Timing-dependent reinforcement learning in larvae of *Drosophila melanogaster*

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Zusammenfassung

Für alle Tierarten ist es unerlässlich, Belohnung und Bestrafung adaptiv zu verarbeiten, um ihr Verhalten an die jeweiligen Umstände anzupassen. Bei Beeinträchtigung dieser Verarbeitung, kommt es zu psychischen Störungen, und Verhalten kann nicht richtig angepasst werden, um eine Belohnung zu erhalten und eine Bestrafung zu vermeiden. Ein wichtiger Aspekt dieser Verhaltensanpassung ist, dass ein und dieselbe Belohnung/Bestrafung gegensätzliche Motivation auslösen und das Verhalten in entgegengesetzte Richtungen lenken kann. Zu erwarten sind Freude über den Beginn einer Belohnung, und Frustration über ihr Ende. Insbesondere das Lernen über das Ende einer Belohnung wurde in der Forschung bisher vernachlässigt. Die Larven von *Drosophila melanogaster* sind in dieser Hinsicht ein attraktives Modell, denn obwohl ihr Gehirn numerisch einfach ist, sind sie zu ausreichend komplexem Verhalten fähig, um diese Lernprozesse zu untersuchen. Angesichts des bekannten Konnektoms des Larvengehirns ist es in der Tat möglich, Schaltkreise im Gehirn auf der Ebene einzelner Zellen zu untersuchen.

Im ersten Teil benutze ich transgene Manipulationen an larvalen *Drosophila*, um das Lernen über den Beginn und das Ende eines zentralen Belohnungssignals im Gehirn, erreicht durch optogenetische Aktivierung des dopaminergen Neurons DAN-i1, zu untersuchen. Je nachdem, wann ein Geruch in Bezug auf die Aktivierung des DANs präsentiert wird, zeigen die Larven Gedächtnisse mit entgegengesetzter Valenz. Diese Ergebnisse werden mit dem Lernen über den Beginn und das Ende der Aktivierung des Bestrafung signalisierenden DAN-f1s verglichen und in den Kontext anderer Formen der Konditionierung gestellt, die zeigen, dass der Zeitpunkt einer Belohnung/Bestrafung für das Lernen entscheidend ist.

Im zweiten Teil stelle ich ein Protokoll zur Fütterung des Dopaminsynthesehemmers 3-Iodo-L-Tyrosin vor. Mit Hilfe dieser akuten systemischen Hemmung der Signalübertragung durch Dopamin, wird die Beteiligung von Dopamin am Belohnungslernen bei larvalen *Drosophila* und am Bestrafungslernen bei Fliegen von *Drosophila* demonstriert. Die Kombination dieses pharmakologischen Ansatzes zusammen mit den genetischen Techniken, die bei *Drosophila* zur Verfügung stehen, um neuronale und verhaltensbezogene Funktionen zu manipulieren, könnten dazu beitragen, Schaltkreisprinzipien zu identifizieren und die neuronalen Mechanismen aufzudecken, die diesen Gedächtnissen mit unterschiedlicher Valenz zugrunde liegen.

Summary

For all animal species, it is essential to process reward and punishment adaptively in order to adapt their behaviour to given circumstances. If this processing is disturbed mental disorders arise and behaviour cannot be properly adapted to obtain reward nor to avoid, or escape, punishment. An important aspect of this behavioural adaptation is that the same reward/punishment can be motivationally opposing and steer behaviour into opposite directions. While the beginning of a reward is something desirable, the termination of a reward is something rather frustrating. In particular, learning about the termination of a reward has been neglected in research. The larvae of *Drosophila melanogaster* serve as an attractive study case in this regard because, although their brain is numerically simple, they are capable of sufficiently complex behaviour to study these learning processes. Given the well-known connectome of the larval brain, it is indeed possible to study brain circuits on the level of single cells.

In the first part, I use transgenic manipulation of larval *Drosophila* to investigate learning about the beginning and termination of a central brain reward signal via optogenetic activation of the dopaminergic neuron DAN-i1. Depending on when an odour is presented with respect to the activation of the DAN, larvae show memories of opposite valence. These results are compared with learning about the beginning and termination of the punishment-signalling DAN-f1 and further placed in the context of other forms of conditioning showing that the timing of a reward/punishment is critical for reinforcement learning.

In the second part, I establish a feeding protocol of the dopamine synthesis inhibitor 3-Iodo-L-tyrosine. Using this for an acute systemic inhibition of dopamine signalling, the involvement of dopamine in odour-sugar reward-learning in larval *Drosophila* and in odour-DAN punishment-learning in adult *Drosophila* is demonstrated. Combining this pharmacological approach together with the genetic techniques available in *Drosophila* to manipulate neuronal and behavioural functions could help identify circuit principles and reveal the neuronal mechanisms underlying the memories of different valences.

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List of abbreviations

3IY	3-Iodo-L-tyrosine
AC	Adenylyl cyclase
AL	Antennal lobe
AM	n-amylacetate
APL	Anterior paired lateral neuron
BA	Benzaldehyde
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CR	Conditioned reaction
CS	Conditioned stimulus
DAN	Dopaminergic neuron
DO	Dorsal organ
DOG	Dorsal organ ganglion
DPO	Dorsal pharyngeal organ
DPS	Dorsal pharyngeal sensillae
EM	Empty
FRU	Fructose
GPCR	G-protein-coupled receptor
GRN	Gustatory receptor neuron
HC	Head cast
ISI	Inter stimulus interval
KC	Kenyon cell
KW	Kruskal-Wallis test
L1	1st instar larval stage
L2	2nd instar larval stage
L3	3rd instar larval stage
L-DOPA	L-3,4-dihydroxyphenylalanine
LN	Local interneuron
LTD	Long-term depression
LTP	Long-term potentiation
MB	Mushroom body
MBIN	Mushroom body input neuron
MBON	Mushroom body output neuron
MWU	Mann-Whitney U-test
NS	Neutral stimulus
NSSI	Non-suicidal self-injury
OAN	Octopaminergic neuron
OCT	3-octanol
OR	Olfactory receptor
ORN	Olfactory receptor neuron
OSS	One-sample sign test
PKA	Protein kinase a
PN	Projection neuron
PPS	Posterior pharyngeal sensillae
PTSD	Post-traumatic stress disorder
S	Solvent control
SEG	Suboesophageal zone
STDP	Spike-timing-dependent plasticity
TH	Tyrosine hydroxylase
TO	Terminal organ
TOG	Terminal organ ganglion
UAS	Upstream activation sequence
UR	Unconditioned reaction
US	Unconditioned stimulus
VO	Ventral organ
VOG	Ventral organ ganglion
VPS	Ventral pharyngeal sensillae

General introduction

Avoiding danger and finding food are essential skills for all animal species. To successfully accomplish these tasks, humans and animals alike are constantly adapting their behaviour to given circumstances while learning signals that mark the beginning and termination of punishing or rewarding events. Or as Pavlov wrote already in the 1920s (Pavlov, 1927):

> *„Conditioned reflexes [learned associations] are phenomena of common and widespread occurrence : their establishment is an integral function in everyday life. We recognize them in ourselves and in other people or animals under such names as " education," " habits," and " training ; " and all of these are really nothing more than the results of an establishment of new nervous connections during the post-natal existence of the organism."* (S. 26).

Pavlov was the pioneer of classical conditioning experiments that show learning of the association between stimuli. In his experiments he trained dogs to associate a neutral stimulus (NS; bell ringing) together with an unconditioned stimulus (US, food). Before training, the US itself, but not the NS, elicited an innate reaction (UR) (including but not limited to salivation). After presenting the NS together with the US for several times, the NS became a conditioned stimulus (CS) which now evoked itself a reaction (CR, including but likewise not limited to salivation). Importantly, although in terms of salivation the CR resembles the UR in this case, the CR can also be quite different, as for example in reward learning, where the UR is the consumption of the US (e.g. sugar) and the CR the approach to but not the consumption of the CS (e.g. light). More generally, one can say that the CR is the behaviour that the animals show when, based on associative learning, they expect the US to occur. As for association formation, Pavlov described the conditions under which it can take place: the nervous system needs to be in an alert state, the properties of the CS and US must fit and the relative timing of CS and US must be correct:

> *"The fundamental requisite is that any external stimulus which is to become the signal in a conditioned reflex must overlap in point of time with the action of an unconditioned stimulus."* (Pavlov, 1927, S. 26) and *"Further, it is not enough that there should be overlapping between the two stimuli ; it is also and equally necessary that the conditioned stimulus should begin to operate before the unconditioned stimulus comes into action. If*

this order is reversed, the unconditioned stimulus being applied first and the neutral stimulus second, the conditioned reflex cannot be established at all." (Pavlov, 1927, S. 27).

Pavlov's assumptions were based on his initial experiments were dogs only responded by saliva production to the CS when it was presented before the US (forward pairing) but not when this order of events was reversed (backward pairing). But is it really true, that '*the conditioned reflex cannot be established at all'* in backward conditioning?

Timing is crucial: Timing-dependent valence reversal

Although in Pavlov's experiments backward pairing did not result in a salivation-response there soon sparked debate because later experiments showed that backward conditioning can indeed take place using for example pigeons, rats or rabbits (Barker and Smith, 1974; Hearst, 1989; Siegel and Domjan, 1974; review about early data: Spetch et al., 1981). Furthermore, based on everyday observations, Solomon and Corbit (1974) postulated the opponent-process theory which says that not only the beginning but also the termination of an US is important to an individual and can be learned about. According to their theory, the beginning of a stimulus evokes a specific primary affective response, and the termination of the same stimulus evokes an after-affect with opposite valence (Figure 1). For example, for a positive US, e.g. giving a child an ice cream, the child is very happy when it gets the ice cream (positive primary affect). However, when the ice cream falls down, the child is very frustrated (negative after-affect). Similarly, at the beginning of an event like skydiving, we experience fear before the jump, which turns into relief at landing safely on the ground.

Learning experiments in different species indeed demonstrated that the memory for a CS changes its valence depending on its timing to a US. Whereas most of the studies about this timing-dependent valence reversal concentrated on the aversive domain, for example using shock pulses in adult *Drosophila* (Diegelmann et al., 2013a; Jacob and Waddell, 2020; König et al., 2018; Niewalda et al., 2015; Tanimoto et al., 2004; Yarali and Gerber, 2010; Yarali et al., 2008, 2009; for more studies in *Drosophila* see 'General discussion - Timing-dependent valence

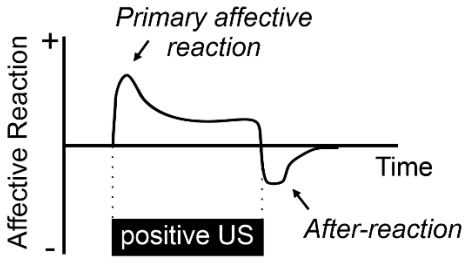

Figure 1. Opponent-process theory. At the beginning of a positive US, a positive primary affective reaction is observed that reaches a steady level while the US maintains and decays and turns into an affective after-reaction of opposite valence when the US is terminated. Adapted from Solomon and Corbit, 1974.

reversal in *Drosophila'*) and later on was also demonstrated in rodents and humans (Andreatta et al., 2010, 2012; Luck and Lipp, 2017; Mohammadi et al., 2014), for the appetitive domain only few studies about such a valence reversal exist (Aso and Rubin, 2016; Felsenberg et al., 2013; Hellstern et al., 1998; Saumweber et al., 2018; Urushihara, 2004).

A closer look into the experimental methods of these studies shows why backward conditioning effects were overlooked in the past and what is needed to investigate backward conditioning. One the one hand, like Pavlov assumed, the time between presentations of CS and US is of relevance. For example, Silva and colleagues (1996) demonstrated that backward conditioning only occurs if the time gap between US and CS is very short and thus, backward conditioning may have been missed if the time between the presentation of US and CS was too long. On the other hand, the behavioural readout of the memory must be carefully considered. Usually, experimenters select CSs that elicit no or only little of the target conditioned response prior to conditioning. This can be a problem for detecting oppositely-valenced effects of backward conditioning because it is difficult or practically impossible to see a decrease in response. For example, in Pavlov's (1927) experiments the target behavioural readout was the salivation of the dog. The innate behaviour of the dog towards the CS (bell) is zero salivation, thus it is impossible to see any motivationally opposite effect, namely a decrease in salivation after backward conditioning. To overcome these difficulties, in vertebrates the bi-valent modulation of the startle response as an indicator of conditioned fear was established (rodents: Fendt and Fanselow, 1999; Koch and Schnitzler, 1997) (monkeys: Davis et al., 2008; Winslow et al., 2002) (humans: Norrholm et al., 2006; van Well et al., 2012).

The startle response reflects an evolutionarily conserved defence reflex towards sudden or threatening stimuli, typically a very loud noise (Koch, 1999). The modulation of this startle response can reveal the implicit valence of a CS (Andreatta et al., 2010; Luck and Lipp, 2017). This means, the amplitude of the startle response can be increased/potentiated by CSs that had been forward-conditioned with a painful event (Davis, 2006; Grillon and Davis, 1997; Schwienbacher et al., 2004) and decreased/attenuated by CSs that had been forward-conditioned with a positive event (Koch et al., 1996; Schneider and Spanagel, 2008; Steidl et al., 2001). Importantly, it was found that startle amplitude is likewise decreased/attenuated by CSs that had been backward-conditioned with a painful event, indicating the positive valence of such stimuli (Andreatta et al., 2012). In the simpler organism *Drosophila melanogaster*, odours are used as a CS and as a bi-valent behavioural readout the animals' decision to approach or

avoid an odour in a preference test is used (adult flies: Quinn et al., 1974; Schwaerzel et al., 2003; Tully and Quinn, 1985) (larvae: Michels et al., 2017; Pauls et al., 2010a; Scherer et al., 2003). Thus, using learning tasks that allow to observe motivationally opposing types of behaviours towards a CS, it is possible to study timing-dependent valence reversal and to reveal memories of opposite valence for a CS based on its association with the occurrence or termination of an US. Using such bivalent tasks revealed timing-dependent valence reversal as an across-species principle. As will be argued in the next section, for investigating its fundamental mechanisms it is beneficial to use a 'simple' brain: for example, of *Drosophila melanogaster*.

An attractive model organism: *Drosophila melanogaster*

Both the adult and the larval stages of *Drosophila melanogaster* are attractive and well-studied model organisms that are used to investigate mechanisms of brain development, neurodegenerative diseases or the circadian clock, to name just a few examples (Jennings, 2011; Lessing and Bonini, 2009; Lu and Vogel, 2009; O'Kane, 2011). Although the fly is a very different animal from humans, findings from studies on flies often can be applied to humans because they share many basic genetic, cellular and neurobiological processes (O'Kane, 2011; Venken et al., 2011) and about 75% of known human disease genes have recognizable match in *Drosophila* (O'Kane, 2011; Reiter et al., 2001; Scaplen and Kaun, 2016; Venken et al., 2011). For this reason, studies in *Drosophila* have influenced neuroscience research in vertebrate species for a long time (Bellen et al., 2010) and helped to understand the basics of neurodegeneration in for example Alzheimer's disease (Fernandez-Funez et al., 2015; Prüßing et al., 2013), Parkinson's disease (Feany and Bender, 2000; Guo, 2012), Huntington's disease (Campesan et al., 2011; Lewis and Smith, 2016) or basic mechanisms of addiction (Kaun et al., 2012; Ryvkin et al., 2018). Studies on the fruit fly played such a key role in uncovering fundamental biological processes that discoveries in flies repeatedly have been rewarded with the Nobel Prize. In 2017, for example, Jeffrey C. Hall, Michael Rosbash and Michael W. Young received the prize for uncovering the molecular mechanisms that control circadian rhythms.

But it is not only because of the shared processes with vertebrates that *Drosophila* is a widely used model organism. Another aspect is their short generation time. The reproductive cycle of *Drosophila* lasts only 10-12 days at 25 °C. About one day after egg laying, the larva hatches (1st instar larval stage; L1). Over the next four to five days, the larva stays in the food, eats a lot

and grows very fast and reaches the 2nd instar larval stage (L2) and the final 3rd instar larval stage (L3). On the 6th -7th day after egg laying, the larva begins to crawl out of the food and develops into an immobile pupa. During the next four to six days, the pupa undergoes metamorphosis into the adult form until it hatches (on the 10th-12th day after egg laying). About 12 h after hatching the adult female can mate and the cycle begins again.

Other aspects of why *Drosophila* is a common model organism are the facts that (1) a rich repertoire of robust, simple learning paradigms is available, (2) a toolbox for transgenic manipulations is available and (3) that they have a fairly small and numerically simple brain that is at least in parts reconstructed. Especially the larva of *Drosophila melanogaster* are experimentally attractive because of their even smaller nervous system, simple handling and still interesting enough behaviour. Thus, I focused my studies on *Drosophila* larvae and will continue to focus on larvae for the rest of this introduction, with repeated comparisons to adult flies and other animals.

How to train a *Drosophila* larva: Olfactory conditioning

Quinn and colleagues (1974) were the first who demonstrated conditioned behaviour in *Drosophila*: adult flies were trained in a classical conditioning paradigm such that one of two odours (CS) was paired with an electric shock (US). When afterwards flies were given the choice between these two odours, they avoided the previously punished odour. This was the first demonstration that adult flies can form an aversive associative memory for a stimulus. Some years later, this paradigm was adapted to an appetitive version, where sugar served as the reward (Tempel et al., 1983). Since then, these paradigms were further developed (Klapoetke et al., 2014; Liu et al., 1999; Putz and Heisenberg, 2002; Wolf and Heisenberg, 1997) and led to investigation of basic principles of learning and memory (Davis, 2005; Heisenberg, 2003; Schwaerzel et al., 2003; Yarali and Gerber, 2010; Zars et al., 2000).

Just a few years later after the first conditioning experiments in adult *Drosophila*, a corresponding classical conditioning paradigm was developed for larval *Drosophila* (Aceves-Piña and Quinn, 1979; Rodrigues, 1980) with increased attention from the end-1990s on (reviewed in: Cobb, 1999; Sokolowski, 2001; Stocker, 1994). Thus, also for larvae it was demonstrated that they can form an aversive or appetitive memory using electric shocks or sugar, respectively (Hendel et al., 2005; Khurana et al., 2009; Neuser et al., 2005; Pauls et al., 2010a; Scherer et al., 2003). In the two-odour paradigm, larvae are presented with two odours,

only one of which is paired with a positive or negative US and afterwards larvae are given the choice between the two presented odours. In the one-odour paradigm (Saumweber et al., 2011; Weiglein et al., 2019) however, only one single odour is used. In half of the cases the odour is paired with a reinforcer ('paired' group), in the other half the odour is not paired with it ('unpaired' group). For the 'paired' group of larvae the odour becomes a predictor for the reinforcer. For the 'unpaired' group the odour becomes a predictor for the absence of the reinforcer, a process that is assumed to work via associations with the experimental context (Saumweber et al., 2011; reviewed and discussed in: Schleyer et al., 2018).

Until today, many substances have been identified as USs (see Table 1 for an overview), different odours were utilised and variations of the paradigm were developed (Chen and Gerber, 2014; Chen et al., 2011; Hendel et al., 2005; Mancini et al., 2019; Mishra et al., 2013; Saumweber et al., 2018; Scherer et al., 2003; Schleyer et al., 2015a; Weiglein et al., 2019). Astonishingly, the memories larvae can form are rather complex: their memory showed to be specific for the quantity and quality of the odour (Chen et al., 2011; Mishra et al., 2013) and the US (Schleyer et al., 2011, 2015a), adapts to changes in CS-US contingency (Mancini et al., 2019) and is dependent on the timing of odour and US presentation (Saumweber et al., 2018; Weiglein et al., 2021). Importantly, it was also shown that instead of using real rewards or punishments optogenetic activation of specific neurons in the larval brain are sufficient to mediate US information (Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006; Weiglein et al., 2021). This optogenetic activation of even single neurons is achieved by transgenic manipulation of the *Drosophila* genome, which will be briefly discussed in the following section.

A method of genetic manipulation: The Gal4-UAS system

The well-established learning paradigms in larval *Drosophila* are especially important in combination with the reconstructed connectome of the larval brain (see next chapter: 'A not-so-simple brain: The brain of larval *Drosophila*') and with the possibilities of genetic manipulation of the fly genome. A widely used approach to selectively express any gene in a tissue specific manner in *Drosophila* is the so called Gal4/UAS system (Figure 2**;** Brand and Perrimon, 1993). The Gal4 protein is a transcription factor in yeast and has no endogenous target in *Drosophila*. By inserting Gal4 in arbitrary locations in the genome of *Drosophila* many transgenic lines of flies were created in which the *Gal4* gene is inserted in the fly genome and,

Table 1. Examples of USs used for associative olfactory learning in larval *Drosophila*.

US	Reference
Rewarding	
Sugars *Sweet & nutritious:* Glucose, Fructose, Sucrose *Only sweet:* Arabinose, Xylose *Only nutritious:* Maltodextrin, Sorbitol	Gerber and Hendel, 2006; Hendel et al., 2005; Honjo and Furukubo-Tokunaga, 2009; Neuser et al., 2005; Rohwedder et al., 2012; Scherer et al., 2003; Schipanski et al., 2008; Schleyer et al., 2011, 2015a
Ethanol	Schumann et al., 2021
Low concentration of salt	Gerber and Hendel, 2006; Niewalda et al., 2008; Schleyer et al., 2011
Punishing	
High concentration of salt	Gerber and Hendel, 2006; Hendel et al., 2005; Niewalda et al., 2008; Schleyer et al., 2011; Widmann et al., 2016
Quinine	Apostolopoulou et al., 2014; El-Keredy et al., 2012; Gerber and Hendel, 2006; Hendel et al., 2005; Scherer et al., 2003; Schleyer et al., 2011
Caffeine	Apostolopoulou et al., 2016
Electric shock	Khurana et al., 2009; Pauls et al., 2010a
Heat shock	Khurana et al., 2012
Light	von Essen et al., 2011
Vibration	Eschbach et al., 2011; Saumweber et al., 2014
Rewarding and punishing	
Amino acids	Kudow et al., 2017; Schleyer et al., 2015a; Toshima et al., 2019
Optogenetic activation of dopaminergic brain neurons	Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006; Weiglein et al., 2021

depending on the local enhancers is expressed in specific regions of the brain ('where'; 'driver'). As long as there are no binding sites for the Gal4 protein it has no or only little effect on the fly (Jenett et al., 2012). The DNA-binding sequence of Gal4 is the Upstream Activation Sequence (UAS). This UAS can be inserted upstream to a target gene of interest ('what'; 'effector') and thus many fly lines were created holding different effectors (see Table 2 for an overview of used effectors; for more details see Owald et al., 2015). If upon crossing driver x effector flies the Gal4 protein and the UAS sequence are present in the same animal of the F1 offspring, such that the Gal4 within the genetically defined brain region binds to the UAS and the gene downstream to it is activated.

