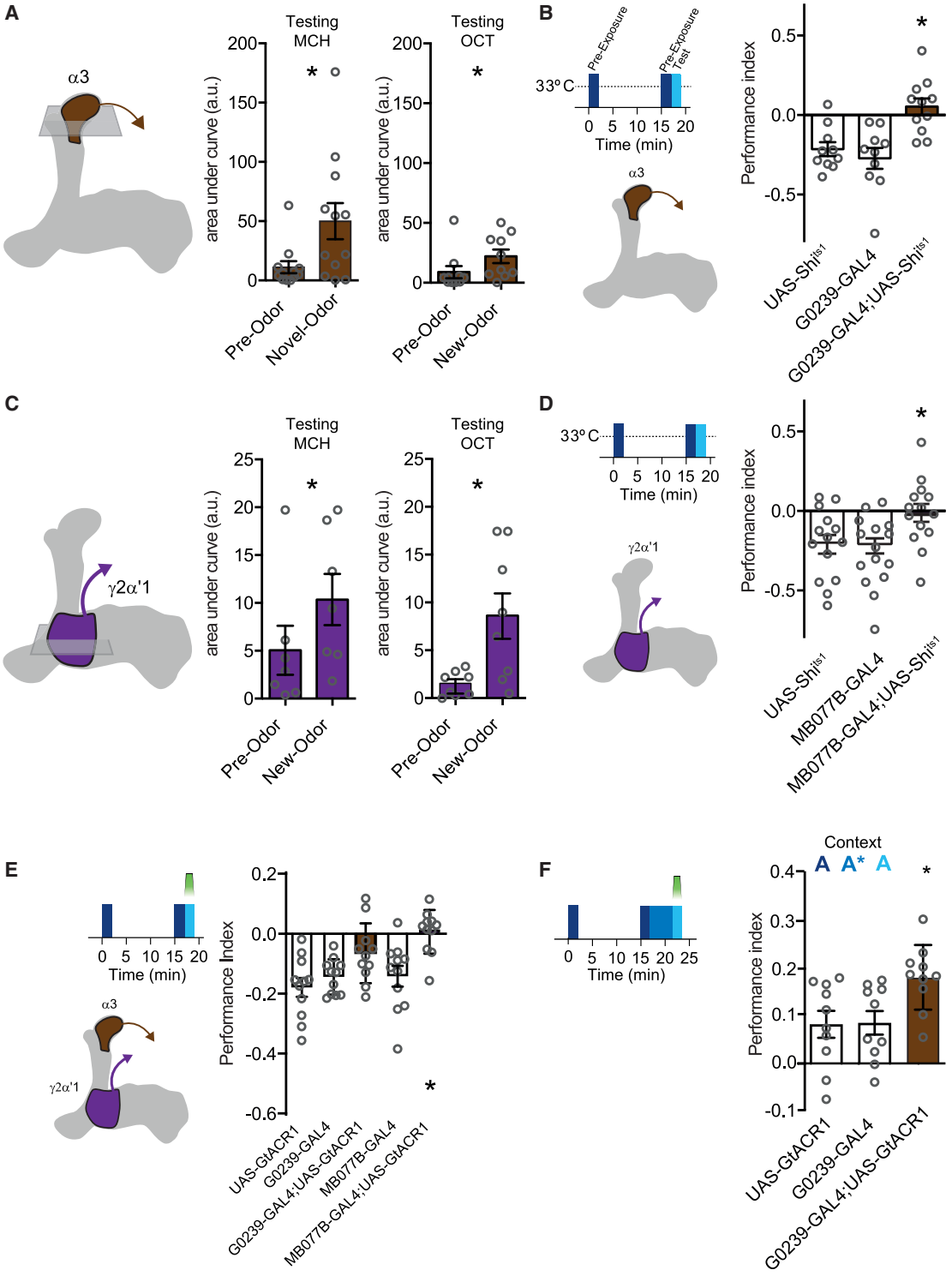


Figure 5. γ2α′1 and α3 MBONs exhibit an odor pre-exposure memory trace and are required for behavioral expression of odor aversion and LI of appetitive memory

(A) Odor pre-exposure reduces the responses of MBON-α3 to that odor compared to a novel odor (for MCH: W(12) = 56, p = 0.0269; for OCT: W(13) = 57, p = 0.0429). Imaging traces are presented in Figure S2.