To achieve a more specific spatial expression of the Gal4, even in single cells, the Split-Gal4/UAS system was developed (Figure 2B; Jenett et al., 2012; Luan et al., 2006; Pfeiffer et al., 2010). It takes advantage of the fact that the Gal4 protein consists of two functional domains: the DNA-binding (DBD) and transcription activation (AD) domains. These can be expressed

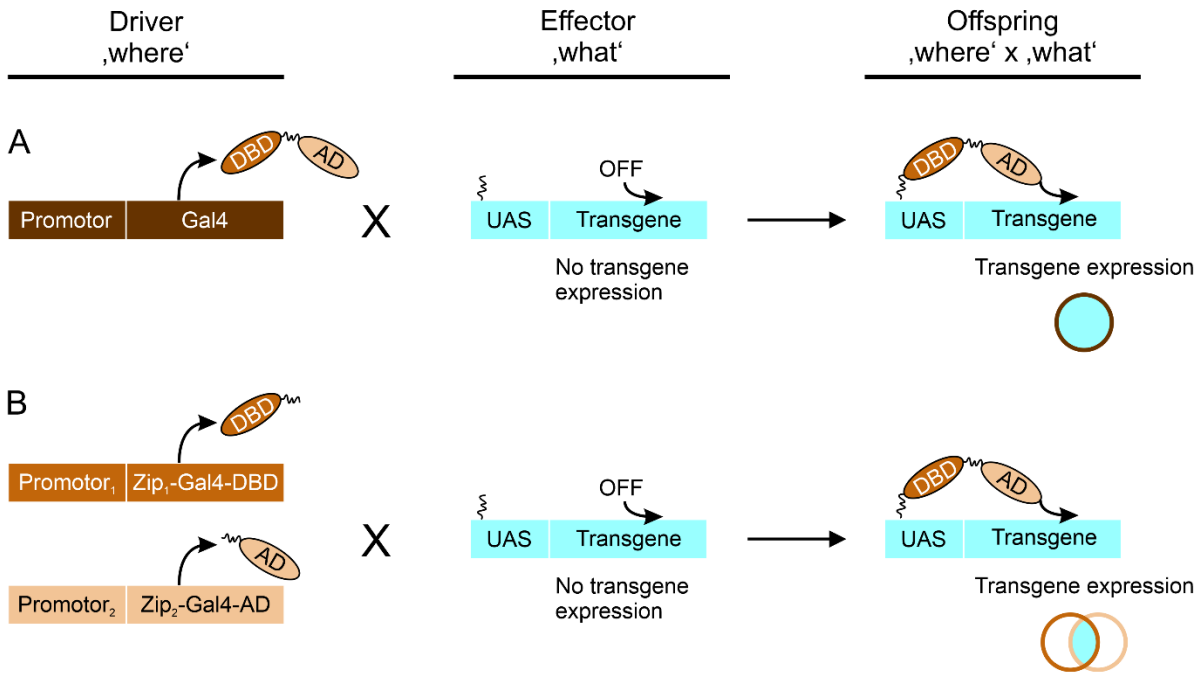

Figure 2. The Gal4-UAS system. (A) One fly in which the Gal4 gene is expressed in a specific region of the brain ('where'; 'driver') is crossed to another fly in which the UAS is placed upstream to a target gene of interest ('what'; 'effector'). In the offspring the Gal4 protein within the specific brain region binds to the UAS and the gene downstream of the UAS is activated. (B) In the split-Gal4-UAS system, the two functional domains of the Gal4 protein (the DNA-binding (DBD) and transcription activation (AD) domain) are expressed in different brain regions and consequently only in cells where both functional domains are expressed, the Gal4 protein is build and can bind to the UAS.

separately in different brain regions and consequently only in cells where both functional domains are expressed, a full, functional Gal4 protein is expressed, can bind to the UAS and the gene downstream to the UAS is activated.

More temporal precision can be achieved by additionally using the $Gal80^{ts}$, a temperature-sensitive protein that normally represses the Gal4 protein in yeast (McGuire et al., 2003). Inserting $Gal80^{ts}$ additionally to the Gal4 construct allows temporal control over the expression of the Gal4 protein. Another way to achieve more temporal precision is the usage of effectors that are light-sensitive or temperature-sensitive. This way, neurons can for example be activated during a specific time window using light or shifts of temperature (Aso and Rubin, 2016; Inada et al., 2011; Schroll et al., 2006).

For example, using the Gal4/UAS system, studies demonstrated that the mushroom body (MB) is the olfactory learning centre in insects (Heisenberg, 2003; Keene and Waddell, 2007; McGuire et al., 2005) and that dopaminergic neurons (DANs) are required for learning and their activation is sufficient to exert rewarding or punishing effects (larvae: Eichler et al., 2017; Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006) (adults: Aso and Rubin, 2016; Aso et al., 2012; König et al., 2019). To sum up, the Gal4/UAS or Split-Gal4/UAS system enables to express any desirable gene ('what'; see Table 2 for an overview of used effectors) in any desirable cell or tissue ('where') and thus to investigate the role of specific neurons in biological processes.

A not-so-simple brain: The brain of larval *Drosophila*

With about 10.000 neurons (Bossing et al., 1996; Larsen et al., 2009), the brain of larval *Drosophila* is in order of magnitudes simpler than that of humans (~$86x10^{12}$, Azevedo et al., 2009), mice (~$70x10^{6}$; Herculano-Houzel et al., 2006) and also adult *Drosophila* (~100.000, Simpson, 2009). Nevertheless, it shares similar organization with that of adult *Drosophila* and in some respects even vertebrates (Apostolopoulou et al., 2015; Cobb, 1999; Gerber and Stocker, 2007; Gerber et al., 2009; Kwon et al., 2011; Vosshall and Stocker, 2007; for more details see Table 3). This 'simple' brain allows the larvae – in order to survive – to cope with their environment in a way that allows them to find food and avoid harmful stimuli. This is, for example, telling apart different chemicals when necessary and ignore differences when there is no danger. Therefore, the chemosensory system of larval *Drosophila* is essential to map the important features of their environment such that they can quickly adjust their behaviour if

Table 2. Examples of effector lines used in *Drosophila*.

Name of effector	What it is	What it does	Reference
Channelrhodopsin-2 (ChR2)	Light-gated cation channel	Activation of neurons	Dawydow et al., 2014
CsChrimson	Light-gated cation channel	Activation of neurons	Klapoetke et al., 2014
dTrpA1	Temperature-sensitive transient receptor potential cation channel	Activation of neurons	Rosenzweig et al., 2005
Guillardia theta anion channel rhodopsin 1 (GtACR1)	Light-gated anion channel	Inhibition/ hyperpolarization of neurons	Mohammad et al., 2017
Halorhodopsin (NpHR)	Light-gated chloride pump	Inhibition/ hyperpolarization of neurons	Inada et al., 2011; Liu et al., 2016
$Shibire^{ts1}$ (shi^{ts1})	Temperature-sensitive dynamin	Temperature-dependent block of synaptic output of neurons by blocking endocytosis and therefore synaptic vesicle recycling	Kitamoto, 2001; Kosaka and Ikeda, 1983
RNAi	RNA interference method	Suppressing gene expression	Dietzl et al., 2007
GCaMP	Calcium-sensitive fluorescent reporter of intracellular Ca^{2+}	Monitoring neuronal activity	Wang et al., 2003
Kir2.1	Inward-rectifier potassium channel	Preventing action potential firing	Hodge, 2009
Reaper (rpr)	Gene coding for protein of apoptosis cascade	Cell death	Busto et al., 1999; McNabb et al., 1997
Head involution defective (hid)	Gene coding for protein of apoptosis cascade	Cell death	Grether et al., 1995

needed. However, it is not only important to 'respond' to stimuli, but also to 'interact' with them. Thus, it is beneficial for larvae to learn where to find as-yet absent food and to avoid as-yet absent noxious stimuli. The learning paradigms explained in the previous chapter show that larvae are indeed capable of learning the kinds of associations underlying such learned 'action'. In the following, I will therefore explain the basics of the gustatory and olfactory systems and take a look at the associative memory centre of *Drosophila* larvae.

The gustatory and olfactory system

The chemosensory system of larval *Drosophila* consists per body side of three external sense organs on the head and four internal sense organs near the pharynx (Figure 3A). Each of the external organs – dorsal (DO), terminal (TO) and ventral organ (VO) – and internal organs – dorsal (DPS), ventral (VPS), posterior pharyngeal sense organ (PPS) and the dorsal pharyngeal organ (DPO) – compromise several sensilla, which in turn contain several sensory neurons (Gendre et al., 2004; Python and Stocker, 2002a). Of these 119 sensory neurons, for half of them it is known that they serve olfaction, taste, mechanosensorics or thermosensorics whereas for the rest, their function is so far unclear (for review see: Apostolopoulou et al., 2015; Gerber and Stocker, 2007; Vosshall and Stocker, 2007). All cell bodies of the sensory neurons of the external organs are located in the ganglion below the respective sensory organ (DOG, TOG, VOG), except for the TO, where some cell bodies are also located in the DOG. From there and from the internal organs, sensory information is transmitted to the brain via the antennal nerve (from DOG), maxillary nerve (from TOG and VOG), labral nerve (from DPS, DPO and PPS) and labial nerve (from VPS).

Gustatory receptor neurons (GRNs) were found to respond to sugar, bitter substances, CO2, water or different concentrations of salt by expressing different G protein-coupled receptors of the *Gr* family, ionotropic glutamate receptors of the *Ir* family, sodium channels of the DEG/ENaC pickpocket receptor gene (*PPK*) family or transient receptor potential (TRP) cation channels (Benton et al., 2009; Clyne et al., 2000; Liu et al., 2003, 2007; reviewed in: Apostolopoulou et al., 2015). Finally, via the maxillary, labral and labial nerve, all information from the GRNs is transmitted to multiple compartments of the suboesophageal zone (SEG) – the primary taste centre in larval *Drosophila* (Colomb et al., 2007) – from where connections exist to pre-motor centres to trigger innate gustatory behaviour and to the MB to modulate

Table 3. Similarities between invertebrates and vertebrates.

Invertebrates (insects)	Vertebrates (mammals)	References
Anatomical similarities		
Transmission of olfactory information		Reviewed in: Ache and Young, 2005; Hildebrand and Shepherd, 1997; Kay and Stopfer, 2006; Strausfeld and Hildebrand, 1999
ORN → Glomeruli (LH) → PNs	Granule/periglomerular cells → mitral/tufted cells	
Processing of olfactory information		
Antennal lobe	Olfactory bulb	
MB as cerebellum like structure: three-layer circuit of input, expansion and output		Reviewed in: Farris, 2011
PNs, KCS, MBONs	Mossy fibres, granule cells, Purkinje cells	
Functional similarities		
Modulation of olfactory behaviour: innate olfactory behaviour *vs* olfactory memory		Vertebrates: Parnas et al., 2013; Root et al., 2014; Sacco and Sacchetti, 2010; Reviewed for *Drosophila*: Schultzhaus et al., 2017; Vosshall and Stocker, 2007
Lateral horn *vs* MB calyx	Amygdala *vs* piriform cortex	
Gustatory receptor neurons are tuned to classify different taste substances as either attractive or aversive		Reviewed in: Yarmolinsky et al., 2009
Functional segregation of DANs: reward *vs* aversion		Reviewed in: Bromberg-Martin et al., 2010; Scaplen and Kaun, 2016; Schultz, 2015; Verharen et al., 2020
DANs innervating the medial lobe *vs* DANs innervating the vertical lobe	Ventral part striatum (nucleus accumbens) *vs* tail of striatum	
Modulation of LTP and LTP in DANs		Huang et al., 2004; Matsuda et al., 2006; Sheynikhovich et al., 2013; Waddell, 2013
The role of dopamine in reinforcement learning		Schultz, 2015, 2016; Waddell, 2013
Reward associative learning		Heffley et al., 2018; Ohmae and Medina, 2015; Wagner et al., 2017; Reviewed in: Caligiore et al., 2017; Cerminara et al., 2015; Van Damme et al., 2021; Schmahmann et al., 2019; Waddell, 2013
MB	Cerebellum	

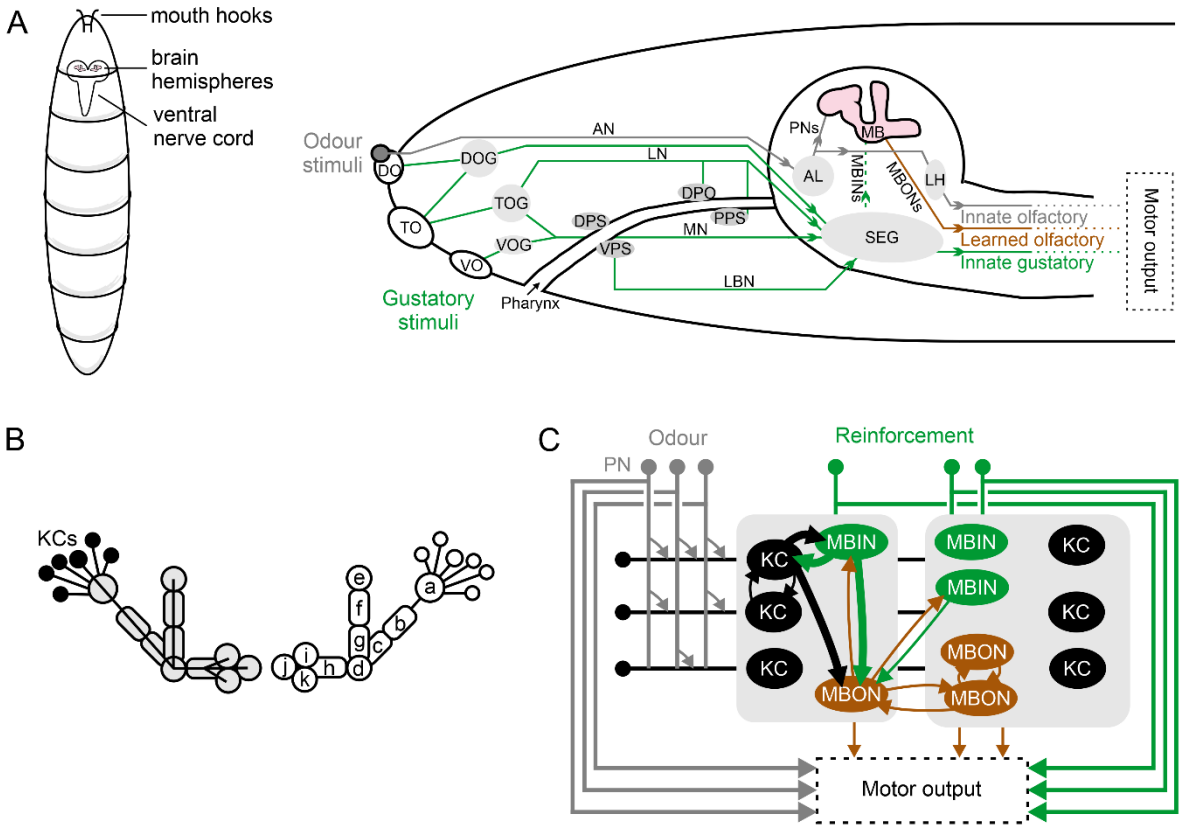

Figure 3. Anatomy of larval *Drosophila*. (A) The olfactory and gustatory pathways in *Drosophila* larva. The olfactory pathway (grey) includes the dorsal organ (DO) and dorsal organ ganglion (DOG) with projection to the antennal lobe (AL) via the antennal nerve (AN) toward the mushroom body (MB; pink) and the lateral horn (LH) via projection neurons (PN; grey). Gustatory pathways (green) include all outer and inner chemosensory organs and ganglions (DO/DOG; TO/TOG; VO/VOG) as well as the dorsal (DPS), ventral (VPS) and posterior (PPS) pharyngeal sensillae and the dorsal pharyngeal organ (DPO). Gustatory afferents are collected in regions of the suboesophageal ganglion (SEG). Taste driven signals are transferred from the SEG towards the MB via an unknown number of synaptic steps to the mushroom body input neurons (MBINS). In the MB gustatory and olfactory signals converge and can be associated to form a memory. The mushroom body output neurons (MBONs) drive learned olfactory behaviour (brown). The LH and SEG drive innate olfactory and gustatory behaviour, respectively. (B) Schematic of the compartmental organization of the larval MB. (C) Simplified illustration of the neural circuit within and between two exemplified compartments of the MB. Thicker lines demonstrate the universal circuitry between KC, MBIN and MBON that exists in every compartment. Thinner lines demonstrate other possible connections found in electron microscope data. Abbreviations: AL, antennal lobe; AN, antennal nerve; DO/DOG, dorsal organ/dorsal organ ganglion; DPO, dorsal pharyngeal organ; DPS, dorsal pharyngeal sensillae; KC, Kenyon cell; LBN, labial nerve; LH, lateral horn; LN, labral nerve; MB, Mushroom body; MBIN, Mushroom body input neurons; MBON, Mushroom body output neurons; MN, maxillary nerve; PNs, projection neurons; PPS, posterior pharyngeal sensillae; SEG, suboesophageal ganglion; TO/TOG, terminal organ/terminal organ ganglion; VO/VOG, ventral organ/ventral organ ganglion; VPS, ventral pharyngeal sensillae. Panel A adapted from Ramaekers et al., 2005, panel B and C from Eichler et al., 2017 and Saumweber et al., 2018.

learned behaviour (Apostolopoulou et al., 2015; Colomb et al., 2007; Gendre et al., 2004; Kwon et al., 2011; Masek and Keene, 2016; Melcher and Pankratz, 2005; Miroschnikow et al., 2018, 2020; Python and Stocker, 2002a; Selcho et al., 2009; Singh and Singh, 1984).

The olfactory system consists of much fewer neurons and is much better understood than the gustatory system (Berck et al., 2016; Gerber and Stocker, 2007). 21 cholinergic olfactory receptor neurons (ORNs) are solely located in the so-called dome of the DO that is the 'nose' of the larva (Fishilevich et al., 2005). Most of the ORNs express one type of 25 different olfactory receptors (ORs) that form the basis for combinatorial activation of ORNs, allowing animals to discriminate a large number of different odours (Clyne et al., 1999; Fishilevich et al., 2005). Additionally, each larval ORN (except the CO_2-sensitive neurons) expresses the co-receptor ORCO (Vosshall and Stocker, 2007). 14 of the larval ORs are larval specific whereas 11 are also expressed in adults (Fishilevich et al., 2005). The ORNs project via the antennal nerve to one of the 21 glomeruli within the antennal lobe (AL) (Berck et al., 2016; Ramaekers et al., 2005; Thum et al., 2011). For 22 ORNs it was found that each of them target a different glomerulus with a one ORN to one glomerulus rule (Fishilevich et al., 2005; Kreher et al., 2005). The glomeruli itself are connected laterally by 14 mostly GABAergic but also glutamatergic local interneurons (LNs), which allow to extract behaviourally relevant information from incoming signals by providing lateral inhibition between other LNs, ORNs and projection neurons (PNs) (Berck et al., 2016; Vosshall and Stocker, 2007). The 21 cholinergic PNs connect the AL to higher order olfactory centres: the MB calyx and lateral horn (Marin et al., 2005; Python and Stocker, 2002a, 2002b; Thum et al., 2011). While the lateral horn displays predominantly the direct pathway to the motor system and is associated with innate behaviour (Schultzhaus et al., 2017), the MB displays predominantly a detour to the motor system and is associated with learned olfactory behaviour (Heisenberg, 2003; Keene and Waddell, 2007; McGuire et al., 2005).

The mushroom body

The essential structure for associative learning in the *Drosophila* brain is the so-called mushroom body (MB) (de Belle and Heisenberg, 1994; Diegelmann et al., 2013b; Heisenberg, 2003; Hige, 2018; Menzel, 2012; Owald and Waddell, 2015; Pauls et al., 2010b; Rohwedder et al., 2016; Waddell, 2013). It is a paired anatomical structure in the central brain present in various invertebrate species (reviewed in: Fahrbach, 2006; Strausfeld et al., 1998). During the

last five years, the chemical-synapse connectome of this associative memory centre has been reconstructed in an L1 larva and transgenic driver strains labelling several or even single neurons were tested to analyse their behavioural relevance (Eichler et al., 2017; Eschbach et al., 2020, 2021; Saumweber et al., 2018). This revealed that the neuronal circuits underlying associative learning in larvae largely parallel those of adult *Drosophila* and other insects (reviews with focus on larvae: Eschbach and Zlatic, 2020; Gerber and Stocker, 2007; Thum and Gerber, 2019; Widmann et al., 2018; with focus on adult flies: Cognigni et al., 2018; Li et al., 2020; McGuire et al., 2005; Takemura et al., 2017).

PNs, encoding predominantly olfactory information but also thermal, gustatory and visual information (Berck et al., 2016; Eichler et al., 2017; Masuda-Nakagawa et al., 2005), innervate the calyx of the MB. The calyx is organized into 34 glomeruli where typically one PN is innervating only one glomerulus (Masuda-Nakagawa et al., 2005; Ramaekers et al., 2005) featuring the dendrites of the MB intrinsic neurons: the Kenyon cells (KC). These integrate input from one up to six mostly randomly selected sensory PNs and thus display a sparse, combinatorial representation of the sensory environment of the larvae (Eichler et al., 2017; Gerber and Stocker, 2007). By parallel axons, the cholinergic KCs (Barnstedt et al., 2016) form the peduncle and two lobes (Diegelmann et al., 2013b; Eichler et al., 2017; Saumweber et al., 2018) along which modulatory MB input neurons (MBINs) that mediate reinforcement information synapse onto the KCs (Diegelmann et al., 2013b; Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018). The KCs in turn synapse onto MB output neurons (MBONs) that forward towards the motor system and thus modulate behaviour (Eschbach et al., 2021; Saumweber et al., 2018).

MBINs and MBONs form local circuits with the KCs which defines separated compartments (named from a to k) with accordingly named MBINs and MBONs (see Figure 3B; Eichler et al., 2017; Saumweber et al., 2018; Schleyer et al., 2020). Each compartment is innervated by one to three MBINs and one to five MBONs. Most of the MBINs and MBONs exist paired with one cell per hemisphere whereas only some can be found unpaired with cell bodies located in the midline of the brain and some are present as double pairs with two cells per hemisphere. The majority of the MBINs and MBONs innervate only a single compartment per hemisphere, however, almost all MBINs and about half of the MBONs provide interhemispheric crosstalk. Overall, within a compartment the KCs integrate input from one to three MBINs and synapse onto one to three MBONs (Eichler et al., 2017).

The electron microscopic analysis of a L1 larvae by Eichler and colleagues (2017) revealed a much more complex larval brain than the former anatomical preparations suggested (reviewed in: Heisenberg, 2003). The data revealed a universal circuitry within a compartment consisting of the already mentioned PN-to-KC, MBIN-to-KC and KC-to-MBON connections, plus the newly discovered KC-to-MBIN and MBIN-to-MBON connections (Figure 3C). In addition, connections were discovered that do not exist in every compartment:

(1) Connections from MBONs to MBINs of the same compartment or of different compartments
(2) Connections from MBINs to MBONS of different compartments
(3) Reciprocal connections between KCs
(4) 'Lateral' connections between MBONs of the same compartment or of different compartments

Combining the electron microscopy data of an L1 larva (Eichler et al., 2017) and the flip-out data of L3 larvae (Saumweber et al., 2018), revealed 16 MBINs in L1. Only 15 of these MBINs can also be found in L3 (not MBIN-e1) plus three additional MBINs exclusively found in L3 that apparently are not yet mature in L1 (MBIN-c1, DAN-h1 and MBIN-m1). These MBINs convey US information onto the KCs using different neurotransmitters (Diegelmann et al., 2013b; Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018): the majority of eight have been shown to be dopaminergic (DAN-c1, -d1, -f1 -g1, -h1, -i1, -j1, -k1), 4 to be octopaminergic (OAN-a1, -a2, e1, -g1) and six are of so far unknown identity (MBIN-b1, -b2, -e1, -e2, -l1, -m1) (Eichler et al., 2017; Pauls et al., 2010b; Rohwedder et al., 2016; Saumweber et al., 2018; Selcho et al., 2014).

From the 24 MBONs existing in L1, 22 were also found in L3 (not MBON-o1 and MBON-q1) plus 3 additional MBONs exclusively found in L3 (MBON-c2, MBON-f2 and MBON-j2). MBONs convey output towards the motor system and cause modulations of parameters of locomotion towards approach or avoidance (Eschbach et al., 2021; Paisios et al., 2017; Schleyer et al., 2020; Thane et al., 2019). In fact, of the 25 MBONs in L3, eight have to been shown to be approach promoting (MBON-a1, -b1, -b2, -d1, e1, -g1, -g2, -m1) and six to be avoidance promoting (MBON-a2, -e2, -h1, -h2, -i1, k1) (Eschbach et al., 2021). Also MBONs use different neurotransmitters: four are cholinergic (MBON-a1, -a2, -c1, -e1), six are GABAergic (MBON-b1, -b2, -d1, -g1, -g2, -m1), five are glutamatergic (MBON-e2, -i1, -j1, -j2, -k1), two are both GABAergic and glutamatergic (MBON-h1, -h2), one is dopaminergic (MBON-c2) and nine are

of so far unknown identity (MBON-b3, -d2, -d3, -f1, -f2, -n1, -o1, -p1, -q1) (Eichler et al., 2017; Pauls et al., 2010b; Saumweber et al., 2018; Selcho et al., 2009; Slater et al., 2015).

Another hemispherically single neuron, the GABAergic anterior paired lateral neuron (APL) receives excitatory input from the cholinergic KCs in the calyx and six of ten compartments whereas it gives back inhibitory output only to the KCs in the calyx, and is thought to exert sparsening the sensory representation of the KCs within the MB (larvae: Eschbach et al., 2021; Mancini et al., 2023; Masuda-Nakagawa et al., 2005; Saumweber et al., 2018) (adults: Amin et al., 2020; Lin et al., 2014).

The knowledge of the complete connectome and available transgenic manipulations (see previous chapter: 'A method of genetic manipulation: The Gal4-UAS system') enabled to use optogenetic activation or inhibition of MBINs or MBONs to investigate their behavioural relevance. In fact, direct activation of DANs was shown to substitute for USs to induce learning in larval and adult *Drosophila* (larvae: Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006) (adults: Aso and Rubin, 2016; Aso et al., 2010; Claridge-Chang et al., 2009; König et al., 2019; Liu et al., 2012). In larvae, two out of the eight DANs in L3 have been shown to mediate rewarding effects (DAN-h1, -i1) and three to mediate punishing effects (DAN-d1, -f1, -g1) (Eschbach et al., 2020; Saumweber et al., 2018). Interestingly, this functional bipartition of the DANs is also mapped on a spatial level within the MB: rewarding effects are mediated by DANs innervating the medial lobe, whereas punishing effects are mediated by DANs innervating the vertical lobe and lateral appendix (Eichler et al., 2017; Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006; Selcho et al., 2009). These dual roles and spatial segregation of DAN target regions is similar to the situation in mammals and may reflect a principle across species (Adel and Griffith, 2021; Groessl et al., 2018; de Jong et al., 2019; Menegas et al., 2018; Waddell, 2013; for more details see Table 3). Together with the possibility for transgenic manipulation of the fly genome and the applicable learning paradigms, this known connectome enables to investigate and understand basic principles of learning and memory in detail.

Behind the scenes: Molecular mechanisms of learning

Being aware of the connectome obviously is not sufficient to explain how learning comes about within the network, as one also needs to have a look 'behind the scenes': what happens inside

the network or neuron? The essential mechanism for learning and memory preserved throughout the animal kingdom is synaptic plasticity (reviewed in: Davis, 2005; Dudai, 2004; Kandel et al., 2014). As explained before, in *Drosophila* activity in a specific subset of KCs within the MB codes for the odour and additionally within a given compartment of the MB, KCs receive information about the US. If both these pieces of information coincide within a given compartment, the synapse from the KC to the respective MBON gets modified, that is, the synaptic strength is altered (larvae: Eschbach et al., 2020, 2021) (adult: Hancock et al., 2019; Hige et al., 2015a; Owald and Waddell, 2015). In the naïve animal, the strength of KC drive to approach promoting MBONs and aversive promoting MBONs is thought to be balanced such that the net output of the MB is neutral and only naïve behaviour is expressed along the lateral horn pathway. However, after learning, this balance of approach and aversion is changed due to the changed synaptic strength in the connection from the odour-coding KCs to the respective MBON. In reward learning for example, the synapse from the KC to an avoidance promoting MBON is weakened. When in the test the odour is encountered again, the KC drive to the avoidance promoting MBON is less, while the KC drive to the approach promoting MBON remains unchanged. As net result approach tendencies prevail and the animal approaches the odour.

Whereas odour information from the PNs is signalled via calcium influx which in turn activates Ca^{2+}/calmodulin-dependent protein kinase, the US signal from the MBINs is transferred via G-protein-coupled receptors (GPCRs) and leads to the dissociation of the G-protein subunits (Levin et al., 1992; Livingstone et al., 1984; Widmann et al., 2018). When both signals coincidence within a KC, the adenylyl cyclase (AC) is super-additively activated, which in turn stimulates the production of the second messenger cyclic adenosine monophosphate (cAMP) (Dudai et al., 1976, 1988). The cAMP activates protein kinase A (PKA), which in turn leads to phosphorylation of multiple downstream proteins what in consequence induces cellular or synaptic plasticity (Gervasi et al., 2010; for a more detailed explanation see: Thum and Gerber, 2019; Widmann et al., 2018).

Studies showed that interference with steps of this cascade or with other genes involved in brain development led to memory deficits in larval and adult *Drosophila* (Tumkaya et al., 2018; Widmann et al., 2016, 2018). A meta-analysis of experiments in adult *Drosophila* investigated 32 genes that have so far been shown as relevant for short term memory formation using odour shock learning (Tumkaya et al., 2018). Genes that are encoding phosphorylation factors (for

example pka-R1, sra), molecules with intracellular-signalling function (rut, dnc, syn, raf, CREB) or extracellular-signalling function (scb, amn) had an impact on memory formation (reviewed in: Tumkaya et al., 2018). The most severe and reliable impact on short term memory formation in adult *Drosophila* were shown in mutants for the *dunce* gene (dnc; encodes a cAMP-specific phosphodiesterase and thus is responsible for cAMP degradation; Dudai et al., 1976) and *rutabaga* gene (rut; encodes the mentioned AC; Livingstone et al., 1984), with reduced short-term memory by 67% to 57%. Furthermore, in larval and adult *Drosophila*, mutation of the *synapsin* gene (syn; Klagges et al., 1996) which encodes a phosphoprotein that is involved in the regulation of neurotransmitter release specifically in the KCs led to memory deficits (larvae: Diegelmann et al., 2013b; Michels et al., 2005; Niewalda et al., 2015) (adults: (Denker et al., 2011; Godenschwege et al., 2004; Knapek et al., 2010).

But not only genes involved in the AC-PKA-cAMP-cascade are important for learning to happen, but also the neurotransmitters which are necessary for representing the reinforcement information by binding to GPCRs were shown to have an impact on memory formation, first and foremost neurotransmitter of the biogenic amines class.

Biogenic amines

Dopamine, serotonin and octopamine are the biogenic amines in the nervous system of insects which have been studied in *Drosophila* in relatively much detail (reviewed in: Monastirioti, 1999). While dopamine and serotonin are also present in the vertebrate brain, octopamine represents the structural and functional equivalent to the vertebrate norepinephrine (reviewed in: Roeder et al., 2003). Each neurotransmitter is present in a specific pattern in a small number of neurons that are widely distributed in the larval (and adult) central nervous system (CNS).

From all the OANs within the larval CNS, four of them innervate the MB (Selcho et al., 2014). From the four different octopamine receptors existing within the larval CNS (Oamb, Oct1ßR, Oct2ßR, Oct3ßR) only Oamb and Oct3ßR are also present within the MB KCs (El-Kholy et al., 2015). In any case, octopamine signalling was identified to be involved in appetitive learning (Honjo and Furukubo-Tokunaga, 2009; Schroll et al., 2006; Selcho et al., 2014), odour sensing/discrimination (Wong et al., 2021) and locomotion in larval *Drosophila* (Saraswati et al., 2004; Selcho et al., 2012). In adult *Drosophila*, with clusters of OANs distributed over the brain and all octopamine receptors expressed within the MB KCs (Busch et al., 2009; El-Kholy et al., 2015), octopamine signalling was identified to regulate memory

formation, ovulation, courtship behaviour and sleep regulation (Crocker and Sehgal, 2008; Kim et al., 2013; Monastirioti, 2003; Zhou et al., 2012).

Serotonergic cells, also present within the whole larval CNS, only weakly innervate the MB calyx but not the MB lobes (Monastirioti, 1999; Vallés and White, 1988). Thus, not surprisingly, in larvae serotonergic cells were shown to be not necessary for associative olfactory learning and memory using fructose and electric shock as positive and negative reinforcers, respectively (Huser et al., 2012). However, specifically the function of the 5-HT_{2A} receptor influences only odour-salt learning but not electric shock learning (Huser et al., 2017) and impaired serotonin receptor and neuron function influences locomotion in larvae (Rodriguez Moncalvo and Campos, 2009; Silva et al., 2014). Interestingly for the other receptors also expressed in the larval brain (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2B} and 5-HT_{7}) of which only 5-HT_{1B} is expressed in a few KCs (Huser et al., 2017; Saudou et al., 1992) data is inconclusive so far or suggest that they are dispensable for olfactory learning in larvae (Huser et al., 2017). In adult *Drosophila* however, with serotonergic neurons spread over various clusters within the whole CNS where some are innervating the MB lobes and only the 5-HT_{1A} and 5-HT_{1B} receptors are expressed in the MB (Nichols, 2007; Saudou et al., 1992; reviewed in: Blenau and Thamm, 2011), serotonin signalling was identified to influence a number of behaviours including sleep, feeding, courtship and memory formation (Haynes et al., 2015; Luo et al., 2012; Pooryasin and Fiala, 2015; Yuan et al., 2005; reviewed in: Kasture et al., 2018).

Dopamine is the most talked about neurotransmitter during at least the last 10 years with over 5000 publications per year (source: PubMed). DANs were identified to play a key role in reinforcement processing during associative olfactory learning in *Drosophila* (larvae: Honjo and Furukubo-Tokunaga, 2009; Rohwedder et al., 2016; Selcho et al., 2009) (adults: Berry et al., 2012; Riemensperger et al., 2011; Schwaerzel et al., 2003) (reviewed in: Adel and Griffith, 2021) and across the animal kingdom (Schultz, 2015; Waddell, 2013). In case of larval *Drosophila*, of the so far tested MBINs in L3, eight are dopaminergic and transfer the reinforcing properties of stimuli. Activation of these neurons were shown to substitute for real rewards and thus can mediate reward or punishment signals (larvae: Eichler et al., 2017; Eschbach et al., 2020; Rohwedder et al., 2016; Saumweber et al., 2018; Schleyer et al., 2020; Schroll et al., 2006) (adults: Aso and Rubin, 2016; König et al., 2019). *Drosophila* possesses four different dopamine receptors: Dop1R1 and Dop1R2 (related to the vertebrate D1-like receptors; Feng et al., 1996; Gotzes et al., 1994; Han et al., 1996; Sugamori et al., 1995), Dop2R (related to the vertebrate D2-

like receptors; Hearn et al., 2002; Vickrey and Venton, 2011) and DopEcR (more related to vertebrate beta-adrenergic receptors; Srivastava et al., 2005). In larval *Drosophila*, only two, the Dop1R1 and Dop1R2 are expressed in the MB (Feng et al., 1996; Gotzes et al., 1994; Han et al., 1996; Sugamori et al., 1995). In adult *Drosophila*, however, all four receptors are expressed in the MB, with Dop1R1 mostly expressed in KCs and DANs, Dop1R2 almost exclusively expressed in KCs, Dop2R mostly expressed in DANs and KCs and DopEcR expressed all over the MB (Aso et al., 2019). In larval *Drosophila*, Dop1R1 but not the others were shown to modulate locomotion (Silva et al., 2020) whereas Dop1R2 mutants showed impaired aversive olfactory memory (Selcho et al., 2009). Flies lacking the Dop1R1 were defective in both aversive and appetitive learning (Kim et al., 2007; Qin et al., 2012). As in the *Drosophila* larva, DANs in adults are organized into two clusters in the CNS that innervate the MB, and activation of these DANs demonstrated to mediate formation of aversive and appetitive memories, respectively (Aso et al., 2012; Burke et al., 2012; Claridge-Chang et al., 2009; Kasture et al., 2018; Liu et al., 2012; Riemensperger et al., 2011). Interestingly, dopamine does not only play a role in memory formation but also has a role in active forgetting and memory retrieval (larvae: Schleyer et al., 2020) (adults: Berry et al., 2012, 2018; Himmelreich et al., 2017).

Overall, in *Drosophila* the biogenic amines were identified to modulate several behaviours like memory formation and forgetting, ovulation, courtship behaviour, aggression, sleep and feeding regulation.

What this thesis is specifically about

The scientific background painted so far showed that different timing of CS and US lead to two different types of memories, more specifically memories of opposite valence. In Chapter I, I provide a comprehensive analysis of timing-dependent valence reversal in the appetitive domain that complements previous research. Using *Drosophila* larvae already Saumweber and colleagues (2018) showed, that depending on when the optogenetic activation of a reward mediating DAN (DAN-i1^{864}) starts with respect to an odour presentation one can observe reward memory when the odour is associated with the occurrence of DAN activation and frustration memory when the odour is associated with the termination of the DAN activation. Based on these results, I present the first detailed characterization of the dynamics of reward and frustration memory and show that these memories have different dynamics. These results

are compared to the published parametric characterization of punishment and relief memory mediated by timed DAN-f1^{2180} activation, a punishment mediating DAN in larval *Drosophila*.

Because dopamine has been shown to be play an important role in reinforcement learning, in Chapter II, I present a pharmacological method to acutely impair dopamine synthesis in larval *Drosophila* that can be used to study the role of dopamine in reinforcement learning, as an alternative method to genetic manipulations. Based on developmental studies in larval *Drosophila* and learning studies in adult *Drosophila*, I developed a method of feeding the dopamine synthesis inhibitor 3-Iodo-L-tyrosine to *Drosophila* larvae. I investigate the effect in a classical conditioning paradigm using an odour as CS and fructose as a rewarding US in larval *Drosophila,* and using two different odours and optogenetic activation of a punishment mediating DAN in adult *Drosophila*. Furthermore, I check for effects on task-related locomotor faculties and innate behaviour.

Chapter I

Optogenetically induced reward and 'frustration' memory in larval *Drosophila melanogaster*

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Introduction

Pleasurable events induce positive affect when they occur, and negative affect when they terminate. Accordingly, humans and other animals alike form appetitive memory for stimuli that are associated with the occurrence of reward ('reward' memory), and aversive memory for stimuli associated with its termination ('frustration' memory) (Hellstern et al., 1998; Solomon and Corbit, 1974) (the same is observed for painful events, with inverted signs: Gerber et al., 2014; Solomon and Corbit, 1974) (see also Figure 1). Although the mechanisms of reward memory are understood in considerable detail (Schultz, 2015; Waddell, 2013), much less is known about frustration memory (Felsenberg et al., 2013; Hellstern et al., 1998). Such an incomplete picture of how pleasurable events are processed may lead both computational modelling of neural networks and the understanding of related pathology astray. In this context, we provide the first characterization of the learning from the occurrence and termination of a central-brain reward signal.

For such an endeavour, larval *Drosophila melanogaster* are an attractive study case. Robust paradigms of associative learning and resources for cell-specific transgene expression are

available (Eschbach et al., 2020; Li et al., 2014; Saumweber et al., 2018). Moreover, their central nervous system is compact and consists of only approximately 10,000 neurons (Bossing et al., 1996; Larsen et al., 2009). This has allowed for the reconstruction of its chemical-synapse connectome, including the mushroom bodies as the associative memory centre of insects (Eichler et al., 2017). It has turned out that the circuits and mechanisms underlying associative learning in larvae largely parallel those of adults (Eschbach and Zlatic, 2020; Thum and Gerber, 2019; adult flies: Li et al., 2020; Takemura et al., 2017). Sensory projection neurons establish a sparse, combinatorial representation of the sensory environment across the mushroom body Kenyon cells (KCs). The KC axons are intersected by mostly dopaminergic modulatory neurons (DANs) and by mushroom body output neurons (MBONs), which eventually connect towards the motor system to adapt the parameters of locomotion (Eschbach et al., 2021; Paisios et al., 2017; Schleyer et al., 2020; Thane et al., 2019). Axonal regions of DANs and MBON dendritic regions are organized as compartments in which matched-up individual DANs and MBONs form local circuits with the KCs (Figure 4A). Of the eight DANs innervating the mushroom body, two mediate rewarding effects and three mediate punishing effects (Eschbach et al., 2020; Saumweber et al., 2018). When an odour is encountered, and a reward or punishment signal reaches a given compartment, the strength of the local synapses from the odour-activated KCs to the MBON(s) of that same compartment is modified. As a result, the animal's behaviour towards the odour in question is changed when the odour is encountered again.

Given this separation of reward and punishment signalling at the level of the DANs, it was striking to observe that in both larval and adult *Drosophila melanogaster*, individual DANs can confer timing-dependent valence reversal. In the case of larvae, presentation of an odour followed by optogenetic activation of the DAN-i1 neuron (as covered by the SS00864-Gal4 strain, henceforth DAN-i1^{864}) (forward training) leads to appetitive reward memory, whereas presentation of the odour upon termination of DAN-i1^{864} activation (backward training) establishes aversive frustration memory (Saumweber et al., 2018) (adults: Handler et al., 2019). Whether the mechanisms of frustration memory established by termination of DAN-i1^{864} activation differ from those of the likewise aversive memory established by unpaired presentations of odour and DAN-i1^{864} activation (Schleyer et al., 2018, 2020) remains to be tested. The DAN-f1 neuron, in contrast, supports aversive punishment memory upon forward training and appetitive 'relief' memory upon backward training (Weiglein et al., 2021; using

SS02180-Gal4 as the driver, henceforth DAN-f1^{2180}) (adults: Aso and Rubin, 2016; Handler et al., 2019; König et al., 2018). For DAN-f1^{2180} Weiglein et al. (2021) have characterized punishment and relief memories in some detail; however, no further analysis has been performed for the reward and frustration memories established by DAN-i1^{864} activation. Here, we undertake such an analysis to provide a more complete picture of the timing-dependent valence reversal conferred by dopaminergic neurons.

Material and Methods

Animals

Experiments were performed on 3rd instar foraging larvae of *Drosophila melanogaster* raised on standard food at 25 °C, 60-70 % relative humidity, in a room running on a 12 h light/dark cycle but in vials wrapped with black cardboard to keep them in darkness.

We used the split-Gal4 driver strain SS00864 (HHMI Janelia Research Campus, Ashburn VA, USA; Saumweber et al., 2018), supporting strong and reliable transgene expression in DAN-i1 of both hemispheres, plus expression from a few additional cells that is stochastic between hemispheres and preparations (Saumweber et al., 2018; Schleyer et al., 2020; Weiglein et al., 2019). As argued before, such stochastic expression is unlikely to cause systematic effects in the behavioural mass assays employed in the present study (Saumweber et al., 2018; Schleyer et al., 2020). We refer to this driver strain and the covered neurons as DAN-i1^{864}.

For optogenetic activation, offspring of the UAS-ChR2-XXL effector strain (Bloomington Stock Centre no. 58374; Dawydow et al., 2014) crossed to DAN-i1^{864} were used. The driver control resulted from DAN-i1^{864} crossed to w^{1118} (Bloomington Stock Centre nos. 3605, 5905, 6326), and the effector control from UAS-CHR2-XXL crossed to a strain carrying both split-Gal4 landing sites (attP40/attP2) but without Gal4 domains inserted ("empty") (HHMI Janelia Research Campus, USA; Pfeiffer et al., 2010).

Odour-DAN associative learning

Procedures followed Saumweber et al. (2018) and Weiglein et al. (2021). Variations in the following procedures will be mentioned in the Results section.

Experiments were performed in a custom-made setup, consisting of a wooden box equipped with a light table featuring 24 x 12 LEDs (peak wavelength 470 nm; Solarox, Dessau-Roßlau, Germany) and a 6 mm thick diffusion plate of frosted acrylic glass on top to ensure

uniform blue light for ChR2-XXL activation (120 µW/cm²). On top of the diffusion plate, Petri dishes were placed into a polyethylene diffusion ring illuminated by 30 infrared LEDs (850 nm; Solarox, Dessau-Roßlau, Germany). For video recording a camera (Basler acA204090umNIR; Basler, Ahrensburg, 196 Germany) equipped with an infrared-pass filter was placed approximately 25 cm above the Petri dish. The Petri dishes were filled with 1% agarose solution (electrophoresis grade; CAS: 9012-36-6, Roth, Karlsruhe, Germany) as the substrate on which cohorts of approximately 30 larvae were free to move once transferred from their food vials.

During training, one set of larvae was presented with an odour paired with optogenetic activation of DAN-i1^{864} at the mentioned inter-stimulus-interval (ISI), whereas a second set of larvae received the odour and the DAN-i1^{864} activation in an unpaired manner (Figure S1). Two different ISIs were used: -10 s for forward training (the 30-s odour presentation preceded the 30-s DAN-i1^{864} activation by 10 s) and 30 s for backward training (the odour presentation occurred 30 s after DAN-i1^{864} activation had started) (Figure S1).

For the presentation of the odour (*n*-amylacetate, AM; CAS: 628-63-7, Merck, Darmstadt, Germany, diluted 1:20 in paraffin oil; CAS: 8042-47-5, AppliChem, Darmstadt, Germany) or of paraffin oil as the solvent control (S), we used Petri dish lids equipped with four sticky filter papers onto which 5 µl of either substance could be applied. Paraffin oil does not have behavioural significance as an odour (Saumweber et al., 2011).

After three training trials, the larvae were placed in the middle of a fresh test Petri dish and given the choice between AM and S on opposite sides. After 3 min, the number of animals (#) on either side and in a 1-cm wide middle stripe was determined and the preference for AM (Odour$_{AM}$ PREF) was calculated as:

$$Odour_{AM}PREF = \frac{\#AM - \#S}{\#Total} \quad (1)$$

Thus, values can range between 1 and -1 with positive values indicating attraction and negative values aversion to AM.

From paired-trained versus unpaired-trained sets of larvae a Memory score was calculated as:

$$Memory\ score = \frac{Odour_{AM}PREF_{Paired} - Odour_{AM}PREF_{Unpaired}}{2} \quad (2)$$

Thus, Memory scores range between 1 and -1, with positive values indicating appetitive associative memory and negative values aversive associative memory. Each sample (N = 1) thus reflects the behaviour of two cohorts of approximately 30 larvae, one paired-trained and the other unpaired-trained.

Video-tracking of locomotion

During the test, larval behaviour was video-recorded and analysed offline (Paisios et al., 2017). The typical zig-zagging larval behaviour was classified as either a head cast (HC) or a run, and was characterized by the HC rate, the change in orientation that results from a HC, and the run speed. All measurements are presented combined for around 30 larvae on a given Petri dish as one sample (N= 1).

The HC rate-modulation was defined as:

$$HC\ rate\text{-}modulation = \frac{HC/s\ (heading\ away) - HC/s\ (heading\ towards)}{HC/s\ \ (heading\ away) + HC/s\ (heading\ towards)} \quad (3)$$

Positive scores indicate that larvae carry out more HCs per second (HC/s) while heading away from the odour than while heading towards it, and thus indicate attraction. In contrast, negative scores indicate aversion.

To calculate the difference in the HC rate-modulation of animals after paired versus unpaired training, we calculated the ΔHC rate-modulation as:

$$\Delta HC\ rate\text{-}modulation = HC\ rate\text{-}modulation_{Paired}\ -\ HC\ rate\text{-}modulation_{Unpaired} \quad (4)$$

The Reorientation per HC was defined as:

$$Reorientation\ per\ HC = abs(heading\ angle\ before\ HC) - abs(heading\ angle\ after\ HC) \quad (5)$$

The absolute heading angle was defined as 0° when the animal's head was pointing towards the odour and 180° when pointing away from it. HCs reorienting the animals towards the odour thus reduce this absolute heading angle and yield positive scores indicative of attraction whereas HCs reorienting the animal away from the odour result in negative scores indicative of aversion.

To calculate the difference in reorientation of animals after paired versus unpaired training, we calculated the ΔReorientation per HC as:

$$\Delta Reorientation\ per\ HC = Reorientation\ per\ HC_{Paired} - Reorientation\ per\ HC_{Unpaired} \quad (6)$$

Run speed-modulation was defined as:

$$Run\ speed\text{-}modulation = \frac{Run\ speed\ (heading\ towards) - Run\ speed\ (heading\ away)}{Run\ speed\ \ (heading\ towards) + Run\ speed\ (heading\ away)} \quad (7)$$

Thus, positive scores result if animals run faster when heading toward the odour than when heading away, indicating attraction, whereas negative scores imply aversion.

To calculate the difference in Run speed-modulation of animals after paired versus unpaired training, we calculated the ΔRun speed-modulation as:

$$\Delta Run\ speed\text{-}modulation = Run\ speed\text{-}modulation_{\ Paired} - Run\ speed\text{-}modulation_{\ Unpaired} \quad (8)$$

In all three cases, positive Δ scores would thus indicate appetitive memory. In turn, negative Δ scores would indicate aversive memory.

Statistics

Non-parametric statistics were performed throughout (Statistica 13, RRID:SCR_014213, StatSoft Inc., Tulsa, OK, USA). For comparisons with chance level (zero), one-sample sign tests (OSS) were used. To compare across multiple independent groups, we conducted Kruskal-Wallis tests (KW) with subsequent pair-wise comparisons by Mann-Whitney U-tests (MWU).