(B) Blocking output from MBON-α3 impairs pre-exposure memory performance (F[2,28] = 10.50, p = 0.0004, n = 10–11; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus G0239-GAL4, p = 0.7412; UAS-*Shi*^{ts1} versus G0239-GAL4;UAS-*Shi*^{ts1}, p = 0.0043; G0239-GAL4 versus G0239-GAL4;UAS-*Shi*^{ts1}, p = 0.0006).

(legend continued on next page)

Repeated odor presentation depresses odor responses of γ2α′1 and α3 MBONs

The predominant model for *Drosophila* learning is dopamine-driven depression of synapses between odor-specific KCs and MBONs.[29,66–70] We therefore used MBON expression of GCaMP6m to test whether KC-MBON connections underlying the PPL1-α3 and PPL1-γ2α′1 DANs were changed following repeated odor exposure (Figures 5 and S2). Odor responses of MBON-α3 and MBON-γ2α′1 were both reduced compared to their responses to a novel odor (Figures 5A, 5C, and S2). In addition, blocking either the α3 or γ2α′1 MBONs throughout an odor exposure experiment with UAS-*Shi*^{ts1}, or specifically during the test phase with UAS-*GtACR1*, abolished exposure-induced avoidance behavior in the T-maze (Figures 5B, 5D, and 5E). Taken together, these data indicate that spaced odor exposure forms aversive memory that manifests as reduced odor-evoked activity of the α3 and MBON-γ2α′1 MBONs resulting from increased odor-evoked activation of the corresponding DANs.

α3 MBON output is required for expression of latent inhibition of appetitive memory

We last tested whether the activity of the γ2α′1 and α3 MBONs was required for the expression of LI. Flies expressing UAS-*GtACR1* in γ2α′1 and α3 MBONs were subjected to the standard pre-exposure regimen followed by appetitive conditioning (Figure 5F). Blocking output from γ2α′1 MBONs impaired the expression of appetitive memory, so their contribution to LI could not be further tested (data not shown). However, silencing the α3 MBONs during testing by illuminating the flies with green light abolished the effect of LI that was observed in the control flies.

DISCUSSION

In this study, we demonstrate a form of latent inhibition (LI) in *Drosophila* and identify an underlying neuronal mechanism. We find that repeated odor presentation can form a labile self-reinforced aversive memory for that odor, which can temporarily compete with the expression of a newly acquired appetitive memory for that same odor. During memory testing, the conditioned odor should therefore activate both the memory of the pre-exposure (odor-self) and that of the appetitive conditioning (odor-sugar). Importantly, the aversive pre-exposure memory is labile, which means that the LI effect is transient. As a result, the appetitive memory performance exhibits "spontaneous recovery." These results demonstrate that an R model underlies this form of LI in the fly. An A model is not supported because flies acquire an associative reward memory for the odor after pre-exposures of that odor. Instead, the expression of the learned approach performance is impeded by the co-expression of a competing aversive pre-exposure memory. In further support of this R model, the same pre-exposure regimen caused facilitation of a subsequently acquired aversive olfactory memory. In this instance, the pre-exposure memory adds to the new aversive associative memory, rather than competes with an appetitive memory. Last, pre-exposure to the to be non-reinforced odor (the CS−) enhanced subsequent appetitive memory performance but inhibited aversive memory performance—a logical expectation of the CS− acquiring negative valence during pre-exposure.

A defining feature of LI is sensitivity to the consistency of the context in which the pre-exposure, learning, and testing are carried out.[71] Changing between the clear and paper-lined tubes did not impair LI, suggesting that the flies likely consider these to be a similar context. However, if odor pre-exposure, learning, and testing were performed in different contexts (i.e., a copper grid-lined versus a paper-lined or clear tube) LI was abolished. Most strikingly, LI could be restored if copper grid tubes were used to provide the same context during pre-exposure and testing. In line with prior theories and studies of LI in other animals,[72,73] these results suggest that flies learn an association between the odor and the context in which it is experienced during the non-reinforced pre-exposure. As a result, the pre-exposure memory gains context-dependence, and our experiments show it is not retrieved and therefore does not interfere with the newer appetitive memory if the context is different when memory is tested. The failure to retrieve the pre-exposure memory in a different context manifests as a loss of LI—the appetitive memory is fully expressed. Our study therefore reveals that the context-dependency of LI results from the ability (correct context, LI evident) or inability (wrong context, no LI) to retrieve the pre-exposure memory. In addition, context only plays a role in the expression of the pre-exposure memory when it is in conflict with a subsequently acquired appetitive memory. Further work will be required to define what the flies recognize as a "change of context." There are many possibilities including background odors, tube/paper texture, relative luminance, and other flies in the group.