To ensure a within-experiment error rate below 5%, Bonferroni-Holm correction (Holm, 1979) was applied. Box plots show the median as the middle line, the 25 and 75 % quantiles as box boundaries, and the 10 and 90 % quantiles as whiskers. Sample sizes were chosen based on previous, similar studies. Experimenters were blind with respect to genotype when applicable. The results of the statistical tests are documented in Table S1 in the Appendix. Experimenters were blind to genotype.

Results

We crossed the DAN-i1^{864} driver to the UAS-ChR2-XXL effector and trained larvae such that the odour was presented either 10 s before DAN-i1^{864} activation started (forward paired training) or 30 s after the start of DAN-i1^{864} activation (backward paired training; Figure S1). To determine the memory score in each case the odour preference of the larvae was compared with the preference of larvae that had undergone presentations of the odour unpaired from DAN-i1^{864} activation (unpaired). Forward training induced appetitive, reward memory, which was not the case in the genetic controls (Figure 4B) (Saumweber et al., 2018). In contrast, backward training resulted in aversive, frustration memory (Figure 4C) (Saumweber et al., 2018). Given the reports of a punishing effect of light (von Essen et al., 2011), the data for the driver control suggest a moderate appetitive relief memory introduced by backward training with light; notably, such an effect would lead us to underestimate the aversive frustration memory in the experimental genotype (Figure 4C).

To further analyse reward and frustration memory by DAN-i1^{864} activation, we next enquired into the impact of the duration of DAN-i1^{864} activation. Despite a trend, this manipulation did not affect reward memory upon forward training (Figure 4D). After backward training, however, we observed that frustration memory increased with longer-lasting DAN-i1^{864} activation before odour presentation (Figure 4E).

Second, given that a single training trial can be sufficient to establish memory for some natural tastant reinforcers (Weiglein et al., 2019), we tested for one-trial memory using DAN-i1^{864} activation. We observed reward memory after one-trial forward training with DAN-i1^{864} activation, but no frustration memory after one-trial backward training (Figure 4F,G). Accordingly, when compared across experiments, reward memory does not benefit from repeated training (Figure 4B, 4D middle plot vs. 4F), whereas frustration memory does (Figure 4C, 4E middle plot vs. 4G**).**

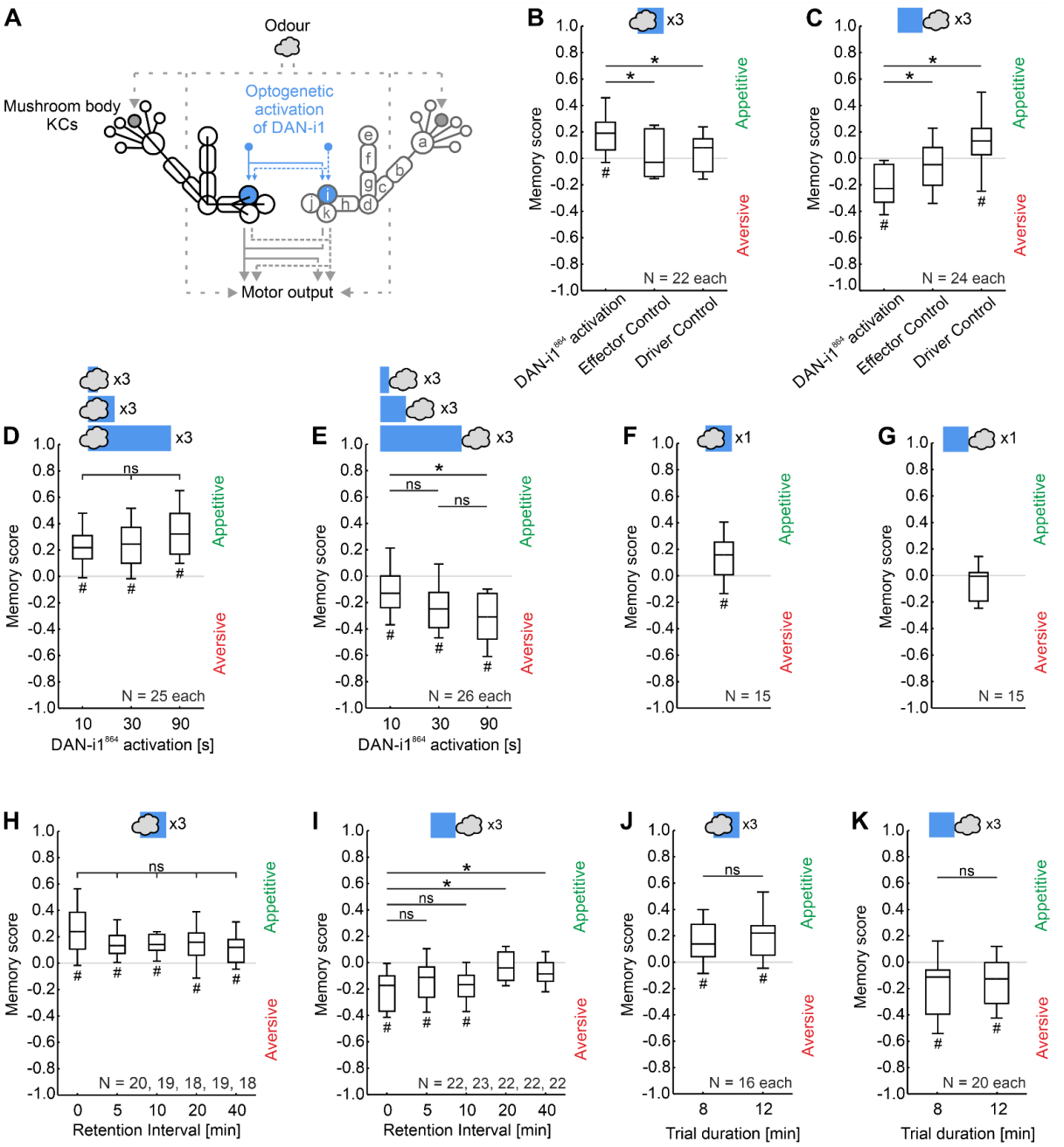


Figure 4. Reward and frustration memory by optogenetic DAN-i1^{864} activation. (A) Schematic of the DAN-i1 neuron (blue) innervating the i-compartment of the mushroom body. Kenyon cells (KCs) feature a higher-order odour representation. (B, C) Larvae were trained with three trials of odour (cloud) and DAN-i1^{864} activation (blue square) and subsequently tested for their odour preference. Positive and negative Memory scores reflect appetitive or aversive associative memory, respectively. (B) Upon forward training, only the experimental genotype showed appetitive memory. (C) Upon backward training, aversive memory was only shown by the experimental genotype. (D, E) Larvae received three trials of forward or backward training with varying durations of DAN-i1^{864} activation. (D) For forward training, odour presentation always preceded DAN-i1^{864} activation by 10 s. Appetitive memory of equal strength was observed in all cases. (E) For backward training, odour presentation always started at the offset of the DAN-i1^{864} activation. Increased durations of DAN-i1^{864} activation supported increased aversive memory. (F, G) With a single training trial, forward training established appetitive memory (F), whereas backward training established no aversive memory (G). (H, I) When three training trials were conducted, larvae showed appetitive memory up to at least 40 min after

Figure 4 continued on the next page

Figure 4 continued
forward training (H), whereas aversive memory after backward training has decayed after just 20 min. Note that the training trial duration in these experiments was 8 min instead of 12 min as in (B-G). (J, K) Results show that such a difference in trial duration does not affect memory scores. Box plots represent the median as the midline and the 25/ 75 % and 10/90 % quantiles as box boundaries and whiskers, respectively. Sample sizes are displayed within the Figure. # Significance relative to chance levels. * Significance in MWU-test or KW-tests (ns, not significant). See Figure S2 for Odour preferences underlying the Memory scores. Statistical results are reported in Table S1 in the Appendix.

Third, we were interested in how temporally stable reward and frustration memories established by DAN-i1^{864} activation are. Reward memory lasted at least 40 min and indeed only tendentially decayed across this time period (Figure 4H). In contrast, frustration memory was observed for only up to 10 min after training (Figure 4I). We note that the experiments shown in Figure 4H,I used a shortened protocol with reduced idle times before and after the actual training events, such that the total trial duration was 8 rather than 12 min. In a direct comparison, this difference in procedure was without effect (Figure 4J,K).

Fourth, we wondered whether reward and frustration memories established via DAN-i1^{864} activation differ in their locomotor 'footprint'; that is, whether associative memories can modulate both rate and direction of lateral head movements (head casts, HCs) (Paisios et al., 2017; Thane et al., 2019; Toshima et al., 2019). In comparison to larvae that underwent unpaired training, forward training promoted head casts when crawling away, rather than when crawling towards the odour, whereas the opposite was the case upon backward training (Figure 5A). Likewise, forward and backward training promoted HCs reorienting the larvae towards and away from the odour, respectively (Figure 5B). In addition, upon forward training we observed that the runs towards the odour were faster than those away from it (Figure 5C); this was surprising given that in 13 previous datasets tendencies for such run speed modulation, which can be recognized in about half of the cases, had never reached statistical significance (Paisios et al., 2017; Saumweber et al., 2018; Schleyer et al., 2015b, 2020; Thane et al., 2019; Toshima et al., 2019; Weiglein et al., 2019, 2021). In any event, these analyses show that reward and frustration memories established by DAN-i1^{864} are behaviourally expressed through opposite modulations of the same aspects of locomotion.

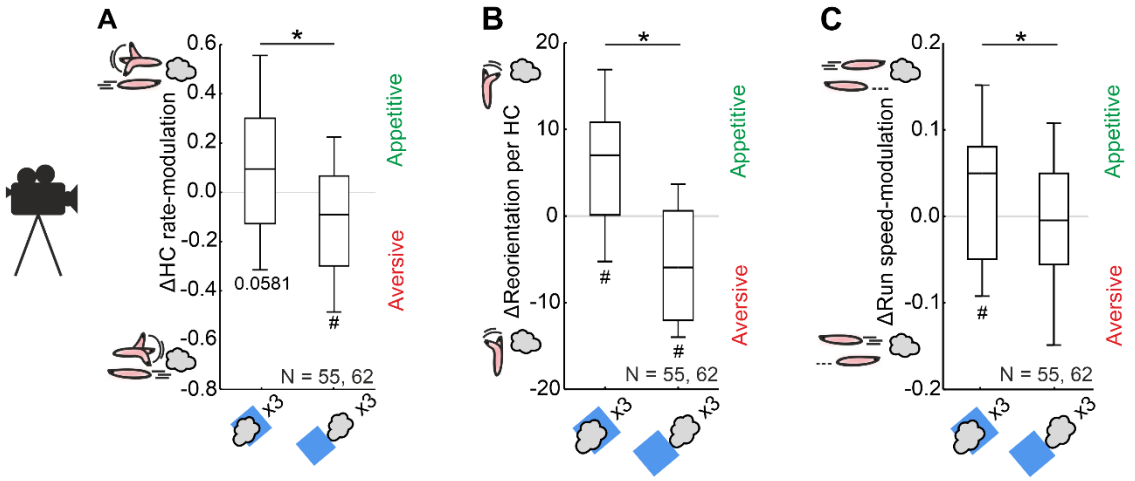


Figure 5. Locomotion footprint of reward and frustration memory by DAN-i1^{864} activation. Larvae were video tracked during testing, and for three behavioural variables the difference between larvae undergoing forward or backward paired training versus unpaired training (Δ) was calculated. (A) Forward training promoted head casts (HCs) when the larvae were crawling away from the odour rather than when crawling towards it. After backward training, the opposite was observed. (B) Forward and backward training prompted larvae to reorient their HCs towards and away from the odour, respectively. (C) Forward training resulted in faster runs towards the odour than away from it, whereas backward training had no effect upon run speed. Data are combined from Figures 4B-E (30-s light duration) and Figure 4 J,K (12-min trial duration). See Figure S3 for results separated for forward- and backward-paired versus unpaired training. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

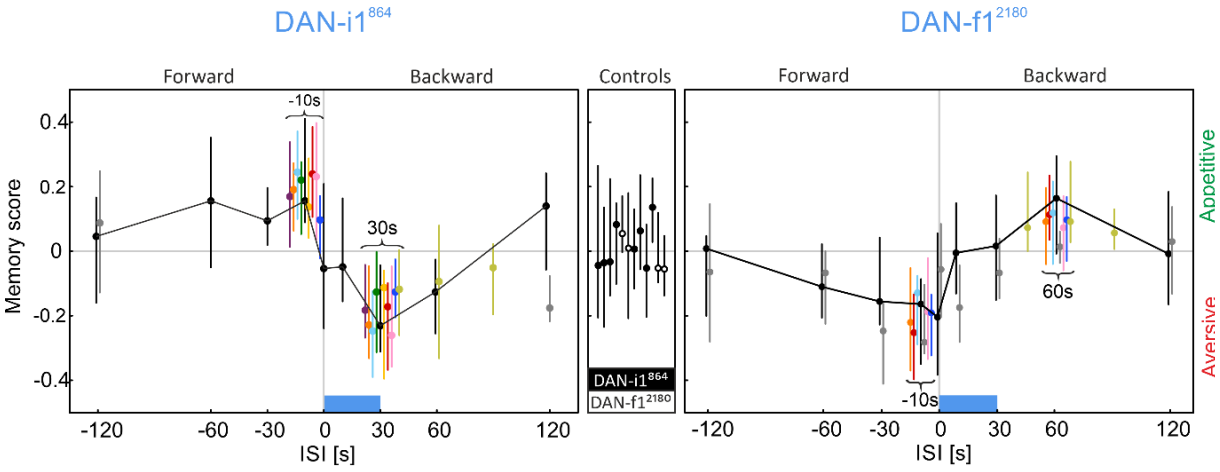
Discussion

We present a detailed characterization of the learning from the occurrence and termination of a central-brain reward signal, using optogenetic DAN-i1^{864} activation as a study case. Together with previous mirror-symmetric results concerning DAN-f1^{2180} (Weiglein et al., 2021), this reveals a 2x2 matrix of memory valence showing memories of opposite valence (appetitive or aversive) for stimuli that are associated with the occurrence or termination of central-brain reinforcement signals (Figure 6A). Such a push-pull organization makes it possible to decipher the predictive, causal structure of events around a target occurrence and could be inspiring for computational modelling. Indeed, using notably broader drivers for effector expression, a similar organization was reported for adult flies (Handler et al., 2019) and may thus reflect a more general principle (Gerber et al., 2019). Elegant and general as such a 2x2 organization appears to be, there are a number of differences in the memories established:

(1) In both life stages, reinforcing effects were strongly determined by the time point of the occurrence/ termination of reinforcement, whereas its duration was only of impact for memories related to reinforcement termination (Figure 4B-E; larval DAN activation: Weiglein

et al., 2021; adult DAN activation: König et al., 2018; electric shock: Diegelmann et al., 2013a; Jacob and Waddell, 2020).

(2) Memories established through the occurrence of an event last longer than those established through its termination, in both larval and adult *Drosophila* (Figure 4H-J; Weiglein et al., 2021; adults: Diegelmann et al., 2013; Yarali et al., 2008). With due caveats concerning rates of acquisition in mind, it might thus be that memories related to the occurrence of reinforcement are more stable than memories related to its termination.



-10 s	+30 s	Optimal ISI	-10 s	+60 s
Appetitive, reward memory	Aversive, frustration memory	3 training trials	Aversive, punishment memory	Appetitive, relief memory
Appetitive, reward memory	No memory	1 training trial	No memory	No memory
...trend to improve reward memory	...improved frustration memory	Longer lasting DAN activation...	...trend to improve punishment memory	...trend to improve relief memory
...up to 40 min	...up to 10 min	Memory detectable...	...up to 10 min	...only immediately after training
...decreased when heading towards odour	...increased when heading towards odour	Head cast rate is...	...increased when heading towards odour	...not modulated
...towards the odour	...away from the odour	Head casts reorient...	...away from the odour	...towards the odour
...mildly increased when heading towards odour	...not modulated	Run speed is...	...not modulated	...not modulated

Figure 6. Summary matrix of timing-dependent valence reversal. Summary of the present and previously published data, showing a 2x2 matrix of timing and valence for DAN-i1^{864} (left panel) and DAN-f1^{2180} (right panel). Larvae underwent pairings of odour and optogenetic activation of the respective DAN (blue box) with different inter-stimulus intervals (ISIs). Odour presentation preceding DAN activation results in negative ISIs (forward training); the reversed sequence results in positive ISIs (backward training). The graph in the middle displays the results of the genetic controls. Shown are medians and 25/ 75 % quantiles of independent experiments from the present study and Saumweber et al. (2018) (top left) as well as Weiglein et al. (2021) (top right). The table summarizes features of reward and frustration memory, and of punishment and relief memory established by pairing odour with the occurrence or termination of DAN-i1^{864} and DAN-f1^{2180}.

(3) Fewer trials were sufficient to establish reward memory than frustration memory (Figure 4F,G), which is in line with the opponent-process theory of Solomon and Corbit (1974). However, punishment and relief memory seem to benefit in a similar way from repeated training (larvae: Weiglein et al., 2021; adults: König et al., 2018).

(4) So far, only two out of three reinforcing DANs tested in larvae and two out of nine reinforcing DANs tested in adult *Drosophila* have been found to mediate opposing memories for stimuli associated with the occurrence versus termination of their activation (larvae: Saumweber et al., 2018; Weiglein et al., 2021; adults: Aso and Rubin, 2016; König et al., 2018). In other words, reinforcement signals that do *not* feature timing-dependent valence reversal need to be considered.

As both DAN-i1 and DAN-f1 are dopaminergic (Eichler et al., 2017), it appears straightforward that reward and frustration learning as well as punishment and relief learning are mediated by dopamine. This is likely to be the case, given related results using broader drivers, at least for reward memory (Rohwedder et al., 2016) and punishment memory (Selcho et al., 2009), and it is consistent with findings in adult *Drosophila* (Aso et al., 2019; Handler et al., 2019) and in mammals (Navratilova et al., 2015; but see König et al., 2018; Niens et al., 2017).

Taken together, our results complete the characterization of memories brought about by the timed activation of different larval DANs. The current data regarding DAN-i1^{864} activation and the data regarding DAN-f1^{2180} (Weiglein et al., 2021) provide a critical step to understand the fundamental features of reinforcement processing, and pave the way both for an improved modelling of neural networks of reinforcement learning (Springer and Nawrot, 2021) and for further research into the underlying molecular mechanisms.

Chapter II

Associative learning in larval and adult *Drosophila* is impaired by the dopamine-synthesis inhibitor 3-Iodo-L-tyrosine

Based on:

Thoener, J., König, C., Weiglein, A., Toshima, N., Mancini, N., Amin, F., & Schleyer, M. (2021). Associative learning in larval and adult *Drosophila* is impaired by the dopamine-synthesis inhibitor 3-Iodo-L-tyrosine. Biology open, 10(6), bio058198.

Author contributions:

Conceptualization: **J.T.**, C.K., A.W., N.T., N.M., M.S.; Methodology: **J.T.**, C.K., A.W., N.T., N.M.; Validation: **J.T.**, C.K., A.W., N.T., N.M., F.A.; Formal analysis: **J.T.**, C.K., M.S.; Investigation: **J.T.**, C.K., A.W., N.T., N.M., F.A., M.S.; Data curation: **J.T.**, C.K.; Writing - original draft: **J.T.**, C.K., A.W., M.S.; Writing - review & editing: **J.T.**, C.K., A.W., N.T., N.M., F.A., M.S.; Visualization: **J.T.**; Supervision: C.K., M.S.; Project administration: M.S.; Funding acquisition: N.T., M.S.

Introduction

Dopamine signalling serves multiple functions, including movement initiation, sleep regulation, motivation, learning, memory extinction and forgetting (Berke, 2018; Meder et al., 2019; Oishi and Lazarus, 2017; Schultz, 2007; Yamamoto and Seto, 2014). In particular, it is crucial for conferring reinforcement signals that teach animals about the causal structure of the world (Ryvkin et al., 2018; Schultz, 2015; Waddell, 2013; Yamamoto and Vernier, 2011). This role of dopamine is found across the animal kingdom, including the fruit fly *Drosophila melanogaster*. For this model organism, a rich genetic toolbox is available to study the functions of the dopaminergic system. Here, we employ a complementary approach using pharmacological intervention.

Since the 1970s, both adult and larval *D. melanogaster* have been established as powerful model organisms to investigate Pavlovian conditioning, using odours as the conditioned stimulus (CS) and various types of rewarding and punishing unconditioned stimuli (US)

(adults: Busto et al., 2010; McGuire et al., 2005; Perisse et al., 2013; Quinn et al., 1974; larvae: Diegelmann et al., 2013b; Gerber and Stocker, 2007; Scherer et al., 2003; Thum and Gerber, 2019; Widmann et al., 2018). The genetic tools available for *D. melanogaster* have allowed the neurogenetic mechanisms of learning and memory to be investigated, and revealed many striking similarities between the dopaminergic systems of flies and mammals, including humans (reviewed in Yamamoto and Seto, 2014). To mention but a few, flies and mammals share the majority of genes involved in dopamine synthesis, secretion and signalling (Clark et al., 1978; Karam et al., 2020; Riemensperger et al., 2011; Yamamoto and Seto, 2014), as well as the crucial role of dopaminergic neurons in reinforcement signalling (Burke et al., 2012; Liu et al., 2012; Schroll et al., 2006; Schwaerzel et al., 2003; Selcho et al., 2009; reviewed in Scaplen and Kaun, 2016). Of note, in *D. melanogaster* different sets of dopaminergic neurons signal appetitive or aversive reinforcement, respectively, to distinct compartments of the insects' memory centre, the mushroom body, which harbours a sparse and specific representation of the olfactory environment (Diegelmann et al., 2013b; Guven-Ozkan and Davis, 2014; Heisenberg, 2003; Owald and Waddell, 2015; Thum and Gerber, 2019). A similar dichotomy of appetitive and aversive reinforcement signals carried by different sets of dopaminergic neurons may also be emerging in vertebrates (Groessl et al., 2018; Lammel et al., 2012; Menegas et al., 2018). Due to the seductive power, ease and elegance of the available genetic tools in *D. melanogaster*, however, other useful techniques are used less often in the field. For example, feeding or injecting drugs, although lacking the neuronal specificity of many transgenic tools, is a convenient way of exerting acute systemic effects. Furthermore, these approaches can be combined with genetic methods like cell specific optogenetic manipulations, allowing greater flexibility in manipulating the animals' nervous system.

Many drugs affecting the dopamine system in mammals are also effective in flies (Nichols, 2006; Pandey and Nichols, 2011). For example, drugs that target mammalian D1 and D2 receptors have already been used pharmacologically to activate and inhibit their *Drosophila* homologs in vivo (Chang et al., 2006; Srivastava et al., 2005; Yellman et al., 1997). Also, drugs that induce dopamine deficiency have been found to influence various brain functions. For example, 3-Iodo-L-tyrosine (3IY; other abbreviations sometimes used are 3-IY and 3-IT) interferes with dopamine synthesis by inhibiting the tyrosine hydroxylase enzyme (TH) that catalyses the conversion of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine. As a result, 3IY reduces dopamine levels (Bainton et al., 2000;

Fernandez et al., 2017; Neckameyer, 1996) (Figure S4A). Feeding 3IY to flies decreases activity/locomotion and increases sleep (Andretic et al., 2005; Cichewicz et al., 2017; Tomita et al., 2015; Ueno and Kume, 2014), increases ethanol preference (Ojelade et al., 2019 preprint), and alters courtship behaviour (Monier et al., 2019; Neckameyer, 1998; Wicker-Thomas and Hamann, 2008). Regarding learning and memory, 3IY feeding impairs visual and olfactory learning, as well as long-term appetitive ethanol memory in adult flies (Kaun et al., 2011; Seugnet et al., 2008; Zhang et al., 2008). Importantly, these effects of 3IY-induced dopamine deficiency can be substantially rescued by additionally feeding L-DOPA to the flies (Cichewicz et al., 2017; Monier et al., 2019; Riemensperger et al., 2011; Zhang et al., 2008).

In larvae, 3IY feeding has been used to study the developmental effects of dopamine (Neckameyer, 1996; reviewed in Verlinden, 2018) as well as the characterization of dopamine synthesis, reuptake and release (Pyakurel et al., 2018; Xiao and Venton, 2015). Furthermore, 3IY has been found to attenuate the increase in sugar feeding elicited by food odours, an effect that likewise was reversed by additional L-DOPA feeding (Wang et al., 2013).