We found that the odor-driven activity of γ2α′1 and α3 DANs increased with repeated odor pre-exposure and that they were required for the formation of the odor pre-exposure memory. In addition, the odor-specific responses of the corresponding

(C) Odor pre-exposure reduces responses of MBON-γ2α′1 to that odor compared to a novel odor (for MCH: W(7) = 24, p = 0.0436; for OCT: t(7) = 2.689, p = 0.0311). Imaging traces are presented in Figure S2.

(D) Blocking output from MBON-γ2α′1 impairs pre-exposure memory performance (F[2,45] = 5.063, p = 0.0104, n = 16; Tukey's multiple comparisons test: UAS-*Shi*^{ts1} versus MB077B-GAL4, p = 0.9823; UAS-*Shi*^{ts1} versus MB077B-GAL4;UAS-*Shi*^{ts1}, p = 0.0284; MB077B-GAL4 versus MB077B-GAL4;UAS-*Shi*^{ts1}, p = 0.0181).

(E) Restricting MBON block to the test phase using GtACR1 reveals a requirement for MBON-γ2α′1 but not MBON-α3 to express the pre-exposure memory (F[4,50] = 7.188, p = 0.0001, n = 11; Šídák's multiple comparisons test: UAS-GtACR1 versus G0239-GAL4, p = 0.9435; UAS-*GtACR1* versus G0239-GAL4;UAS-*GtACR1*, p = 0.0337; G0239-GAL4 versus G0239-GAL4;UAS-*GtACR1*, p = 0.2668; UAS-*GtACR1* versus MB077B-GAL4, p = 0.9249; UAS-*GtACR1* versus MB077B-GAL4;UAS-*GtACR1*, p = 0.0001; MB077B-GAL4 versus MB077B-GAL4;UAS-*GtACR1*, p = 0.0027).

(F) Blocking MBON-α3 during memory testing impairs the expression of LI of appetitive memory. The inhibitory effect of odor X pre-exposure on appetitive memory is not observed when α3 MBONs are blocked at retrieval (F[2,27] = 5.063, p = 0.0136, n = 10; Tukey's multiple comparisons test: UAS-*GtACR1* versus G0239-GAL4, p = 0.9965; UAS-*GtACR1* versus G0239-GAL4;UAS-*GtACR1*, p = 0.0248; G0239-GAL4 versus G0239-GAL4;UAS-*GtACR1*, p = 0.0297). All behavioral pre-exposure experiments were performed in the context A (clear tubes) for pre-exposure and testing phases.

MBONs were depressed following pre-exposure. We therefore conclude that ramping odor-driven DAN activity assigns negative value to the odor itself by depressing odor-specific KC connections onto the γ2α′1 and α3 MBONs. In support of this model, repeated pre-exposure of flies to a lower and less innately aversive odor concentration did not increase the activity of the α3 DANs or form an aversive pre-exposure memory. Importantly, reduced odor activation of the approach-directing γ2α′1 and α3 MBONs is sufficient to account for the aversive nature of pre-exposure memory. Moreover, both expression of pre-exposure memory and LI are abolished if the α3 MBONs are blocked during testing, confirming the model that LI is produced by the expression of the aversive pre-exposure memory competing with that of the associative reward memory believed to be represented as depression of conditioned odor responses of γ5, β′2, and α1 MBONs.[33,68,74]

Several prior *Drosophila* studies have documented changes in odor-driven behavior following different regimens of odor exposure, many of which employed longer durations or more trials than those employed here, and that produced shorter-lived inhibitory effects.[17,18,75] One of these studies described odor-driven activity of the PPL1-α′3 DANs, and subsequent depression of odor-specific responses of the α′3 MBONs to underlie how flies can become familiar with an odor following repeated short exposures.[17] In contrast, we show that two longer and spaced odor exposures produce an aversive memory that manifests as plasticity of γ2α′1 and α3 DANs and MBONs. Moreover, whereas we show a retrieval defect underlies LI of appetitive memory, a reduced attention/familiarity/habituation to the odor following pre-exposures would be expected to result in a subsequent acquisition defect (and A model), likely of both appetitive and aversive learning. It will nevertheless be important to understand how these different types of olfactory experience, and their supporting plasticity mechanisms, are represented and combined in the brain.