Here, we provide the first investigation of the effects of feeding 3IY and/or L-DOPA on Pavlovian conditioning in larval *D. melanogaster*, and report detailed protocols of drug application and behavioural controls. Furthermore, we also feed 3IY and/or L-DOPA to adult flies. We study the drugs' impact on learning about optogenetic activation of an identified dopaminergic neuron to exemplify the potential of combining genetic and pharmacological approaches, as the drugs' effects on wild-type behaviour has previously been shown.

Material and Methods

General

Drosophila melanogaster were raised in mass culture on standard cornmeal-molasses food and maintained at 25°C, 60–70% relative humidity, and a 12:12 h light/dark cycle.

For larval behaviour experiments, we used third instar, feeding-stage wild-type Canton Special larvae of either sex, aged 4 or 5 days after egg laying, as mentioned along with the results. For adult behaviour experiments, the split-GAL4 driver strain MB320C (detailed information can be found in the relevant database http://splitgal4.janelia.org/cgi-bin/splitgal4.cgi as well as in Aso et al., 2014), covering the PPL1-γ1pedc neurons (alternative nomenclatures: PPL1-01 and MB-MP1), was crossed to UAS-ChR2-XXL (Bloomington, stock

number: 58374, Dawydow et al., 2014) as the effector and kept in darkness throughout to avoid optogenetic activation by room light. Flies of either sex, aged 1 to 4 days after hatching, were used.

Prior to behavioural experiments, animals were fed with solutions of 3-Iodo-L-tyrosine (3IY; stored at −20°C; CAS: 70-78-0, Sigma- Aldrich, Steinheim, Germany) and/or 3,4-dihydroxyphenylalanine (L-DOPA; CAS: 59-92-7, Sigma-Aldrich) at concentrations of 5 mg/ml and 10 mg/ml, respectively, as explained in more detail below. To facilitate reproducibility, we measured the absorption of the solutions in the UV-visible spectrum, using a NanoDrop 2000c spectrometer (ThermoFisher Scientific, Dreiich, Germany). For 5 mg/ml 3IY in distilled water, we found the wavelength of maximal absorption to be 280 nm, and the average absorption at this wavelength to be 4.46. For 10 mg/ml L-DOPA in distilled water, we determined a wavelength of maximal absorption of 280 nm, and an absorption at this wavelength of 7.66.

Feeding of 3IY to larval *D. melanogaster*

A 0.5 mg/ml yeast solution was prepared from fresh baker's yeast (common supermarket brands) diluted in tap water and stored for up to 5 days at 4°C in a closed bottle. Samples of 2 ml yeast solution were filled into a 15 ml Falcon tube and kept for a few minutes in a warm water bath. 3IY was added at a concentration of 5 mg/ml to the respective sample, if not mentioned otherwise. Notably, in contrast to earlier studies using 10 mg/ml or more (Neckameyer, 1996; Wang et al., 2013), we were not able to dissolve concentrations higher than 5 mg/ml. In some experiments, L-DOPA was added at a concentration of 10 mg/ml, either to pure yeast solution, or to yeast solution with 5 mg/ml 3IY.

The solutions were thoroughly mixed by attaching the Falcon tubes to a shaker at high speed for approximately 60 min. Empty vials of 5 cm diameter were equipped with two layers of mesh (PET, 500 μm mesh size). Samples of the mixed yeast solution with or without additional substances were distributed onto the mesh of one vial. Larvae of the third instar feeding stage were collected from the fly food by adding 15% sucrose solution (D-Sucrose; CAS: 57-50- 1, Roth, Karlsruhe, Germany; in dH_2O) so that the larvae floated up and could be transferred to a Petri dish filled with tap water using a tip-cut plastic pipette. After being rinsed in water, the larvae were loaded onto a filter (pluriStrainer 70 μm, pluriSelect Life Science, Leipzig, Germany) to separate them from water and small food particles, and transferred with a brush to one of the prepared vials. For yeast solutions containing different

drugs and/or concentrations, different brushes were used. The larvae were left to feed on the respective yeast solution for 24 or 4 h at 25°C and 60–70% relative humidity. The desired number of larvae were collected with a brush, briefly rinsed in water, and afterwards used in the respective experiment.

Larval behaviour

Odour-fructose associative learning

Experiments for appetitive odour-fructose associative memory (Saumweber et al., 2011; Scherer et al., 2003) were performed using a one-odour, single-training-trial protocol described in Weiglein et al. (2019) (Figure 7A, left). For example, two custom made Teflon containers of 5 mm diameter were filled with 10 µl of odour substance (n-amylacetate, AM; CAS: 628-63-7, Merck, Darmstadt, Germany; diluted 1:20 in paraffin oil; CAS: 8042-47-5, AppliChem, Darmstadt, Germany) and closed with lids perforated with 5–10 holes, each of approximately 0.5 mm diameter. These odour containers were located on opposite sides of a Petri dish (9 cm inner diameter; Nr. 82.1472 Sarstedt, Nümbrecht, Germany) filled with 1% agarose solution (electrophoresis grade; CAS: 9012-36-6, Roth, Karlsruhe, Germany) and additionally containing fructose (FRU; 2 M; purity 99%; CAS: 57-48-7 Roth, Karlsruhe, Germany) as a taste reward (+). Cohorts of approximately 30 larvae were placed at the centre of the Petri dish and allowed to move about it for 2.5 min. Subsequently, the larvae were transferred with a brush to a fresh Petri dish that was filled with plain, tasteless agarose and equipped with two empty Teflon containers (EM). For each cohort trained in such a paired way (paired training; AM+/EM), a second cohort of larvae received the odour unpaired from the fructose reward (unpaired training; EM+/AM). In half of the cases the sequence of events was reversed (EM/AM+, AM/EM+, respectively).

After one training trial, the larvae were transferred to a fresh, tasteless test Petri dish with AM on one side and an EM container on the opposite side. The larvae were left to distribute for 3 min and then counted to evaluate their preference for AM. The number of larvae (#) on the AM side, on the EM side, and in a 10 mm-wide middle zone was counted. Larvae crawling up the sidewalls of the Petri dish were counted for the respective side, whereas larvae on the lid were excluded from the analysis (<5%). A preference for AM ($Odour_{AM}PREF$) was calculated:

$$Odour_{AM}PREF = \frac{\#AM - \#EM}{\#Total} \quad (9)$$

Odour$_{AM}$PREF range from +1 to −1, with positive values indicating AM preference and negative values indicating avoidance of AM.

From the Odour$_{AM}$PREF scores after paired and unpaired training, a Memory score was calculated as follows:

$$Memory\ score = \frac{Odour_{AM}PREF_{Paired} - Odour_{AM}PREF_{Unpaired}}{2} \quad (10)$$

Memory scores range from +1 to −1 with positive values indicating appetitive associative memory; negative values indicate aversive associative memory.

Innate odour preference tests

Cohorts of approximately 20–30 experimentally naïve larvae were collected, briefly washed in tap water, and placed onto a Petri dish with an AM container on one side and an EM container on the other side (Figure 7A, second from left). After 3 min, the odour preference (Odour$_{AM}$PREF$_{innate}$) was determined as detailed in Equation 9.

Innate fructose preference tests

Split Petri dishes were prepared freshly approximately 4 h before the experiment, following the procedures described in (König et al., 2014) such that one half of the Petri dish (9 cm diameter) was filled with agarose with 2 M FRU, and the other half with plain agarose (Figure 7A, second from right). Approximately 20–30 larvae were collected, rinsed in tap water, and placed onto the centre of a split Petri dish. After 3 min, the number of larvae (#) on the fructose side, on the pure agarose side, and in a 10 mm-wide middle zone was counted. Fructose preference (FRU PREF$_{innate}$) was calculated as follows:

$$FRU\ PREF_{innate} = \frac{\#Fructose - \#Agarose}{\#Total} \quad (11)$$

FRU PREF$_{innate}$ scores range from +1 to −1, with positive values indicating approach to the fructose and negative values indicating avoidance.

Analyses of locomotion

Cohorts of approximately 20 larvae were placed on an empty, plain-agarose-filled Petri dish without odour or reward (Figure 7A, right). For 3 min, they were video recorded while they freely moved in the dish. The videos were analysed offline using custom made tracking software described in Paisios et al. (2017). In brief, larvae alternately perform relatively straight forward-locomotion, called runs, and lateral head movements, called head casts (HC) that are often followed by changes in direction. This leads to a typical zigzagging pattern of locomotion (Gershow et al., 2012; Gomez-Marin and Louis, 2014; Gomez-Marin et al., 2011). As described in detail by (Paisios et al., 2017), an HC was detected whenever the angular velocity of a vector through the animal's head exceeded a threshold of 35°/s and ended as soon as that angular velocity dropped below the threshold again. The time during which an animal was not doing a HC was regarded as a run, deducting 1.5 s before and after an HC to exclude the decelerating and accelerating phases that usually happen before and after an HC, respectively. Three aspects of behaviour were analysed: the run speed was determined as the average speed (mm/s) of the larval midpoint during runs; the rate of HCs was determined as the number of HCs per second (HC/s); and the size of HCs was determined by the HC angle. Accordingly, the animal's bending angle as the angle between vectors through the head and tail was determined before and after an HC. Then, the HC angle was calculated as the difference between the animal's bending angle after an HC and the bending angle before an HC. For a detailed description, see Paisios et al. (2017).

To analyse the HC behaviour in more detail, we determined the HC rate and HC angle separately for small and large HCs. The discriminatory threshold for large HCs of an HC angle >20° was based on previous studies (Paisios et al., 2017; Schleyer et al., 2015b; Thane et al., 2019).

Feeding of 3IY to adult *D. melanogaster*

For 3IY feeding in adult flies, a 5 % sucrose solution (CAS: 57-50-1, Hartenstein, Würzburg, Germany) was prepared. This solution was either used pure, or mixed with 5 mg/ml 3IY, or with 10 mg/ml L-DOPA, or with both, in an analogous manner to that described above for the larval case. Hatched adults of the genotype MB320C; ChR2-XXL were collected in fresh food vials and kept under the normal culture conditions mentioned above, at least overnight and at most until 4 days after hatching. Flies were transferred to new vials containing a tissue

(Fripa, Düren, Germany) soaked with 1.8 ml of sucrose solution that either did or did not contain 3IY and/ or L-DOPA, as mentioned in the results section. After 40–48 h under otherwise normal culture conditions, the flies were trained and/or tested en masse.

Adult behaviour

Odor-PPL1-γ1pedc associative learning

For the memory assays, we followed the procedures described in König et al. (2018), unless mentioned otherwise (Figure 10A, left). Approximately 100 flies were loaded into a small transparent tube in a custom-made setup (CON-ELEKTRONIK, Greussenheim, Germany), and were trained and tested at 23–25°C and 60–80% relative humidity. Training was performed in dimmed red light, which is largely invisible to flies and does not stimulate the ChR2-XXL effector; testing was performed in darkness. For the application of blue light, a 2.5 cm-diameter and 4.5 cm-length hollow tube with 24 LEDs mounted on the inner surface was placed around the transparent training tubes harbouring the flies. As odorants, 50 µl benzaldehyde (BA) and 250 µl 3-octanol (OCT) (CAS 100-52-7, 589-98-0; both from Fluka, Steinheim, Germany) were applied to 1 cm-deep Teflon containers of 5 and 14 mm diameter, respectively. From these containers, odour-loaded air was shunted into the permanent air stream flowing through the apparatus. During training, the flies were presented with both odours for 1 min with a 3 min resting interval in between, but only one of the odours was paired with 1 min of blue light (465 nm) for optogenetic activation of PPL1-γ1pedc, whereas the other odour was presented alone (either BA-paired or OCT-paired training, respectively). In half of the cases training started with the odour paired with light (CS+); in the other half training started with the odour without light activation (CS-; for details see electronic supplement Fig. S1B of König et al., 2019). For the subsequent test, the flies were given a 3 min accommodation period, after which they were transferred to the T-maze-like choice point. The test configuration between the two odours used during training was prepared and balanced so that either BA or OCT were present at front versus rear position over the course of all experiments. After 2 min testing time, the arms of the maze were closed and the flies on each side were counted to calculate a benzaldehyde preference index ($Odour_{BA}PREF$):

$$Odour_{BA}PREF = \frac{\#BA - \#OCT}{\#Total} \quad (12)$$

Thus, positive scores indicate preference for BA and negative scores preference for OCT. From the Odour$_{BA}$PREF scores of two independently trained fly groups after BA-paired and OCT-paired training, a Memory score was calculated as follows:

$$Memory\ score = \frac{Odour_{BA}PREF_{BA-paired} - Odour_{BA}PREF_{OCT-paired}}{2} \quad (13)$$

Positive scores thus reflect appetitive associative memory, negative values aversive associative memory.

Innate odour preference tests

Cohorts of approximately 50 flies were loaded into the setup. After a 5 min resting interval, they were transferred to the choice point of a T-maze between an arm equipped with either BA or OCT (in the same manner as described above), and an arm with an empty Teflon container, and allowed to distribute for 2 min (Figure 10A, right). A preference was calculated as:

$$Odour_{BA}PREF_{innate} = \frac{\#BA - \#EM}{\#Total} \quad (14)$$

Or

$$Odour_{OCT}PREF_{innate} = \frac{\#OCT - \#EM}{\#Total} \quad (15)$$

Each data point in Figure 10D, E represents the mean value of two runs tested with the odour in the front or rear T-maze position.

Statistics

Two-tailed, non-parametric statistics were used throughout to analyse the behavioural data. For comparisons of a group's scores with chance levels (zero), one-sample sign tests (OSS) were applied. To compare across multiple independent groups, Kruskal–Wallis tests (KW) with subsequent pair-wise Mann–Whitney U-tests (MWU) were used (Statistica 13, StatSoft Inc, Tulsa, USA). To ensure a within-experiment error rate below 5%, a Bonferroni–Holm correction for multiple comparisons was employed (Holm, 1979). Sample sizes (biological replications) were estimated based on previous studies with small to medium effect sizes

(König et al., 2018; Weiglein et al., 2019). None of the specific experiments reported here had previously been performed in our laboratory, although the basic behavioural paradigms are regularly used. Experimenters were blind to treatment condition during the experiments with larvae, and during the fly counting for the experiments with adults. Data are presented as box plots showing the median as the middle line, the 25 and 75% quantiles as box boundaries, and the 10 and 90% quantiles as whiskers. All statistical results from the behavioural experiments are documented in Table S1 in the Appendix.

Results

Feeding 3IY for 24 h induces broad behavioural impairments in larvae

We first investigated the effects of 3IY feeding on *D. melanogaster* larvae. In an approach modified from Neckameyer (1996), cohorts of 4-day-old larvae were placed on a PET mesh soaked with a yeast solution mixed with 3IY at the indicated concentrations, or without 3IY. After 24 h, the larvae underwent a single-trial Pavlovian training with an odour and a fructose reward, following established protocols (Michels et al., 2017; Saumweber et al., 2011; Scherer et al., 2003; Weiglein et al., 2019): one cohort of larvae was trained by a paired presentation of odour and reward, and a second cohort was trained reciprocally, i.e. with separated, unpaired presentations of odour and reward. In control larvae that were kept on a yeast solution without 3IY, an appetitive associative memory was revealed by higher odour preferences after paired than after unpaired training in a subsequent test (Figure S4B), indicated by positive Memory scores (Figure 7B, left-most box plot). When we performed the same learning experiment with larvae fed with various concentrations of 3IY, we observed decreased Memory scores with increased 3IY concentrations. Significantly reduced scores were found for a concentration of 5 mg/ml (Figure 7B; Figure S4B), a result we replicated in an independent experiment (Figure 7C; Figure S4C). However, we noticed that many larvae had died due to the treatment, and the cuticle of many of the surviving animals was darkened (not shown). We therefore wondered whether the treatment may generally impair behavioural faculties. Indeed, innate odour preference was found to be impaired in 3IY-fed larvae (Figure 7D). This prompted us to test their basic locomotion on an empty, tasteless Petri dish without odour or sugar, and to analyse their behaviour using custom-made analysis software (Paisios et al., 2017). Typically, larvae move by relatively straight runs, interrupted by turning manoeuvres indicated by

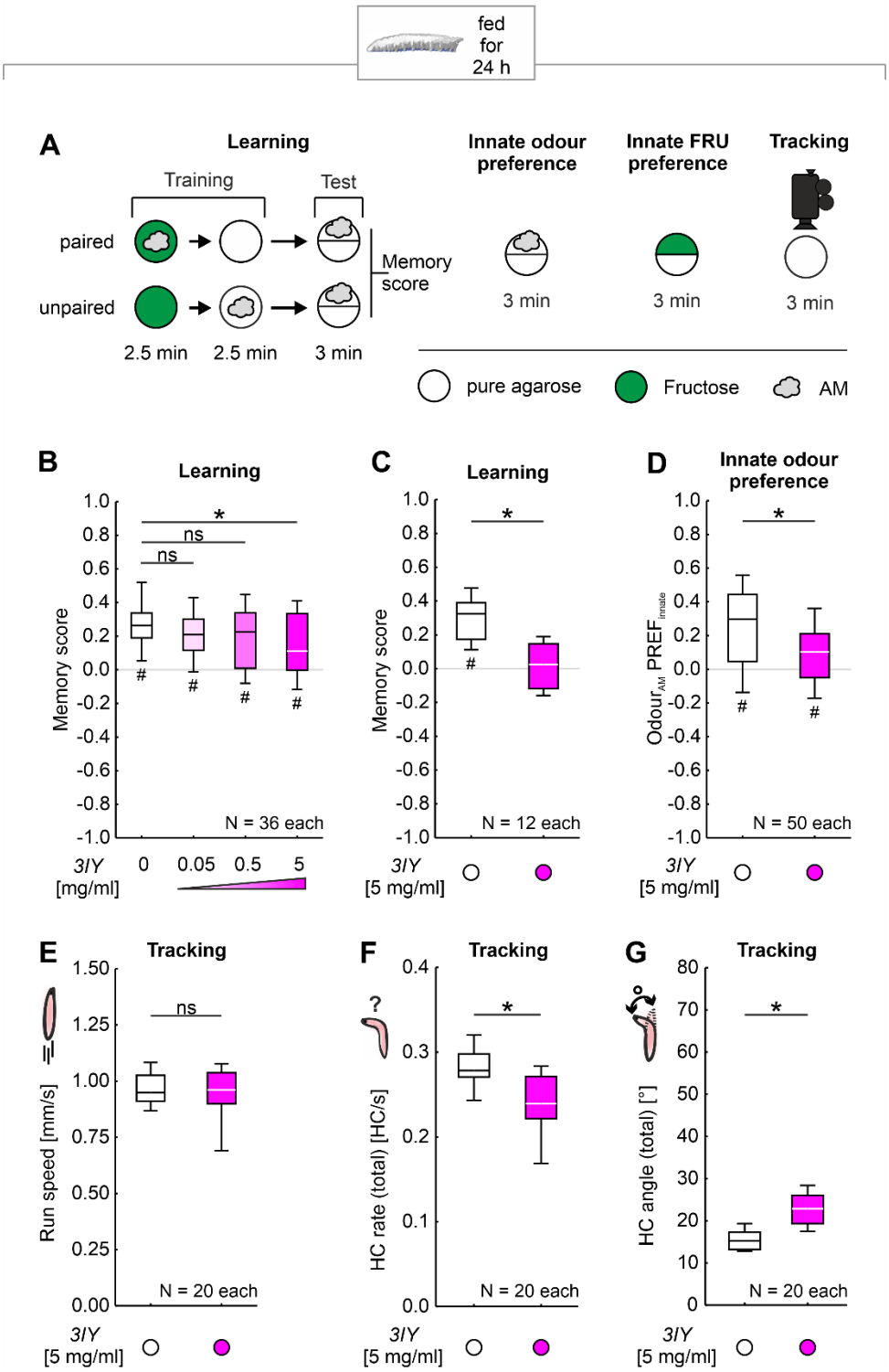


Figure 7. **Feeding 3IY to *D. melanogaster* larvae for 24 h broadly impairs behaviour.** (A) Larvae were either trained in a learning paradigm, tested for their innate odour or fructose (FRU) preference, or behaviour was analysed offline using video recording. In the one-odour learning paradigm, cohorts of larvae were trained by either paired or unpaired presentations of an odour (grey cloud) and sugar (green circle), and subsequently tested for odour preference. Note that in every other experiment the training sequence was reversed to what is depicted. To test the innate odour preference larvae had the choice between odour on one side of the Petri dish and no odour on the other. Likewise, innate FRU preference was tested by presenting FRU (green semicircle) on one half of the Petri dish and pure agarose (white semicircle) on the other half of the Petri dish. To track the locomotion, larvae were video

Figure 7 continued on next page

Figure 7 continued
recorded on a dish filled with agarose, without any particular stimuli. (B) Feeding different concentrations of 3IY for 24 h led to memory impairment, with a significant reduction compared to the control only in the group with the highest tested concentration of 5 mg/ml 3IY. All other tested concentrations did not affect memory scores compared to the control group. (C) As seen in B, larvae fed with 5 mg/ml 3IY showed impaired memory in an independent repetition. (D) An innate preference test revealed lower preference for the tested odour in the group fed with 5 mg/ml 3IY compared to the control group. (E) Offline analysis of larval behaviour revealed no difference in run speed between control larvae and larvae fed with 5 mg/ml 3IY. Regarding head casts, larvae fed with 5 mg/ml 3IY compared to control larvae showed (F) fewer head casts but (G) made larger head casts. See Figure S4 for Odour preferences underlying the Memory scores and detailed head cast analysis. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

lateral head movements called head casts (HCs) (Figure S4D; Gershow et al., 2012; Gomez-Marin and Louis, 2014; Gomez-Marin et al., 2011; Paisios et al., 2017; Thane et al., 2019). Analysis of these parameters of locomotion revealed that the animals' run speed was unchanged by 3IY feeding (Figure 7E). However, the larvae fed with 3IY systematically performed fewer and larger HCs than control animals (Figure 7F,G; Figure S4E-H).

Thus, feeding the larvae with 5 mg/ml 3IY for 24 h seemed to impair their basic behavioural faculties, suggesting that the reduced Memory scores that we observed after the treatment might be secondary to such general impairment. Therefore, we next sought to reduce the 'side effects' of 3IY feeding.