LI has often been compared to memory extinction,[76] and our work in the fly shows that very similar neuronal mechanisms and learning models account for both of these phenomena. Pre-exposure learning in *Drosophila* appears to follow similar rules to extinction learning following aversive olfactory conditioning; 2 spaced trials with 15-min ITI are more efficient than massed training with 1-min ITI,[34] and in both cases, a resulting parallel opposing odor-nothing memory inhibits the retrieval/expression of the odor-punishment or odor-reward memory.[34,77] The obvious difference is that the interfering non-reinforced odor memory is formed before learning for LI and after learning for extinction.

Our studies of learning, extinction, and LI suggest that flies acquire and store all of their experience (rewarded and unrewarded, punished and unpunished) as parallel memory traces.[34,35,37,77,78] As a result, when evoked by an appropriate cue, the relevant experiences are compared/combined at the time of retrieval to determine the most fitting behavioral outcome. Such a model is reminiscent of the Miller and Matzel[79] comparator hypothesis, devised mostly from experiments in rodents.[11] Because recent studies suggest similar processes underlie extinction of fear in flies, rodents, and humans,[34,80–84] it seems likely that the form and mechanism of LI we describe here will also be relevant across phyla.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Fly *strains*
- METHOD DETAILS
  - Behavioral experiments
  - Two-Photon Calcium Imaging
- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Statistical Analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2021.05.056.

ACKNOWLEDGMENTS

We thank G. Rubin, FlyLight, B. Dickson, and the Bloomington Stock Center for flies. We are grateful to Geraldine Wright and members of the Waddell group for discussion. P.V.-G. is funded by the Sloane Robinson/Clarendon Scholarship given by the University of Oxford Clarendon Fund scheme and Keble College, and by the Consejo Nacional de Ciencia y Tecnología, Mexico (CONACYT). S.W. is funded by a Wellcome Principal Research Fellowship (200846/Z/16/Z) and an ERC Advanced Grant (789274).

AUTHOR CONTRIBUTIONS

Designed Research, P.F.J., Z.O., P.V.-G., J.F., and S.W.; Performed Research, P.F.J., Z.O., P.V.-G., J.F., and S.V.-M.; Analyzed Data, P.F.J., Z.O., J.F., P.V.-G., and S.V.-M.; Resources, S.W.; Writing, S.W., P.F.J., Z.O., and J.F.; Supervision, S.W.; Funding Acquisition, S.W.

DECLARATION OF INTERESTS

S.W. is a member of the advisory board of *Current Biology*.

Received: February 19, 2021
Revised: April 29, 2021
Accepted: May 26, 2021
Published: June 18, 2021

REFERENCES

1. Tolman, E.C., and Honzik, C.H. (1930). Introduction and removal of reward, and maze performance in rats. Univ. Calif. Publ. Psychol. *4*, 257–275.
2. Blodgett, H.C. (1929). The effect of the introduction of reward upon the maze performance of rats. Univ. Calif. Publ. Psychol. *4*, 113–134.
3. Lubow, R.E., and Moore, A.U. (1959). Latent inhibition: the effect of non-reinforced pre-exposure to the conditional stimulus. J. Comp. Physiol. Psychol. *52*, 415–419.
4. Weiss, K.R., and Brown, B.L. (1974). Latent inhibition: a review and a new hypothesis. Acta Neurobiol. Exp. (Warsz.) *34*, 301–316.
5. Mackintosh, N.J. (1975). A theory of attention: variations in the associability of stimuli with reinforcement. Psychol. Rev. *82*, 276–298.