Feeding 3IY for 4 h specifically impairs associative sugar learning in larvae

Given the reported role of dopamine and the TH enzyme in development and cuticle formation (Friggi-Grelin et al., 2003; Hsouna et al., 2007; Neckameyer, 1996; Neckameyer and White, 1993; reviewed in Verlinden, 2018), the timing of 3IY feeding is likely to have an impact. In order to minimize developmental effects, it seems desirable to apply 3IY as late as possible in the larval life cycle (and yet early enough to be able to finish the experiment before the larvae start to pupate). We therefore reduced the duration of 3IY feeding to 4 h, which allowed for the feeding of 3IY to 5-day-old animals. After this shortened feeding protocol too, memory scores were reduced compared to controls (Figure 8A; Figure S5A). Critically, the animals' basic behavioural faculties turned out to be intact: no impairment in innate odour preference (Figure 8B) or sugar preference (Figure 8C) was detectable. Thus, the shortened feeding of 3IY specifically impaired associative memory without impairing task-relevant behavioural faculties (nor did we observe any dead or darkened larvae; not shown). This conclusion was also supported by a more detailed analysis of locomotion that revealed only very mild

differences to controls (Figure 8D–F, for more details, see Figure S5B–E). However, we cannot rule out the possibility of impairments in locomotion or other basic behavioural faculties after the animals underwent the training procedure, caused e.g. by fatigue or adaptation to the stimuli used. Given that we used a very short one-trial training paradigm (about 6 min in total), such effects seem not too likely. Notably, we detected a small increase in the HC rate after 4 h of 3IY feeding (Figure 8E). This effect seems to be contradictory to the decrease in the HC rate after 24 h of 3IY feeding (Figure 7F). A closer look revealed that after 4 h feeding the HC rate is increased only for large HC (Figure S5B,C). After 24 h feeding, the same effect is

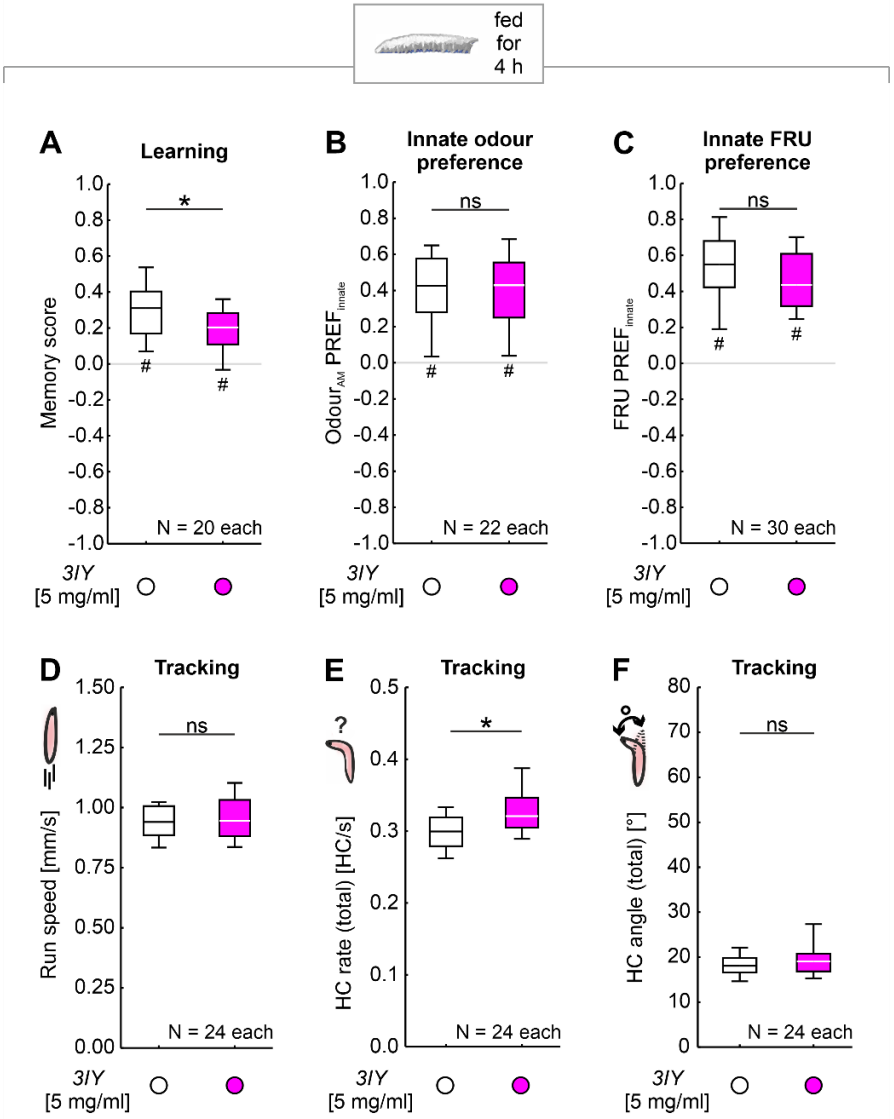


Figure 8. Feeding 3IY to *D. melanogaster* larvae for 4 h impairs memory but leaves innate behaviour intact. (A) Larvae fed with 5 mg/ml 3IY for 4 h showed impaired memory compared to the control group. Innate preference for (B) the odour or (C) FRU was not affected. Video tracking of the larvae revealed (D) no difference in run speed, (E) a slight increase in HC rate for larvae fed with 5 mg/ml 3IY, and (F) no difference in HC angles. See Figure S5 for Odour preferences underlying the Memory scores and detailed analysis of head casts. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

observed, but additionally the rate of small HC is reduced (Figure S4E,F), resulting in a total decrease of the HC rate. How these effects of 3IY feeding exactly come about remains unclear.

We next tried to rescue the effect of 3IY on the TH enzyme by additionally feeding the animals with L-DOPA (Figure S4A). To this end, we fed animals either with plain yeast solution (control), or 5 mg/ml 3IY, or with both 5 mg/ml 3IY and 10 mg/ml L-DOPA. The memory scores were impaired in larvae fed with 3IY alone (Figure 9A; Figure S6A), replicating the results from Figure 8A. These reduced memory scores were restored to control levels by additionally feeding L-DOPA to the larvae (Figure 9A; Fig S6A). Innate odour and sugar preferences were not affected by either 3IY or combined 3IY and L-DOPA feeding, confirming that both effects were specific for associative learning (Figure 9B,C). Importantly, while a repetition of the experiment from Figure 9A replicated the finding that L-DOPA feeding can restore memory scores upon 3IY treatment, we also showed that the feeding of L-DOPA alone did not increase memory scores (Figure 9D; Figure S6B).

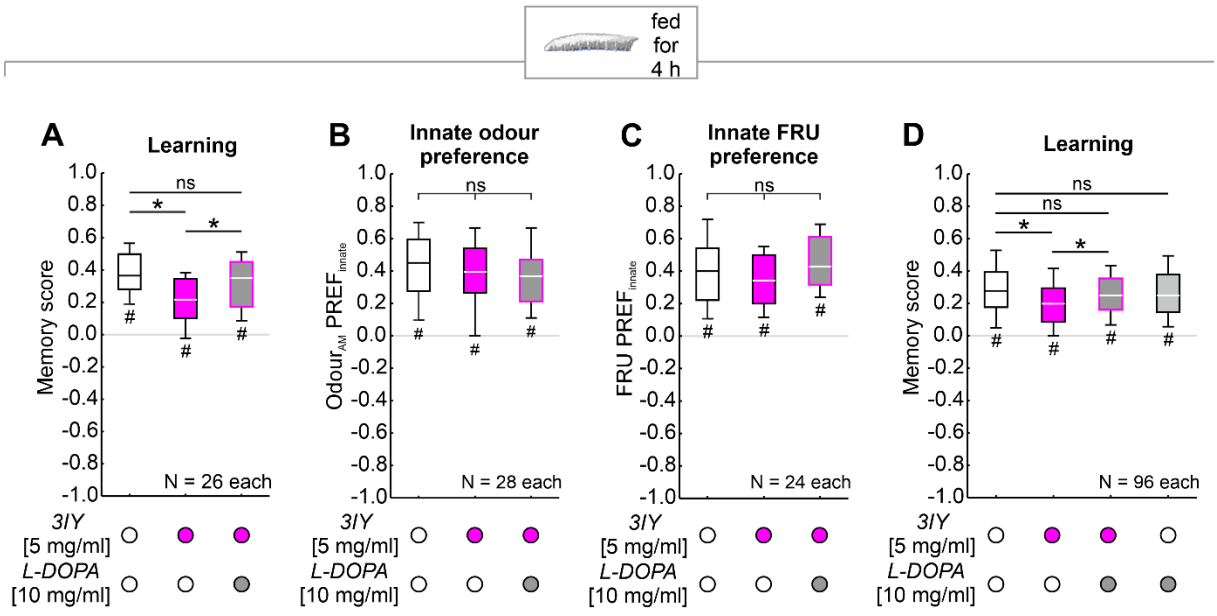


Figure 9. Memory impairment in *Drosophila* larvae due to 3IY can be rescued by additionally feeding L-DOPA. (A) Feeding L-DOPA in addition to 3IY rescued the memory impairment. Feeding 5 mg/ml 3IY alone for 4 h impaired memory, whereas additionally feeding 10 mg/ml L-DOPA rescued memory impairment and led to memory scores comparable to the control group. Feeding either drug did not affect innate approach to (B) odour or (C) FRU. (D) As shown in A, feeding 3IY impaired memory scores, and this impairment was rescued by additional L-DOPA feeding. Feeding L-DOPA alone had no effect on memory scores. Given this lack of effect of feeding L-DOPA alone, we did not perform an additional control for innate odour and sugar preference for this experimental condition. See Figure S6 for Odour preferences underlying the Memory scores. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

Feeding of 3IY specifically impairs associative learning via PPL1-γ1pedc activation in adults

After demonstrating the effect of 3IY feeding on associative learning about natural sugar rewards in larvae, we sought to combine 3IY feeding with genetic manipulations of the dopaminergic system, and at the same time to study how broadly applicable the 3IY approach might be. Therefore, we applied it to a different learning paradigm, by using (i) adult flies instead of larvae; (ii) a two-odour differential paradigm instead of a one-odour, 'absolute' paradigm; and (iii) an optogenetic punishment instead of a natural taste reward (Figure 10). Specifically, we expressed the blue-light-gated cation channel channelrhodopsin-2-XXL as the optogenetic effector (ChR2-XXL; Dawydow et al., 2014) in a single dopaminergic neuron per brain hemisphere, called PPL1-γ1pedc (alternative nomenclatures PPL1-01 and MB-MP1), as covered by the Split-GAL4 driver strain MB320C (Aso et al., 2014). This neuron, when optogenetically activated, carries an internal punishment signal sufficient to establish an aversive associative memory when paired with an odour (Aso and Rubin, 2016; Hige et al., 2015a; König et al., 2018) (Figure 10B, left-most box plot). Upon feeding 3IY for 48 h before training, memory scores were decreased, an effect that was restored by L-DOPA feeding (Figure 10B; Figure S7A). The effect of 3IY in reducing memory scores increased with increasing 3IY concentrations (Figure 10C; Figure S7B), and was equally observed in female and male flies (Figure 10D; Figure S8). Critically, 3IY feeding left innate odour preference to either odour unaffected (Figure 10E,F), which also implies that the animals' locomotor abilities were intact to an extent that allowed normal odour preferences. We therefore did not perform detailed locomotion analyses. Furthermore, feeding L-DOPA alone did not increase memory scores (Figure 10G; Figure S7C). Thus, feeding 3IY specifically impaired associative learning via PPL1-γ1pedc activation in adult flies, but kept their task-relevant behavioural capacities intact.

Discussion

The present study demonstrates that both in larval and adult D. melanogaster, and in two very different kinds of tasks, feeding 3IY can specifically impair associative learning while innate task relevant behaviour remains intact. In either case, the observed memory impairment was rescued by feeding L-DOPA, suggesting that the 3IY-impairment was indeed caused by an

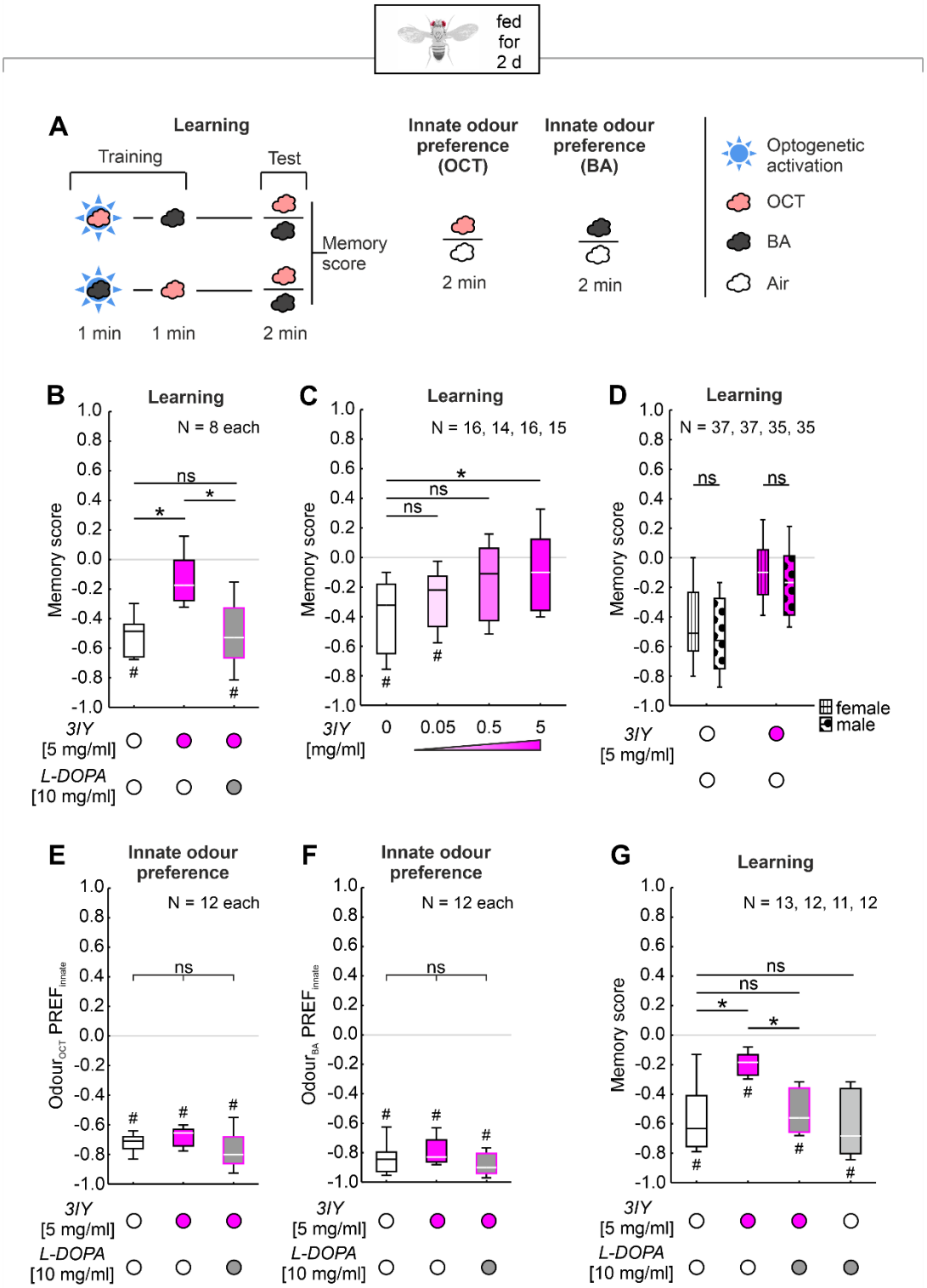


Figure 10. Feeding 3IY to adult *D. melanogaster* impairs optogenetically induced memory but leaves innate behaviour intact. (A) Flies were either trained in a learning paradigm and tested afterwards or were tested for their innate odour preference. In the learning paradigm, cohorts of flies were trained by pairing one of two odours (peach/black cloud) with optogenetic activation of PPL1-γ1pedc (blue star), and subsequently tested for their choice between the two odours. Note that the sequences of the odours and the optogenetic activation was shuffled across experiments as explained in the methods section. In the innate odour preference test, flies were given the choice between an odour (peach/black cloud) and air (white cloud). (B) 3IY feeding led to an impaired performance index compared to the

Figure 10 continued on next page

Figure 10 continued

control group. Additional L-DOPA feeding rescued this impairment of memory scores to the control level. (C) 3IY concentrations significantly influenced Memory score values. The highest concentration of 5 mg/ml 3IY significantly reduced memory compared to the control group. All other tested concentrations of 3IY had no significant effect with the given sample sizes. (D) Analysis of gender differences of pooled data from B,C,G revealed no gender specific effects of 3IY. Striped and dotted boxes represent females and males, respectively. (E,F) Innate odour avoidance of (E) OCT and (F) BA was not affected by 3IY and/or L-DOPA feeding. (G) In a repetition of the experiment shown in B, feeding L-DOPA in addition to 3IY rescued the 3IYinduced memory impairment. Importantly, L-DOPA alone had no effect on the memory scores. Given this lack of effect of feeding L-DOPA alone, we did not perform an additional control for innate odour preference for this experimental condition. See Figure S7 for Odour preferences underlying the Memory scores, and Figure S8 for a full display of all adult fly behavioural results separated by gender. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

inhibition of the TH enzyme that catalyses the synthesis of L-DOPA. Regarding adult flies, these results are in line with previous studies that showed that 3IY feeding impairs associative learning about ethanol, quinine or electric shock (Kaun et al., 2011; Seugnet et al., 2008; Zhang et al., 2008). Here, we find a similar impairment of learning about optogenetic PPL1-γ1pedc activation. Previously, a constitutive RNA-interference knockdown of TH in PPL1-γ1pedc revealed that punishment learning by PPL1-γ1pedc activation is dependent on dopamine synthesis in this same neuron (König et al., 2018). Using the more acute, albeit systemic approach of feeding 3IY, we provided an independent confirmation of these results (Figure 10). Regarding larvae, genetic approaches have uncovered an important role of dopamine for odour-taste associative learning (Rohwedder et al., 2016; Selcho et al., 2009). This is further supported here by an independent pharmacological approach (Figs 8 and 9). Although not unexpected, these results are interesting in themselves by demonstrating for the first time that an acute inhibition of TH impairs associative learning in larvae. This is critical to disentangle acute effects from potential developmental impairments or their compensation.

Indeed, our experiments demonstrate why drug feeding offers a valuable additional approach to manipulate the dopaminergic system of *Drosophila melanogaster*. It is easy to apply, quick, comparably cheap, and it allows inducing the desired effect shortly before the experiment. The approach also does not require generating new fly strains, but can be easily combined with the use of already available genetic tools. As an example, for the experiments shown in Figure 10 we optogenetically activated a specific dopaminergic neuron, while inhibiting the TH enzyme in a both systemic and inducible manner. In order to perform the same type of manipulation by genetic means alone, one would have to combine at least five

genetic constructs for driving expression of channelrhodopsin-2- XXL in the neuron of interest, as well as of an RNAi against TH in the whole body, plus e.g. a $Gal80^{ts}$ construct to make the expression of the RNAi inducible. Although that is certainly possible, feeding 3IY is the quicker and easier option. Also, the effects of the drugs can be titrated relatively conveniently by adjusting the concentration and the duration of feeding (Figures 7 and 8). This makes it possible to find a trade-off between maximizing the intended effect on learning and memory and minimizing developmental side effects, or effects on locomotion or sensory function. Furthermore, drugs with comparable effects in different organisms allow for elegant translational research across different species.

The obvious drawback of drug feeding in comparison to present genetic tools is the lack of spatial specificity. However, in some situations, this may actually be advantageous, for example when asking whether a newly discovered process is dependent on synthesis of dopamine at all. In this case, drugs can be used as a first screening, followed up by spatially specific genetic approaches (see also Ojelade et al., 2019 preprint). To give an example, using the genetic driver strain TH-Gal4, which then was believed to cover all dopaminergic neurons, Schwaerzel et al. (2003) suggested that dopaminergic neurons were responsible only for punishment, but not reward signalling (see also Schroll et al., 2006, regarding larvae). This was reconsidered about 10 years later, when refined genetic reagents became available showing that TH-Gal4 largely missed a cluster of dopaminergic neurons that do indeed signal reward (Burke et al., 2012; Liu et al., 2012; larvae: Rohwedder et al., 2016). A systemic pharmacological approach could have made the discovery that dopaminergic neurons carry punishment as well as reward signals possible right away.

Taken together, pharmacological approaches like the one used here enrich the neurogenetic toolbox available for *Drosophila* and should be considered by the community when investigating the principles of dopaminergic system function.

General discussion

Already Solomon and Corbit noted in 1974 that the same event can evoke affective states of opposite valence. More specifically, the beginning and termination of an aversive or appetitive event induce different affective states (for more details see Figure 1 and 'General introduction - Timing is crucial: Timing-dependent valence reversal'). In the context of associative learning, such timing-dependent valence reversal has been studied mainly using aversive USs, and there are fewer data using appetitive USs (for an overview of data in *Drosophila* see Table 4**;** humans, rats and bees: Andreatta et al., 2010, 2012; Hellstern et al., 1998; Luck and Lipp, 2017; Mohammadi et al., 2014; Urushihara, 2004). The present work in Chapter I provides the most detailed account yet of the parametric features of timing-dependent valence reversal in the appetitive domain by investigating learning about the onset and offset of a central brain reward signal in larval *Drosophila*. Using optogenetic activation of the reward mediating DAN-i1^{864}, *Drosophila* larvae showed opponent memories depending on when the odour presentation started relative to optogenetic activation of DAN-i1^{864}. When the odour was presented before optogenetic activation of DAN-i1^{864}, larvae showed reward memory indicated by approach to the trained odour (Figure 4B; Saumweber et al., 2018). On the other hand, when the odour was presented at the end of optogenetic DAN-i1^{864} activation, larvae showed frustration memory indicated by avoidance of the trained odour (Figure 4C; Saumweber et al., 2018). In addition to this, Chapter I shows the dynamics of both reward and frustration memory. The results are in line with the opponent-process theory (for more details see Figure 1 and 'General introduction - Timing is crucial: Timing-dependent valence reversal') claiming that processes connected with the termination of an event are strengthened with repetition while processes connected to the beginning of an event are not (Solomon and Corbit, 1974). Indeed, after one training trial only reward memory was observed whereas after three training trials both reward and frustration memory were observed (Figure 4B,C vs 4F,G). Similar to repetition, also longer-lasting activation of DAN-i1^{864} increased only frustration memory but not reward memory (Figure 4D,E).

Timing-dependent valence reversal in *Drosophila*

Several studies in larval and adult *Drosophila* investigated timing-dependent valence reversal by testing different ISIs for USs of both the aversive and appetitive domain (for an overview

see Table 4). Collectively these studies suggested that the ability to detect timing-dependent valence reversal depends on several factors.

In larval *Drosophila*, testing multiple ISIs for an appetitive US, more specifically using optogenetic activation of DAN-i1^{864}, resulted in reward and frustration memory (Saumweber et al., 2018; Figure 4 B,C). For none of the other known rewarding DANs, and neither for any of the 'real-world' rewards used in larvae, an ISI curve was investigated so far. Using activation of the APL neuron, which has been shown to have a rewarding effect, resulted in reward memory only for an ISI of 0 sec and in no memory for other ISIs tested (Mancini et al., 2023). Regarding aversive USs, punishment and relief memory were observed using optogenetic activation of DAN-f1^{2180} or electric shock (Khurana et al., 2009; Weiglein et al., 2021). Using heat shock or the optogenetic activation of another punishing DAN, DAN-d1, resulted in punishment memory but not relief memory (Khurana et al., 2012; Weiglein et al., 2021).

Likewise in adult *Drosophila*, regarding timing-dependent valence reversal in the appetitive domain, only optogenetic activation of a subset of rewarding DANs but not of single DANs resulted in reward and frustration memory (Aso and Rubin, 2016; Handler et al., 2019). Punishment and relief memory were shown using as US electric shock (Diegelmann et al., 2013a; Jacob and Waddell, 2020; König et al., 2018; Luck and Lipp, 2017; Tanimoto et al., 2004; Yarali et al., 2008, 2009) or activation of single DANs or subsets of DANs (Aso and Rubin, 2016; Handler et al., 2019; König et al., 2018) and also using visual cues as CS and electric shock as US (Vogt et al., 2015). Note that there are also DANs that only mediate punishment memory or only reward but not relief or frustration memory (Aso and Rubin, 2016; König et al., 2018). The opposite case, i.e., any real-world US or any DAN or set of DANs mediating only relief memory or only frustration memory, has never been observed.

Overall, the data in larval and adult *Drosophila* demonstrates that some but not all DANs and not all types of USs induce timing-dependent valence reversal. Furthermore, in cases where timing-dependent valence reversal is observed different stimuli can have different time scales and absolute memory values but share a general form of the function. In these cases, the parametric analysis of the memories established through the occurrence/termination of a reward/punishment revealed different dynamics (see also 'Chapter I – Discussion') and point to important boundary conditions for detecting timing-dependent valence reversal, and specifically effects of US termination:

(1) *Timing between US and CS*: The ISI is depending on the used USs and CSs. For example, when heat is used as US, the time between both stimuli needs to be longer than when the optogenetic activation of DANs is used as a US because the heat needs more time to act (adults: Aso and Rubin, 2016; Diegelmann et al., 2013b; Handler et al., 2019; Jacob and Waddell, 2020; König et al., 2018; Niewalda et al., 2015; Tanimoto et al., 2004; Yarali and Gerber, 2010; Yarali et al., 2008, 2009) (larvae: Khurana et al., 2009, 2012; Saumweber et al., 2018; Weiglein et al., 2021). In most studies, the ideal ISI for forward conditioning was shorter than for backward conditioning. This means that to detect a timing-dependent valence reversal effect multiple forward and backward ISIs should be tested and not just single time points. Furthermore, it was shown that backward conditioning does not work when US and CS overlap (adults: König et al., 2018; Tanimoto et al., 2004; Vogt et al., 2015) (larvae: Saumweber et al., 2018; Weiglein et al., 2021), while forward conditioning works regardless of an overlap only in adult *Drosophila* but not larval *Drosophila* (adults: König et al., 2018; Niewalda et al., 2015; Tanimoto et al., 2004) (larvae: Khurana et al., 2009; Saumweber et al., 2018; Weiglein et al., 2021).