6. Lubow, R.E., Schnur, P., and Rifkin, B. (1976). Latent inhibition and conditioned attention theory. J. Exp. Psychol. Anim. Behav. Process. *2*, 163–174.
7. Wagner, A.R. (1976). Priming in STM: an information-processing mechanism for self-generated or retrieval-generated depression in performance. In Habituation: Perspectives from Child Development, Animal Behavior, and Neurophysiology, T.J. Tighe, and R.N. Leaton, eds. (Erlbaum), pp. 95–128.
8. Miller, R.R., Kasprow, W.J., and Schachtman, T.R. (1986). Retrieval variability: sources and consequences. Am. J. Psychol. *99*, 145–218.
9. Weiner, I. (1990). Neural substrates of latent inhibition: the switching model. Psychol. Bull. *108*, 442–461.
10. Bouton, M.E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. Psychol. Bull. *114*, 80–99.
11. Escobar, M., and Miller, R.R. (2010). Latent inhibition: acquisition or performance deficit? In Latent Inhibition: Cognition, Neuroscience and Applications to Schizophrenia, R.E. Lubow, and I. Weiner, eds. (Cambridge University Press), pp. 62–93.
12. Wagner, A.R. (1981). SOP: a model of automatic memory processing in animal behavior. In Information Processing in Animals: Memory Mechanisms, N.E. Spear, and R.R. Miller, eds. (Erlbaum), pp. 5–47.
13. Wagner, A.R. (2003). Context-sensitive elemental theory. Q. J. Exp. Psychol. B *56*, 7–29.
14. Dudai, Y. (1977). Properties of learning and memory in *Drosophila melanogaster*. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *114*, 69–89.
15. Tempel, B.L., Bonini, N., Dawson, D.R., and Quinn, W.G. (1983). Reward learning in normal and mutant *Drosophila*. Proc. Natl. Acad. Sci. USA *80*, 1482–1486.
16. Colomb, J., Grillenzoni, N., Stocker, R.F., and Ramaekers, A.R.I. (2007). Complex behavioural changes after odour exposure in *Drosophila* larvae. Anim. Behav. *73*, 587–594.
17. Hattori, D., Aso, Y., Swartz, K.J., Rubin, G.M., Abbott, L.F., and Axel, R. (2017). Representations of novelty and familiarity in a mushroom body compartment. Cell *169*, 956–969.e17.
18. Semelidou, O., Acevedo, S.F., and Skoulakis, E.M. (2018). Temporally specific engagement of distinct neuronal circuits regulating olfactory habituation in *Drosophila*. eLife *7*, 1–25.
19. Chandra, S.B.C., Wright, G.A., and Smith, B.H. (2010). Latent inhibition in the honey bee, Apis mellifera: is it a unitary phenomenon? Anim. Cogn. *13*, 805–815.
20. Fernández, V.M., Giurfa, M., Devaud, J.M., and Farina, W.M. (2012). Latent inhibition in an insect: the role of aminergic signaling. Learn. Mem. *19*, 593–597.
21. Bazhenov, M., Huerta, R., and Smith, B.H. (2013). A computational framework for understanding decision making through integration of basic learning rules. J. Neurosci. *33*, 5686–5697.
22. Beck, C.D.O., Schroeder, B., and Davis, R.L. (2000). Learning performance of normal and mutant Drosophila after repeated conditioning trials with discrete stimuli. J. Neurosci. *20*, 2944–2953.
23. Yu, D., Akalal, D.-B.G., and Davis, R.L. (2006). *Drosophila* α/β mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. Neuron *52*, 845–855.
24. Kim, Y.-C., Lee, H.-G., and Han, K.-A. (2007). D1 dopamine receptor dDA1 is required in the mushroom body neurons for aversive and appetitive learning in *Drosophila*. J. Neurosci. *27*, 7640–7647.
25. Tomchik, S.M., and Davis, R.L. (2009). Dynamics of learning-related cAMP signaling and stimulus integration in the *Drosophila* olfactory pathway. Neuron *64*, 510–521.
26. Qin, H., Cressy, M., Li, W., Coravos, J.S., Izzi, S.A., and Dubnau, J. (2012). Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in *Drosophila*. Curr. Biol. *22*, 608–614.
27. Zhang, S., and Roman, G. (2013). Presynaptic inhibition of gamma lobe neurons is required for olfactory learning in Drosophila. Curr. Biol. *23*, 2519–2527.
28. Boto, T., Louis, T., Jindachomthong, K., Jalink, K., and Tomchik, S.M. (2014). Dopaminergic modulation of cAMP drives nonlinear plasticity across the *Drosophila* mushroom body lobes. Curr. Biol. *24*, 822–831.
29. Hige, T., Aso, Y., Modi, M.N., Rubin, G.M., and Turner, G.C. (2015). Heterosynaptic plasticity underlies aversive olfactory learning in Drosophila. Neuron *88*, 985–998.
30. Owald, D., and Waddell, S. (2015). Olfactory learning skews mushroom body output pathways to steer behavioral choice in Drosophila. Curr. Opin. Neurobiol. *35*, 178–184.
31. Burke, C.J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M.J., Das, G., Gohl, D., Silies, M., Certel, S., and Waddell, S. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. Nature *492*, 433–437.
32. Lin, S., Owald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. Nat. Neurosci. *17*, 1536–1542.
33. Huetteroth, W., Perisse, E., Lin, S., Klappenbach, M., Burke, C., and Waddell, S. (2015). Sweet taste and nutrient value subdivide rewarding dopaminergic neurons in *Drosophila*. Curr. Biol. *25*, 751–758.
34. Felsenberg, J., Jacob, P.F., Walker, T., Barnstedt, O., Edmondson-Stait, A.J., Pleijzier, M.W., Otto, N., Schlegel, P., Sharifi, N., Perisse, E., et al. (2018). Integration of parallel opposing memories underlies memory extinction. Cell *175*, 709–722.e15.
35. Jacob, P.F., and Waddell, S. (2020). Spaced training forms complementary long-term memories of opposite valence in Drosophila. Neuron *106*, 977–991.e4.
36. McCurdy, L.Y., Sareen, P., Davoudian, P.A., and Nitabach, M.N. (2021). Dopaminergic mechanism underlying reward-encoding of punishment omission during reversal learning in Drosophila. Nat. Commun. *12*, 1115.
37. Das, G., Klappenbach, M., Vrontou, E., Perisse, E., Clark, C.M., Burke, C.J., and Waddell, S. (2014). Drosophila learn opposing components of a compound food stimulus. Curr. Biol. *24*, 1723–1730.
38. Galili, D.S., Dylla, K.V., Lüdke, A., Friedrich, A.B., Yamagata, N., Wong, J.Y.H., Ho, C.H., Szyszka, P., and Tanimoto, H. (2014). Converging circuits mediate temperature and shock aversive olfactory conditioning in Drosophila. Curr. Biol. *24*, 1712–1722.
39. Kirkhart, C., and Scott, K. (2015). Gustatory learning and processing in the *Drosophila* mushroom bodies. J. Neurosci. *35*, 5950–5958.
40. Tomchik, S.M. (2013). Dopaminergic neurons encode a distributed, asymmetric representation of temperature in Drosophila. J. Neurosci. *33*, 2166, 76a.
41. Krashes, M.J., and Waddell, S. (2008). Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in Drosophila. J. Neurosci. *28*, 3103–3113.
42. Domjan, M. (1972). CS preexposure in taste-aversion learning: effects of deprivation and preexposure duration. Learn. Motiv. *3*, 389–402.
43. Clarke, M.E., and Hupka, R.B. (1974). The effects of stimulus duration and frequency of daily preconditioning stimulus exposures on latent inhibition in Pavlovian conditioning of the rabbit nictitating membrane response. Bull. Psychon. Soc. *4*, 225–228.
44. De la Casa, G., and Lubow, R.E. (1995). Latent inhibition in conditioned taste aversion: the roles of stimulus frequency and duration and the amount of fluid ingested during preexposure. Neurobiol. Learn. Mem. *64*, 125–132.
45. Morillas, E., González, F., and Hall, G. (2019). Facilitation and retardation of flavor preference conditioning following prior exposure to the flavor conditioned stimulus. Learn. Behav. *47*, 177–186.
46. Gaztañaga, M., Aranda-Fernández, P.E., Díaz-Cenzano, E., and Chotro, M.G. (2015). Latent inhibition and facilitation of conditioned taste aversion in preweanling rats. Dev. Psychobiol. *57*, 96–104.