(2) *Duration of the US*: A longer duration of the US can lead to stronger effects especially after backward pairing. Note that also this effect is depending on the type of stimuli (adults: König et al., 2018) (larvae: Figure 4D,E; Weiglein et al., 2021).

(3) *Number of training trials*: As Solomon and Corbit (1974) stated within their opponent process theory, data suggests that especially the backward process gets stronger with repetition. This is in line with the observation that backward memories were observed in the majority only after multiple training trials (adults: Diegelmann et al., 2013a; Jacob and Waddell, 2020; König et al., 2018; Niewalda et al., 2015; Tanimoto et al., 2004; Vogt et al., 2015; Yarali and Gerber, 2010; Yarali et al., 2008, 2009) (larvae: Figure 4B,C vs 4F,G; Khurana et al., 2009; Saumweber et al., 2018; Weiglein et al., 2021) and after a single training trial exclusively in adults using optogenetic DAN activation (Aso and Rubin, 2016; Jacob and Waddell, 2020; König et al., 2018; Naganos et al., 2022).

(4) *When to test the memory*: Backward memories are only detectable for a short retention time while memories after forward conditioning last longer (adults: Diegelmann et al., 2013a; Jacob and Waddell, 2020; König et al., 2018; Yarali et al., 2008) (larvae: Figure 4H,I; Weiglein et al., 2021).

(5) *Type of CS and US:* As already mentioned above, depending on the type of stimuli the timing of both needs to be considered and the stimuli must be chosen in such a way that it is

Table 4. Overview of studies of opponent memories in larval and adult *Drosophila*.

CS	US Aversive/appetitive	Observed memories	Reference
Larvae			
Odour	Heat shock	Punishment	Khurana et al., 2012
Odour	Electric shock	Punishment; relief	Khurana et al., 2009
Odour	Optogenetic activation of DAN-d1	Punishment	Weiglein et al., 2021
Odour	Optogenetic activation of DAN-f1^{2180}	Punishment; relief	Weiglein et al., 2021
Odour	Optogenetic activation of DAN-i1^{864}	Reward; frustration	Saumweber et al., 2018
Adults			
Odour	Electric shock	Punishment; relief	Diegelmann et al., 2013a; Jacob and Waddell, 2020; König et al., 2018; Niewalda et al., 2015; Tanimoto et al., 2004; Yarali and Gerber, 2010; Yarali et al., 2008, 2009
Visual cues	Electric shock	Punishment; relief	Vogt et al., 2015
Odour	Optogenetic activation of PPL1-01	Punishment; relief	Aso and Rubin, 2016; König et al., 2018
Odour	Optogenetic activation of PPL1-03	Punishment	Aso and Rubin, 2016; König et al., 2018
Odour	Optogenetic activation of PPL1-06	Punishment	König et al., 2018
Odour	Optogenetic activation of PAM-12	Punishment	König et al., 2018
Odour	Optogenetic activation of PPL-DANs	Punishment; relief	Handler et al., 2019
Odour	Optogenetic activation of MB043C	Reward	Aso and Rubin, 2016
Odour	Optogenetic activation of MB213B	Reward	Aso and Rubin, 2016
Odour	Optogenetic activation of MB315C + MB109B	Reward	Aso and Rubin, 2016
Odour	Optogenetic activation of PAM-DANs	Reward; frustration	Handler et al., 2019

possible to detect oppositely-valenced memories (for more details see 'Introduction - Timing is crucial: Timing-dependent valence reversal'). In addition, especially when using aversive USs, it is important that they are not chosen too strongly to avoid effects such as an amnestic knockout.

Different timing? Different memories!

As mentioned earlier, different types of memories are observed depending on the timing of CS and US. Forward pairing of an appetitive/aversive stimulus leads to reward/punishment memory whereas backward pairing leads to frustration/relief memory (Figure 4; Table 4). Thus, depending on the timing of the two stimuli, the CS is associated with something positive or negative, even when the same US is used (see also discussion in Box-1 of Gerber et al., 2014). Interestingly, however, it seems that a view more nuanced of training effects with different ISIs than just by forward and backward pairing should be adopted. That is, there seem to be at least five different types of timing between CS and US that should be distinguished (Figure 11; for underlying mechanisms see next chapter).

First, in *forward trace conditioning* the CS is presented before the US, with no overlap of the two stimuli but a temporal gap between CS and US. In this case, the CS is predicting the occurrence of the US. Using aversive USs in a trace conditioning paradigm resulted in punishment memory (*Drosophila*: Galili et al., 2011; Shuai et al., 2011; Tomchik and Davis, 2009; reviewed in: Dylla et al., 2013) (vertebrates: Misane et al., 2005; Woodruff-Pak and Disterhoft, 2008). Studies in rats, bees or moths resulted in reward memory using appetitive USs (Ito et al., 2008; Miyazaki et al., 2011; Szyszka et al., 2011) (note that in the vertebrate literature also the term 'delayed reward' is used for this type of conditioning).

Second, in *forward delay conditioning* the beginning of the CS presentation starts slightly before presentation of the US resulting in overlapping of both stimuli and often their co-termination. Here, the CS is associated with the beginning of the US. As in trace conditioning, forward delay conditioning using aversive stimuli results in punishment memory (for data in *Drosophila* see Table 4; vertebrates: Fendt and Fanselow, 1999; Johansen et al., 2011) whereas using appetitive stimuli results in reward memory (for data in *Drosophila* see Figure 4 and Table 4; vertebrates: Koch et al., 1996; Schneider and Spanagel, 2008).

Third, in *simultaneous conditioning* the presentation of CS and US start and often terminate at the exact same time. Thus, the CS is associated with the presence of the US. As in trace

conditioning and forward delay conditioning, simultaneous conditioning results in reward or punishment memory using appetitive or aversive stimuli, respectively (adults: König and Gerber, 2022; Shuai et al., 2011; Yarali and Gerber, 2010) (larvae: El-Keredy et al., 2012; Schleyer et al., 2015a; Weiglein et al., 2019) (vertebrates: Burkhardt and Ayres, 1978; Heth and Rescorla, 1973; Madden et al., 2016; Rescorla, 1980). Although Pavlov's studies assumed that simultaneous conditioning works less good or not at all (Pavlov, 1927), the standard odour-learning paradigm in larval *Drosophila* as described in Chapter II and particular the sugar-learning experiments in adult *Drosophila* (König and Gerber, 2022; Shuai et al., 2011; Yarali and Gerber, 2010) represent simultaneous conditioning paradigms and show that this form of conditioning works well. However, the different ISI curves in *Drosophila* reveal mostly lower memory strength for simultaneous presentation of US and CS (ISI = 0) than for delay conditioning (for underlying studies see Table 4; see also Rescorla, 1988).

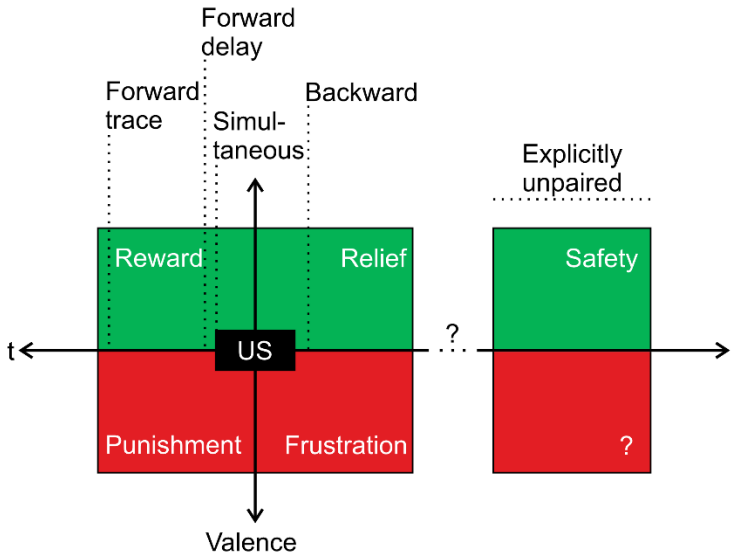


Figure 11. **Memories of different valences depending on the type of US and the timing of CS and US.** Using appetitive or aversive USs different type of memories are established depending on the time interval between US and CS. The stippled line represents beginning of the CS. Using aversive USs one can observe an aversive punishment memory after forward trace and delay and simultaneous conditioning, but appetitive relief memory after backward conditioning and appetitive safety memory after explicit unpairing of CS and US. When using appetitive USs, after forward trace and delay and simultaneous conditioning an appetitive reward memory is observed whereas after backward conditioning an aversive frustration memory is observed. For the aversive memory observed after explicit unpairing of an appetitive US and a CS, to date, no explicit term in the literature was established.

Fourth, in *backward conditioning* the CS is presented after US termination, that is with no overlap between both stimuli (backward conditioning with an overlap of CS and US usually do not result in memory formation; adults: König et al., 2018; Tanimoto et al., 2004; Vogt et al., 2015; larvae: Saumweber et al., 2018; Weiglein et al., 2021). In such backward conditioning, the CS thus is associated with the after-affect that is caused by the termination of the US. When using aversive USs one can observe relief memory (for data in *Drosophila* see Table 4; vertebrates: Andreatta et al., 2010; Mayer et al., 2018; Navratilova et al., 2015) whereas when using appetitive USs frustration memory is observed (for data in *Drosophila* see Table 4; vertebrates: Seitz et al., 2022; Urushihara, 2004). Note that in the vertebrate literature, instead of frustration, the term "reward loss" is also used (reviewed in: Ortega et al., 2017), whereas frustration is also defined as the consequence of the decrease or omission of an expected appetitive reward (Amsel, 1992) and is mostly investigated using the successive negative contrast paradigm (Pellegrini et al., 2004).

Fifth, in *explicit unpairing* CS and US are presented unpaired, i.e., with a long temporal interval between presentation of CS and US and therefore without overlap of both stimuli. In this type of conditioning, the CS is associated with the non-occurrence of the US. Using aversive USs an appetitive safety memory is observed after explicit unpairing (*Drosophila*: Jacob and Waddell, 2020; Naganos et al., 2022) (vertebrates: Mohammadi et al., 2014; Pollak et al., 2010; reviewed in: Christianson et al., 2012). Regarding appetitive USs, in larval *Drosophila* it was found that explicitly unpaired training with either taste rewards or the activation of DAN-i1^{864} leads to an aversive memory (Schleyer et al., 2018, 2020). In vertebrates, however, this type of memory is not very well studied and yet no term has been established for memories seen after explicit unpairing of a reward.

Underlying mechanisms

Little is known in larvae, but studies in adults and other animals allow some insights into the underlying mechanisms of the five different types of conditioning mentioned above. Trace and delay conditioning represent different types of forward conditioning, where the presentation of the CS starts before the presentation of the US. Both forms of conditioning can reach the same memory strength, although trace conditioning (where there is a temporal gap between CS and US) usually needs more training trials and has a higher sensitivity to distraction (adult *Drosophila*: Galili et al., 2011; Grover et al., 2022) (vertebrates: Han et al., 2003). This may be

due to the fact that in delay conditioning CS and US presentation do overlap, whereas in trace conditioning this is not the case, so that a neural representation of the CS must be maintained after its termination. In adult *Drosophila,* different molecular basis for trace and delay conditioning were shown. Rutabaga adenylyl cyclase is dispensable in trace but not delay conditioning (Dudai et al., 1983; Duerr and Quinn, 1982), inhibition of the Rac expression in MB enhances trace conditioning whereas Dop1R1 receptor mutants do not show trace conditioning (Shuai et al., 2011; reviewed in: Dylla et al., 2013). Also, a study using a visual conditioning paradigm in flies demonstrated different roles of dopamine receptors of the ring neurons in trace and delay conditioning: flies with impaired Dop1R1 receptors had impairment in both types of conditioning, impairment of Dop1R2 had no effect and flies with impaired Dop2R showed only delay conditioning (Grover et al., 2022). Moreover, studies in mammals show some differential involvement of brain regions in trace and delay conditioning. Both forms of conditioning share the requirement for the respectively task-specific brain regions, but trace conditioning additionally requires the hippocampus (Beylin et al., 2001; Knight, 2004; McEchron et al., 1999), which may serve the neural representation of the CS. Moreover, serotonergic neurons of the dorsal raphe nucleus showed increased firing while rats were waiting for a reward suggesting that higher activation of serotonergic neurons enables longer waiting (Miyazaki et al., 2011). In summary, although trace and delay conditioning enable the formation of memories of the same valence and the same types of CS and US, different neuronal mechanisms are required to establish these forms of memory.

Also, backward conditioning and explicit unpairing establish memories of the same, i.e. appetitive, valence by using the same types of USs and CSs. However, a backward paired CS is associated with the after-affect that results from US termination of the US whereas an explicitly unpaired CS predicts the absence of the US. The absence or termination of an aversive stimulus has different meanings as it has different consequences for the individual in nature. In both types of conditioning, the presentation of the CS begins after the onset of the US presentation and between both stimuli must be a temporal gap. This raises the question how long this temporal gap between the US and CS must be for the CS to be associated with the absence rather than the after-affect caused by termination of the US, so when it is explicit unpairing and not backward pairing? This question cannot be answered easily. Clearly this depends on the type of experimental procedure, the model organism and the used US. Moreover, it is not clear whether the difference between backward and relief learning is only

quantitative in nature, meaning that it can be disentangled by studying multiple ISIs using the same paradigm (similar to forward trace and delay conditioning), or whether the difference is qualitative in nature, meaning that different types of paradigms, e.g., massed versus spaced training, are required to explore it.

That relief and safety memories are indeed distinct is, however, reflected by the involvement of different brain regions in vertebrates. Studies in rats demonstrated that the nucleus accumbens and specifically dopaminergic neurons of the posterior medial ventral tegmental area that are projecting into the medial shell region of the nucleus accumbens are necessary for relief memory but not safety learning (Bruning et al., 2016; Josselyn et al., 2005; Mayer et al., 2018; Mohammadi et al., 2014). Safety learning on the other hand, involves the basolateral amygdala, prelimbic and infralimbic cortex and the bed nucleus of the stria terminalis (Foilb et al., 2021; Kreutzmann et al., 2020; Sangha et al., 2013; reviewed in: Kong et al., 2014). Data in *Drosophila* comparing the underlying mechanisms of safety and relief memory are difficult to interpret because of the diversity of paradigms used and the consequently ambiguous use of the terms relief and safety (Jacob and Waddell, 2020; König et al., 2018; Naganos et al., 2022). Nevertheless, also for backward conditioning and explicit unpairing it is true that both forms of conditioning enable the formation of memories of the same valence with different underlying neuronal mechanisms.

Overall, trace, delay, simultaneous, backward conditioning and explicit unpairing are distinct types of conditioning that differ in the timing of the presentation of CS and US. Although the behavioural read-out of these memories varies only between two valences (appetitive memories: reward, relief and safety memory; aversive memories: punishment and frustration memory) they are based on different neural mechanisms displayed by the involvement of different brain regions and mechanisms.

How timing-dependent valence reversal comes about

The opponent-process theory proposed by Solomon and Corbit in 1974 offers a theoretical framework to the experiments performed in Chapter I. However, this theory does not serve an explanation of how the associations between the US and a CS that signals either the beginning or termination of reward/punishment are build. For this it is worth to look into the classical conditioning experiments from Pavlov (1927) where he already tested backward conditioning as well as simultaneous, forward delay and trace conditioning. Based on his

observations that responses were higher after delay than trace conditioning and that no responses were observed after simultaneous presentation of US and CS, he concluded that temporal proximity of the conditioned stimuli (contiguity) is an important factor in the formation of associations. Thus, forward conditioning was best when there was a slight temporal asynchrony between CS and US and relatively poor when the temporal gap was too short or too long. Furthermore, based on the finding that simultaneous and backward conditioning did not elicit a response, it was concluded that the CS must have predictive value for the organism (contingency). More precisely, positive contingency was defined as the probability that the US occurs in the presence of the CS but not in its absence, so that the CS becomes a predictor of the US. Consequently, the individual learns that the CS causes the US. Negative contingency, on the other hand, means the opposite: since the US occurs in the absence of the CS but not in its presence, the CS becomes a predictor that the US does not occur, and thus the individual learns that the CS does not cause the US. The rationale was, that the CS achieves a predictive value and will be learned only if the probability of the presence versus absence of the US is different. In sum, based on Pavlov's initial findings, two important variables for associative learning were claimed: contiguity and contingency (reviewed in: Schultz, 2006, 2015).

Qualitative learning theories: Contiguity or contingency?

The traditional associative learning theories (Mackintosh, 1975; Pearce and Hall, 1980; Rescorla, 1972) from the 1960s proclaimed *contingency* as the most important factor. These theories were mostly driven by the observation of the so-called Kamin-blocking effect (Kamin, 1969) and the absence of memories after backward conditioning (reviewed in: Cautela, 1965). In blocking experiments as introduced by Leon Kamin, animals show no response to a CS (CS2) when it is trained in a compound with another CS (CS1) that had been previously paired with the US. Thus, if the US is presented along with CS1 and CS2 as a compound, the US will not be unexpected for the animals because it is already expected through CS1 and therefore no learning will occur for CS2. Time, however, was not considered as an important factor in the traditional associative learning theories and it was assumed that the temporal information itself does not become part of the association but only serves as a facilitator for conditioning. The rationale was that good temporal contiguity facilitates the association of CS and US only if the CS has predictive value.

However, the existence of associative memories after forward, backward and simultaneous conditioning provide evidence that contingency is not the only important factor (see also previous chapter: 'Different timing? Different memories!'). Indeed, already Pavlov's experiments demonstrated the importance of time: when dogs were trained in a delay conditioning paradigm, it was observed that the dogs' salivation on CS presentation shifted away from the time of CS onset more toward the time of US onset. Thus, the longer the interval between the presentation of CS and US, the more delayed was the salivation. This demonstrates that animals not only learn that an outcome will occur, but also when it will occur. Associations are not just simply mental links between two events, but the probability of their joint occurrence and their specific temporal relationship must be considered.

Thus, since the 1980s other approaches claimed *contiguity* as the most important factor (Gallistel, 1990; Gibbon and Balsam, 1981; Sutton and Barto, 1990), first of all the temporal coding hypothesis (Arcediano and Miller, 2002; Matzel et al., 1988; Miller and Barnet, 1993; reviewed in: Molet and Miller, 2014; Savastano and Miller, 1998). This theory assumes that contiguity alone is necessary and sufficient for the formation of an association and that the temporal relationship between events is automatically encoded as part of the association. Additionally, learning and performance were seen as distinct phenomena, meaning that not all learned associations lead to performance in the test, which is also due to the type of testing. It was assumed that learning already occurs when two stimuli have good temporal proximity, regardless of the predictive value of the stimulus, but that the predictive value is necessary to see a performance in the test. Thus, the missing effects after, e.g., simultaneous and backward conditioning were seen as deficits in performance rather than deficits in the formation of an association. These ideas were driven by studies using higher-order forms of associations, namely Second-order conditioning and Sensory preconditioning (for more information and data in larval *Drosophila* see 'Appendix - Technical summary of higher-order forms of learning in larval *Drosophila*'). These forms of conditioning consist of two training phases and allowed to solve the problem of the deficit in performance. For example, in a Second-order conditioning paradigm, in a first training phase a CS1 is paired with the US followed by a second training phase in which CS1 is presented together with CS2 as a compound. Interestingly, associative memories for both CSs can be observed in the test, although CS2 was itself never paired with the US and thus there was no predictive relationship. When researchers varied the timing of CS and US during the first phase of a Second-order conditioning phase, they found effects for

a number of phenomena of conditioning: forward delay, forward trace, simultaneous and backward conditioning, blocking, overshadowing and latent inhibition (Barnet et al., 1993, 1997; Blaisdell et al., 1998; Cole et al., 1995; Lubow and Moore, 1959). The fact these higher-order forms of learning exist at all, such as Sensory preconditioning and Second-order conditioning, is an important indication that it is not only a mere predictive relationship with any US that leads to an association but that even a stimulus that itself was never rewarded and thus had no biological significance at the time of training can elicit a conditioned response. Another important statement by the temporal coding hypothesis is that the temporal information acquired during training determines the magnitude and timing of the conditioned response. Thus, both temporal orders (forward and backward pairing) should provide a good temporal relationship and should lead to associative learning.

Overall, the traditional associative learning theories give an explanation of forward learning but fail to explain backward learning. However, the temporal coding hypothesis provides good theoretical explanations at a qualitative level for the fact that and how associations are formed in forward and also backward learning, but it does not provide formalized mathematical terms for it. For this, one must look into the quantitative learning theories.

Quantitative learning theories: The prediction error

One approach to a formalized mathematical explanation of conditioning phenomena is the prediction error theory established by Wagner and Rescorla (1972). According to this theory, learning occurs to the extent that a reinforcer was unpredicted. Consequently, learning takes place as long as there is a discrepancy between the actual US and its prediction, and slows down the more the US is predicted. These ideas were supported by observations that the prediction error is displayed in the neuronal response: first and foremost, in vertebrates it was shown that DANs in the ventral tegmental area and substantia nigra show higher activation (over baseline level) when a reward is better than predicted, depression when a predicted reward is omitted or worse than predicted and no response when a fully predicted reward is received at the predicted time, in kind and strength (Bayer and Glimcher, 2005; Fiorillo et al., 2003; Pan, 2005; Schultz et al., 1993; Tobler et al., 2005; reviewed in: Schultz, 1998, 2015). Similar for conditioning with punishments, prediction errors can be observed in distinctly different brain regions (for more details see: Garrison et al., 2013; Gueguen et al., 2021; Roy et al., 2014).

Not only in vertebrates, but also in *Drosophila*, modelling studies suggest the presence of a prediction error that may be implemented in e.g. the MB within the MBON to DAN connections (Bennett et al., 2021; Juergensen et al., 2022 preprint; Zhao et al., 2021; reviewed in: Adel and Griffith, 2021; Horiuchi, 2019). However, the prediction error theory works on the basis of the predictive value of the US and treats learning and prediction at the level of trials. Thus, in its initial form this theory is unable to explain the temporal structure of prediction (errors) over several trials and cannot explain for example Second-order conditioning, Sensory preconditioning or memories brought by different timings of US and CS (Sutton, 1988; Sutton and Barto, 1981; reviewed in: Miller et al., 1995).

To overcome these difficulties, the theory of temporal difference learning serves another approach (Sutton, 1988; Sutton and Barto, 1981). This theory takes into account the relative timing of events within each trail. It considers the quantitative difference between received and predicted US value within each trial, rather than only between trials like the Rescorla-Wagner model. According to this theory, a stimulus induces a prediction error whenever it provides new information about future US expectations which means that not only US delivery or non-delivery elicit prediction errors. Thus, US expectations are changed throughout the whole trial whenever a stimulus provides new information about future US expectations, for example a CS that is presented after the US as it is the case in backward conditioning. Altogether, the theory of temporal difference learning is able to explain backward learning and other phenomena of conditioning that cannot be explained by the prediction error theory stated by Wagner and Rescorla (1972).

In summary, temporal difference learning provides a formalized mathematical approach for explaining how associations are formed in forward and also backward learning whereas the prediction error theory by Wagner and Rescorla provides no explanation for associations formed by backward conditioning.

How timing-dependent valence reversal is implemented on a cellular level

The observation of reward/ frustration learning and punishment/ relief learning by activation of a single neuron (larvae: Figure 4; Saumweber et al., 2018; Weiglein et al., 2021) (adults: Aso and Rubin, 2016; König et al., 2018) leads to the question what is actual happening within the

brain: how is timing-dependent valence reversal implemented on a cellular level? As mentioned previously (see also 'General introduction - Behind the scenes: molecular mechanisms of learning'), the MB influences the observed behaviour in such a way that during learning synaptic plasticity takes place at the synapse from the KC to the respective MBON, thereby altering the balance between approach and avoidance (Figure 12). This is thought to be done with the help of the AC, which acts as a molecular coincidence detector of odour- and shock-initiated signalling at the KC (Gervasi et al., 2010; Livingstone et al., 1984; Tomchik and Davis, 2009). The further course of the AC-cAMP-PKA-cascade finally leads to phosphorylation of multiple proteins that can impact cellular or synaptic plasticity. Importantly, whether this will induce a depression or potentiation of the connection between two neurons would depend on the net effect that the phosphorylation of these multiple proteins, plus other proteins modulated by other kinases, would have. At least in theory forward and backward conditioning can affect the AC-cAMP-PKA cascade and plasticity in opposite ways (Heisenberg, 2003; Yarali et al., 2012). However, mutations in the AC seem to have selective effects on punishment- but not relief-learning (personal communication with Thomas Niewalda), providing no experimental support to this idea.