47. DasGupta, S., and Waddell, S. (2008). Learned odor discrimination in Drosophila without combinatorial odor maps in the antennal lobe. Curr. Biol. *18*, 1668–1674.

48. Sandoz, J.C., Laloi, D., Odoux, J.F., and Pham-Delègue, M.H. (2000). Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. Anim. Behav. *59*, 1025–1034.

49. Locatelli, F.F., Fernandez, P.C., Villareal, F., Muezzinoglu, K., Huerta, R., Galizia, C.G., and Smith, B.H. (2013). Nonassociative plasticity alters competitive interactions among mixture components in early olfactory processing. Eur. J. Neurosci. *37*, 63–79.

50. Dolzer, J., Fischer, K., and Stengl, M. (2003). Adaptation in pheromone-sensitive trichoid sensilla of the hawkmoth Manduca sexta. J. Exp. Biol. *206*, 1575–1588.

51. Zufall, F., and Leinders-Zufall, T. (2000). The cellular and molecular basis of odor adaptation. Chem. Senses *25*, 473–481.

52. Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in Drosophila. Science *288*, 672–675.

53. Dubnau, J., Grady, L., Kitamoto, T., and Tully, T. (2001). Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature *411*, 476–480.

54. McGuire, S.E. (2001). The role of Drosophila mushroom body signaling in olfactory memory. Science *293*, 1330–1333.

55. Krashes, M.J., Keene, A.C., Leung, B., Armstrong, J.D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during Drosophila odor memory processing. Neuron *53*, 103–115.

56. Trannoy, S., Redt-Clouet, C., Dura, J.M., and Preat, T. (2011). Parallel processing of appetitive short- and long-term memories in Drosophila. Curr. Biol. *21*, 1647–1653.

57. Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. J. Neurobiol. *47*, 81–92.

58. Mohammad, F., Stewart, J.C., Ott, S., Chlebikova, K., Chua, J.Y., Koh, T.W., Ho, J., and Claridge-Chang, A. (2017). Optogenetic inhibition of behavior with anion channelrhodopsins. Nat. Methods *14*, 271–274.

59. Wang, J.W., Wong, A.M., Flores, J., Vosshall, L.B., and Axel, R. (2003). Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. Cell *112*, 271–282.

60. Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., and Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J. Neurosci. *23*, 10495–10502.

61. Claridge-Chang, A., Roorda, R.D., Vrontou, E., Sjulson, L., Li, H., Hirsh, J., and Miesenböck, G. (2009). Writing memories with light-addressable reinforcement circuitry. Cell *139*, 405–415.

62. Aso, Y., Siwanowicz, I., Bräcker, L., Ito, K., Kitamoto, T., and Tanimoto, H. (2010). Specific dopaminergic neurons for the formation of labile aversive memory. Curr. Biol. *20*, 1445–1451.

63. Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A.B., Ito, K., Scholz, H., and Tanimoto, H. (2012). Three dopamine pathways induce aversive odor memories with different stability. PLoS Genet. *8*, e1002768.

64. Liu, C., Plaçais, P.Y., Yamagata, N., Pfeiffer, B.D., Aso, Y., Friedrich, A.B., Siwanowicz, I., Rubin, G.M., Preat, T., and Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. Nature *488*, 512–516.

65. Aso, Y., and Rubin, G.M. (2016). Dopaminergic neurons write and update memories with cell-type-specific rules. eLife *5*, 1–15.

66. Séjourné, J., Plaçais, P.-Y., Aso, Y., Siwanowicz, I., Trannoy, S., Thoma, V., Tedjakumala, S.R., Rubin, G.M., Tchénio, P., Ito, K., et al. (2011). Mushroom body efferent neurons responsible for aversive olfactory memory retrieval in *Drosophila*. Nat. Neurosci. *14*, 903–910.

67. Bouzaiane, E., Trannoy, S., Scheunemann, L., Plaçais, P.Y., and Preat, T. (2015). Two independent mushroom body output circuits retrieve the six discrete components of *Drosophila* aversive memory. Cell Rep. *11*, 1280–1292.

68. Owald, D., Felsenberg, J., Talbot, C.B., Das, G., Perisse, E., Huetteroth, W., and Waddell, S. (2015). Activity of defined mushroom body output neurons underlies learned olfactory behavior in Drosophila. Neuron *86*, 417–427.

69. Perisse, E., Owald, D., Barnstedt, O., Talbot, C.B., Huetteroth, W., and Waddell, S. (2016). Aversive learning and appetitive motivation toggle feed-forward inhibition in the Drosophila mushroom body. Neuron *90*, 1086–1099.

70. Handler, A., Graham, T.G.W., Cohn, R., Morantte, I., Siliciano, A.F., Zeng, J., Li, Y., and Ruta, V. (2019). Distinct dopamine receptor pathways underlie the temporal sensitivity of associative learning. Cell *178*, 60–75.e19.

71. De la Casa, L.G., and Pineño, O. (2010). Inter-stage context and time as determinants of latent inhibition. In Latent Inhibition, R.E. Lubow, and I. Weiner, eds. (Cambridge University Press), pp. 40–61.

72. Bouton, M.E., and Swartzentruber, D. (1989). Slow reacquisition following extinction: context, encoding, and retrieval mechanisms. J. Exp. Psychol. Anim. Behav. Process. *15*, 43–53.

73. Westbrook, R.F., Jones, M.L., Bailey, G.K., and Harris, J.A. (2000). Contextual control over conditioned responding in a latent inhibition paradigm. J. Exp. Psychol. Anim. Behav. Process. *26*, 157–173.

74. Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.Y., Friedrich, A.B., Sima, R.J., Preat, T., Rubin, G.M., and Tanimoto, H. (2015). Distinct dopamine neurons mediate reward signals for short- and long-term memories. Proc. Natl. Acad. Sci. USA *112*, 578–583.

75. Das, S., Sadanandappa, M.K., Dervan, A., Larkin, A., Lee, J.A., Sudhakaran, I.P., Priya, R., Heidari, R., Holohan, E.E., Pimentel, A., et al. (2011). Plasticity of local GABAergic interneurons drives olfactory habituation. Proc. Natl. Acad. Sci. USA *108*, E646–E654.

76. Westbrook, R.F., and Bouton, M.E. (2010). Latent inhibition and extinction: their signature phenomena and the role of prediction error. In Latent Inhibition: Cognition, Neuroscience and Applications to Schizophrenia, R.E. Lubow, and I. Weiner, eds. (Cambridge University Press), pp. 23–39.

77. Felsenberg, J., Barnstedt, O., Cognigni, P., Lin, S., and Waddell, S. (2017). Re-evaluation of learned information in *Drosophila*. Nature *544*, 240–244.

78. Senapati, B., Tsao, C.-H.H., Juan, Y.-A.A., Chiu, T.H., Wu, C.L., Waddell, S., and Lin, S. (2019). A neural mechanism for deprivation state-specific expression of relevant memories in Drosophila. Nat. Neurosci. *22*, 2029–2039.

79. Miller, R.R., and Matzel, L.D. (1988). The comparator hypothesis: a response rule for the expression of associations. In The Psychology of Learning and Motivation: Advances in Research and Theory, G.H. Bower, ed. (Academic Press), pp. 51–92.

80. Luo, R., Uematsu, A., Weitemier, A., Aquili, L., Koivumaa, J., McHugh, T.J., and Johansen, J.P. (2018). A dopaminergic switch for fear to safety transitions. Nat. Commun. *9*, 2483.

81. Salinas-Hernández, X.I., Vogel, P., Betz, S., Kalisch, R., Sigurdsson, T., and Duvarci, S. (2018). Dopamine neurons drive fear extinction learning by signaling the omission of expected aversive outcomes. eLife *7*, 1–25.

82. Kalisch, R., Gerlicher, A.M.V., and Duvarci, S. (2019). A dopaminergic basis for fear extinction. Trends Cogn. Sci. *23*, 274–277.

83. Zhang, X., Kim, J., and Tonegawa, S. (2020). Amygdala reward neurons form and store fear extinction memory. Neuron *105*, 1077–1093.e7.

84. Thiele, M., Yuen, K.S.L., Gerlicher, A.V.M., and Kalisch, R. (2021). A ventral striatal prediction error signal in human fear extinction learning. Neuroimage *229*, 117709.

85. Aso, Y., Hattori, D., Yu, Y., Johnston, R.M., Iyer, N.A., Ngo, T.-T.T.B., Dionne, H., Abbott, L.F.L., Axel, R., Tanimoto, H., and Rubin, G.M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. eLife *3*, e04577.

86. Aso, Y., Sitaraman, D., Ichinose, T., Kaun, K.R., Vogt, K., Belliart-Guérin, G., Plaçais, P.-Y.Y., Robie, A.A., Yamagata, N., Schnaitmann, C., et al.

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

87. Pai, T.-P., Chen, C.-C., Lin, H.-H., Chin, A.-L., Lai, J.S.-Y., Lee, P.-T., Tully, T., and Chiang, A.-S. (2013). *Drosophila* ORB protein in two mushroom body output neurons is necessary for long-term memory formation. Proc. Natl. Acad. Sci. USA *110*, 7898–7903.
88. Krashes, M.J., DasGupta, S., Vreede, A., White, B., Armstrong, J.D., and Waddell, S. (2009). A neural circuit mechanism integrating motivational state with memory expression in Drosophila. Cell *139*, 416–427.
89. Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto, T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., et al. (2014). Independent optical excitation of distinct neural populations. Nat. Methods *11*, 338–346.
90. Chen, T.-W., Wardill, T.J., Sun, Y., Pulver, S.R., Renninger, S.L., Baohan, A., Schreiter, E.R., Kerr, R.A., Orger, M.B., Jayaraman, V., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. Nature *499*, 295–300.
91. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.
92. Pologruto, T.A., Sabatini, B.L., and Svoboda, K. (2003). ScanImage: flexible software for operating laser scanning microscopes. Biomed. Eng. Online *2*, 13.
93. Tully, T., and Quinn, W.G. (1985). Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. *157*, 263–277.
94. Shang, Y., Claridge-Chang, A., Sjulson, L., Pypaert, M., and Miesenböck, G. (2007). Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. Cell *128*, 601–612.