Plasticity of the same synapse: Spike-timing-dependent plasticity

An alternative explanation is offered by the model of spike-timing-dependent plasticity (STDP; Brzosko et al., 2019; Liu et al., 2021; Pawlak, 2010) which additionally includes causality when determining the direction of synaptic plasticity (Bi and Poo, 1998; Masuda and Kori, 2007; Vogt and Hofmann, 2012). STDP is based on the Hebbian rule paraphrased by Shatz in 1992: 'what fires together, wires together' (Shatz, 1992). More precisely Hebb stated that repeated activation of a presynaptic cell before the postsynaptic cell spikes induces long-term potentiation (LTP) (Hebb, 1949). However, STDP assumes that not only LTP, but also long-term depression (LTD) is induced depending on the spike order (presynapse vs postsynapse) on a millisecond-scale.

Applied to the experiments presented in Chapter I, based on the findings that the KC-to-MBON synapses undergo LTD in associative olfactory learning (Hige et al., 2015b; Perisse et al., 2016) it can be assumed that the KC (activated by the odour) represents the presynapse whereas the MBON (in this case MBON-i1, avoidance promoting; Eschbach et al., 2021) represents the postsynapse and forward and backward learning could be explained as follows.

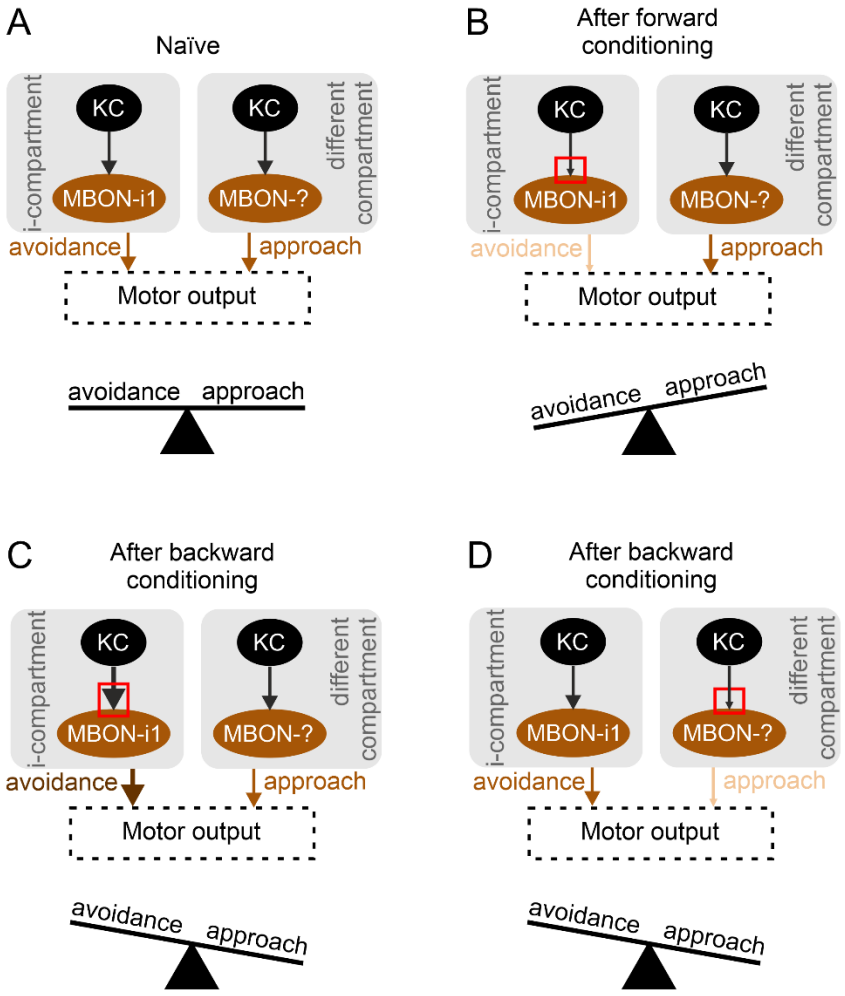


Figure 12. How timing-dependent valence reversal could be implemented in the mushroom body. Displayed is a simplification of the KC-to-MBON connection within the i-compartment and another compartment and the resulting motor output. See Figure 3 for a more detailed circuit of the MB. (A) In a naïve animal avoidance and approach is balanced between the MBONs, thus larvae show the innate behaviour towards an odour. (B) After forward training with DAN-i1 activation, larvae approach the odour in the test (reward memory) due to LTD at the KC-to-MBON-i1 synapse and thus less signalling of avoidance by MBON-i1. (C) After backward training with DAN-i1 activation, the connection between the KC and MBON-i1 is strengthened (LTP) and thus larvae avoid the odour in the test (frustration memory) because avoidance tendencies signalled by MBON-i1 prevail. (D) An alternative explanation of how backward learning is implemented in the mushroom body is based on the post-inhibitory rebound effect in DANs (see text for further details). Briefly, the end of the optogenetic activation of DAN-i1 releases another DAN (with opposite valence) from inhibition and leads to its activation. This would lead to depression of the KC-to-MBON-? synapse (LTD), resulting results in less approach, causing the larvae to avoid the odour in the test.

In forward conditioning, presentation of the odour results in activation of the KC that in turn leads to activation of the MBON-i1. Shortly afterwards DAN-i1^{864} is optogenetically activated which also leads to activation of the MBON-i1 and the KC. Because in this case the presynapse (KC) is activated before the postsynapse (MBON-i1), LTD takes place at the KC-to-MBON-i1 synapse and approach behaviour is observed (Figure 12B). For the case of backward

conditioning, the direct connection from DAN-i1^{864} to MBON-i1 found out in the electron microscope comes into place (Eichler et al., 2017). Thus, in backward conditioning the optogenetic activation of DAN-i1^{864} comes first and leads to activation of MBON-i1 and the KC. The following odour presentation leads again to KC activation. If it is the case that the postsynapse (MBON-i1) is activated before the presynapse (KC) LTP would take place at the KC-to-MBON-i1 synapse and avoidance behaviour is observed (Figure 12C). However, this is only true under one important prerequisite: the DAN-to-MBON-i1 activation is faster than the DAN-to-KC activation. However, this was so far experimentally not investigated and needs to be further tested. Also, whether and what role KC output plays during conditioning would need to be checked. Work in adult *Drosophila* suggests that KC output is dispensable for at least the formation of aversive short-term memories (Dubnau et al., 2001; McGuire et al., 2001; Pribbenow et al., 2022; Schwaerzel et al., 2002). If this is indeed the case, it suggests that postsynaptic activations (i.e. activation of the MBON) could occur via the DAN-to-MBON connection (Takemura et al., 2017).

Overall, the change of the KC-to-MBON-i1 synapse could implement a reward memory after forward pairing with DAN-i1^{864} activation and to frustration memory after backward pairing with DAN-i1^{864} activation due to the altered balance of approach-promoting and avoidance-promoting MBONs. For DAN-f1^{2180} (Weiglein et al., 2021) the same could be assumed only with reversed valences of the DAN and MBON and thus the change of the KC-to-MBON-f1 synapse leads to avoidance of the trained odour after forward pairing with DAN-f1^{2180} activation and in approach of the odour after backward pairing with DAN-f1^{2180} activation. Thus, opposing types of plasticity at the same KC-to-MBON synapse would lead to memories of opposite valence.

Importantly, the general assumption of STDP is that LTP is triggered when the presynapse spikes before the postsynapse, and LTD when the postsynapse spikes before the presynapse (reviewed in: Brzosko et al., 2019) which is thus the exact opposite of the proposed model. However, this 'reversed' STDP could be achieved by neuromodulation, meaning all kind of changes (reversible) of the functional properties of neurons and synapses that are induced by signalling molecules like neurotransmitters (for review see: Brzosko et al., 2019; Pawlak, 2010). These changes can have impact on a whole network, synapses or only at intracellular level. Studies in vertebrates for example demonstrated dopaminergic or serotonergic modulation of STDP (Dale et al., 2014; Lahiri and Bevan, 2020; Zhang et al., 2009). In adult *Drosophila*, Aso

and colleagues (2019) saw a modulatory effect on learning by the co-transmitter nitric oxide: activating a subset of punishing or rewarding DANs lead to memories of inverted valence even when the dopamine synthesis was downregulated within the whole system, an effect that was rescued by restoring the dopamine synthesis within the activated DANs. Additionally, there is evidence in locusts and adult Drosophila that synapses made by Kenyon cells onto downstream targets undergo STDP (Cassenaer and Laurent, 2007; Cohn et al., 2015). Cohn and colleagues (2015) showed bidirectional modulation of the *same* KC-to-MBON connection in adult Drosophila: while paired activation of KC and DAN lead to depression of the KC-to-MBON synapse, the unpaired activation of KC and DAN strengthened the same KC-to-MBON synapse.

Another general assumption for STDP is that the temporal correlation of KC activation and MBON activation must be within a millisecond-scale. However, in the experiments of Chapter I, the exact time of when the odour is sensed by the larvae and therefore KCs are spiking is unknown, but most likely not within milliseconds. It was also observed that odour responses in KCs are sparse and long-lasting (Ito et al., 2008; Murthy et al., 2008; Turner et al., 2008). In addition, it is not clear when the light-activated ion channel ChR2-XXL returns to the closed state and thus the activation of the DAN stops. It has been reported that the channel closes with a time constant of several seconds (Dawydow et al., 2014). Thus, a temporal correlation of milliseconds is most likely not achieved. However, behavioural experiments show that animals can learn to associate a stimulus and reward that are presented seconds apart. Against the general assumption that the temporal correlation must be within a millisecond range, Drew and Abbott (2006) demonstrated in a simulation that sustained activity that decays slowly can enable STDP on a time frame larger than milliseconds. This 'delayed' STDP could again be achieved by neuromodulation (for review see: Brzosko et al., 2019; Pawlak, 2010). For example a study in adult *Drosophila* suggests the role of the serotonergic DPM neuron in regulating the coincidence time window of STDP by providing inhibitory feedback to KCs (Zeng et al., 2023).

Overall, STDP gives reliable explanations of how these opposite memories can be implemented on the same MBON. However, further studies are needed to validate these assumptions in *Drosophila*.

Plasticity at different synapses: Post-inhibitory rebound

Another alternative explanation of how timing-dependent valence reversal is implemented on a cellular level is based on the post-inhibitory rebound effect as seen in neurons in vertebrates. It was demonstrated that neurons that were inhibited by punishment show rebound activation upon the termination of the punishment which in turn results in activation of reward coding areas (Becerra et al., 2013; Budygin et al., 2012; Navratilova et al., 2012). Simply put, the hypothesis about the post-inhibitory rebound effect is based on connections between DANs and assumes that activation of a DAN 'A' leads to inhibition of a DAN 'B', with the consequence that when activation of 'A' terminates, inhibition of 'B' also ends, leading to rebound firing of 'B' (Adel and Griffith, 2021; König et al., 2018).

Applied to the experiments presented in Chapter I, the explanation of forward learning as explained in the previous section remains the same, however, backward learning is explained differently. Overall, it is based on the assumption that activation of DAN-i1^{864} leads to inhibition of another opposite valenced DAN (punishing DAN). In backward conditioning, termination of the DAN-i1^{864} activation leads to termination of the inhibition of the punishing DAN, which in turn starts spiking. If this rebound activation of the punishing DAN coincides together with KC activation by the presentation of the odour, LTD takes place at the KC-to-MBON synapse (in this case an approach promoting MBON connected to the punishing DAN) and thus, larvae show avoidance of the odour (Figure 12D). The same logic can be applied for memories brought by DAN-f1^{2180} (Weiglein et al., 2021) activation with reversed valences of DANs and MBONs. To sum up, in comparison to the explanation through STDP where the formation of reward/frustration and punishment/relief memories was based on different types of plasticity at the same KC-to-MBON synapse (MBON-i1 or MBON-f1), here the memory formation in both cases is based on LTD - but at *different* KC-to-MBON synapses, and in different compartments.

As mentioned before, the explanation based on the post-inhibitory rebound is assuming connections between DANs. Electron microscope data of larval *Drosophila* showed no direct connections between DANs, however, indirect connections via the MBON layer or directly via the US were shown that could serve for inhibition of an oppositely-valenced DAN (Eichler et al., 2017; Eschbach et al., 2020). However, electron microscope data can only show chemical but no electrical synapses. Thus, direct inhibitory DAN-to-DAN connections via electrical synapses would also be conceivable. At least in adult *Drosophila*, there is limited evidence of a

post-inhibitory rebound effect in DANs (Felsenberg et al., 2018), thus, also the model of a post-inhibitory rebound effect serves a reliable explanation of how these opposite memories can be implemented on a cellular level. Nevertheless further evidence is needed to confirm this hypothesis in *Drosophila*.

It is all about dopamine!?

As already mentioned in Chapter II, the dopaminergic system of flies and humans is very similar in that they share most of the genes involved in dopamine synthesis, secretion and signalling (Clark et al., 1978; Karam et al., 2020; Riemensperger et al., 2011; Yamamoto and Seto, 2014). Therefore, it is not surprising that dopaminergic neurons play a crucial role in reinforcement learning in vertebrates and invertebrates (Burke et al., 2012; Liu et al., 2012; Schroll et al., 2006; Schwaerzel et al., 2003; Selcho et al., 2009; reviewed in: Scaplen and Kaun, 2016) and that drugs that act on the vertebrate dopaminergic system are also effective in flies (Nichols, 2006; Pandey and Nichols, 2011).

Chapter II provides a pharmacological approach of interfering with the dopamine synthesis in larval and adult *Drosophila*. Feeding the dopamine synthesis inhibitor 3-Iodo-L-tyrosine (3IY) to *Drosophila* gives the opportunity to downregulate dopamine synthesis in a graded manner and timing-specific in the whole animal. In an appetitive conditioning paradigm using odour and sugar, larvae fed with 3IY for 24 h showed impaired memory scores (Figure 7B) but also their innate odour preference was affected (Figure 7D). However, a shorter duration of feeding 3IY for only 4 h also impaired memory while leaving innate odour preference (Figure 8B) and sugar preference (Figure 8C) unaffected. This memory decrease after feeding 3IY was rescued by additionally feeding the dopamine precursor L-DOPA (Figure 9B,C,D). In an aversive conditioning paradigm using odour and optogenetic PPL1-γ1pedc activation in adult *Drosophila*, the same effects can be observed: after 2 d of feeding 3IY, flies showed impaired memory (Figure 10B,C) while innate odour preferences were left unaffected (Figure 10E,F). This memory impairment was rescued by additionally feeding L-DOPA (Figure10G). Thus, using 3IY it was demonstrated that dopamine is involved in larval reward learning and adult optogenetic punishment learning (Kaun et al., 2011; Seugnet et al., 2008; Zhang et al., 2008). These findings are in line with studies that had observed the need for dopamine for associative learning (larvae: Honjo and Furukubo-Tokunaga, 2009; Rohwedder et al., 2016; Schroll et al.,

2006; Selcho et al., 2009) (adults: Berry et al., 2012; Riemensperger et al., 2011; Schwaerzel et al., 2003; reviewed in: Adel and Griffith, 2021; Waddell, 2013).

Regarding timing-dependent valence reversal, only few studies investigated the requirement of dopamine for forward and backward learning. So far, data suggest the involvement of dopamine in punishment learning but not relief learning. Blocking synaptic output from punishment mediating DANs in the adult brain in an odour shock learning paradigm impaired punishment learning but not relief learning (Yarali and Gerber, 2010). Also, König and colleagues (2018) found that dopamine synthesis in the DAN that is activated by the US is dispensable for the formation of relief memory but not punishment memory at distinct ISIs. However, using optogenetic activation of the rewarding PAM-DANs in adult *Drosophila*, Handler and colleagues (2019) observed that the different dopamine receptors Dop1R1 and Dop1R2 or the components of their signalling cascades are sensitive to the relative timing of KC and DAN input, respectively. Therefore, both receptors influence forward and backward learning in different ways. Behavioural tests in Dop1R1 mutants and Dop1R2 mutants demonstrated that one receptor alone cannot mediate memories of opposite valences but both receptors must work in concert to mediate timing-dependent valence reversal in the appetitive domain. Thus, dopamine and the different dopamine receptors were shown to be involved in the memories brought by forward and/or backward pairing of an appetitive/aversive stimulus and an odour.

In sum, the mentioned data demonstrates the involvement of dopamine in classical conditioning and timing-dependent valence reversal. Of note is, however, the results in Chapter II show only an impairment of the memory but no complete abolishment. Thus, either the inhibition of dopamine synthesis using 3IY was incomplete, or associative learning is not exclusively dependent on dopamine. Also, in timing-dependent valence reversal dopamine was only partially involved, if at all, in memory formation. Thus, associative learning is not exclusively dependent on dopamine; other neuromodulators may also have an influence. Indeed, this could be the case given the different types of synaptic vesicles in MBINs in *Drosophila* (larva: Eichler et al., 2017) (adults: Takemura et al., 2017). However, data on the involvement of other neurotransmitters or co-transmitter are so far inconclusive. Blocking synaptic output of DANs and serotonergic neurons together or different subsets of OANs had no effect on either punishment or relief learning (Yarali and Gerber, 2010). Another study suggests that nitric oxide acts as a co-transmitter in DANs to diversify memory dynamics in

adult *Drosophila* (Aso et al., 2019). Fly mutants lacking dopamine in the PPL1-DAN cluster (punishing DANs) or PAM-DAN cluster (rewarding DANs) showed inverted memories which was supported by nitric oxide. However, this effect was only seen when no dopamine was around and only in some of the tested DANs.

Overall, there is no conclusive evidence as to which neurotransmitters or co-transmitters is involved in timing-dependent valence reversal. However, in adult *Drosophila* dopamine seems to be involved in forward trace and delay conditioning and backward conditioning whereas serotonin seems to be involved in only forward trace and delay conditioning but not backward conditioning when using optogenetic activation of a punishing DAN (personal communication with Christian König and Fatima Amin). In the larval case, data about the involvement of dopamine in forward and backward learning using DAN-i1^{864} and DAN-f1^{2180} activation are inconclusive so far and need further testing (see Figure S9-10).

Timing-dependent valence reversal as a common principle with clinical relevance

Timing-dependent valence reversal is a principle that can be observed across animal species (reviewed in: Gerber et al., 2014; see also 'Introduction - Timing is crucial: Timing-dependent valence reversal'). Depending on whether a CS is associated with the occurrence or termination of an appetitive or aversive US, memories of opposite valence are formed: reward and frustration memory using appetitive USs, punishment and relief memory using aversive USs. This also means that CSs can induce different affective states depending on the timing of CS and US. An imbalance of these affective states can cause mental disorders.

Regarding the processing of reward and frustration, too strong reward learning and too weak frustration learning can turn into addiction. Addictive drugs are reinforcing like natural rewards and affect the same brain regions (reviewed in: Kelley and Berridge, 2002). It is suggested that substance abuse causes permanent changes in the reward circuit (especially the dopaminergic system) and thus leads to different processing of rewards (reviewed in: Baskin-Sommers and Foti, 2015; Volkow et al., 2019; Wassum and Izquierdo, 2015). As a result, the threshold at which something is rewarding is altered, leading to overall reduced sensitivity to rewards and increased sensitivity to drug predicting stimuli (García-García et al., 2014; Schacht et al., 2013; Tang et al., 2012). This could point to an imbalance between reward and

frustration learning. Humans and animals can learn the associations between (contextual) cues and the availability of addictive drugs (reviewed in: Hyman et al., 2006) and drug use and relapses often occur after exposure to such cues (O'Brien et al., 1998; Wikler and Pescor, 1967; reviewed in: Tiffany, 1990). The pathological potential of learning cues that predict the beginning of the reward lies in its reinforcing nature itself. Cues that are associated with the affective state after termination of a reward, however, are important because they energize escape and avoidance behaviour which consequently lead to drug use in order to get rid of the negative symptoms of withdrawal. Thus, also cues that predict withdrawal should be considered when treating addiction (reviewed in: Solinas et al., 2019), as it is already realized in some approaches (reviewed in: Koob and Mason, 2016).

On the other hand, it was shown that the loss of a reward like a long-lasting relationship can induce depressive symptoms (reviewed in: Tennant, 2002) which also depend on the magnitude of the reward (reviewed in: Rolls, 2013). It was also observed that continuous frustration experiences increase the likelihood of developing mental disorders like anxiety, depression or substance abuse (Harrington, 2006), e.g. after a frustration or other stress situations an increased alcohol consumption can be observed (Manzo et al., 2015; Wille-Bille et al., 2017). Reward loss was even described as a type of psychological and physical pain (reviewed in: Eisenberger and Lieberman, 2004; Papini et al., 2006). In fact, animal studies have shown that the omission of rewards triggers behaviour that depends on the activation of brain regions that also play a role in physical pain, stress, fear or anxiety (Flaherty, 1999; Gray, 1987; Papini et al., 2006). Not only can reward omission trigger depression, but processing of rewards and frustrations has been shown to be impaired in patients with depression and anxiety disorders. Depressive patients showed a maladaptive response to punishment (Elliott et al., 1997; Steffens et al., 2001; reviewed in: Mathews and MacLeod, 2005), a low frustration tolerance (Deater-Deckard et al., 2010; Mahon et al., 2007; reviewed in: Blair, 2010) and hyposensitivity to rewards (Admon and Pizzagalli, 2015; Bylsma et al., 2008; Pizzagalli et al., 2009). Furthermore, in children that experienced maltreatment or early-life stress an altered reward processing was observed (Armbruster-Genç et al., 2022; Dennison et al., 2016; Goff et al., 2013) and in rats chronic stress exposure disrupted the brain reward system (Berton et al., 2006; Vialou et al., 2010; reviewed in: Slattery and Cryan, 2017). Overall, this could point to an imbalance of forward and backward learning of appetitive CSs, namely too weak reward learning and too strong frustration learning in depression.

However, patients with depression and anxiety disorders show also impaired processing of relief and safety predicting signals (Jovanovic et al., 2010; reviewed in: Kong et al., 2014; Lohr et al., 2007). Thus, also the imbalance of punishment and relief learning could contribute to mental disorders. Chronic aversive situations can lead to post-traumatic stress disorder (PTSD) which is characterized, among other things, by reduced activation of brain areas associated with fear inhibition (for an overview of underlying learning mechanisms see: Lissek and van Meurs, 2015). Individuals with Panic disorder also show a dysregulation in several fear network structures that could contribute to the pathophysiology of the disease (reviewed in: Goddard, 2017); with e.g. enhanced resistance to fear extinction (Michael et al., 2007; reviewed in: Duits et al., 2015). Non-suicidal self-injury (NSSI), the intentional and deliberate damage to one's own body without suicidal intentions, is a behaviour seen in individuals with depression, anxiety or post-traumatic stress disorder and is associated with feelings of relief from distress (Briere and Gil, 1998; Franklin et al., 2013a; reviewed in: Bentley et al., 2014). Thus, whereas in PTSD or Panic disorder punishment learning could be too strong and relief learning too weak, in NSSI punishment learning could be too weak and relief learning too strong. This shows that CSs that are associated with the beginning and termination of aversive events are also equally important. For example the attraction to dangerous activities or non-suicidal self-injury could be explained by the relief at the offset of these activities (Franklin et al., 2013b; Hamza et al., 2021; Kiekens et al., 2019) and thus, not only CSs associated with punishment, but also CSs associated with relief should be considered in therapy.

Conclusion

The parametric analysis of reward and frustration learning in this work is the most detailed analysis to date regarding timing-dependent valence reversal in the appetitive domain and may help to understand the underlying principles of learning about the onset or termination of reward. It is argued that the imbalance of these learning processes, and of the 'mirrored' processes in the aversive domain, should be considered to understand various kinds of mental disorders and their treatment of these mental disorders. However, further research is needed in particular on how exactly memories related to the termination of rewards and of punishment are implemented at the molecular and cellular level. Thanks to the simultaneous possibility of transgenic and pharmacological manipulation of *Drosophila* larvae and their rather 'simple' brain, they are a promising model organism for such an endeavour. Given the

similarities between flies and humans, these results may help to understand the neural mechanisms of timing-dependent valence reversal in humans as well and to treat mental disorders associated with an imbalance of these mechanisms.

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Appendix

Supplemental figures

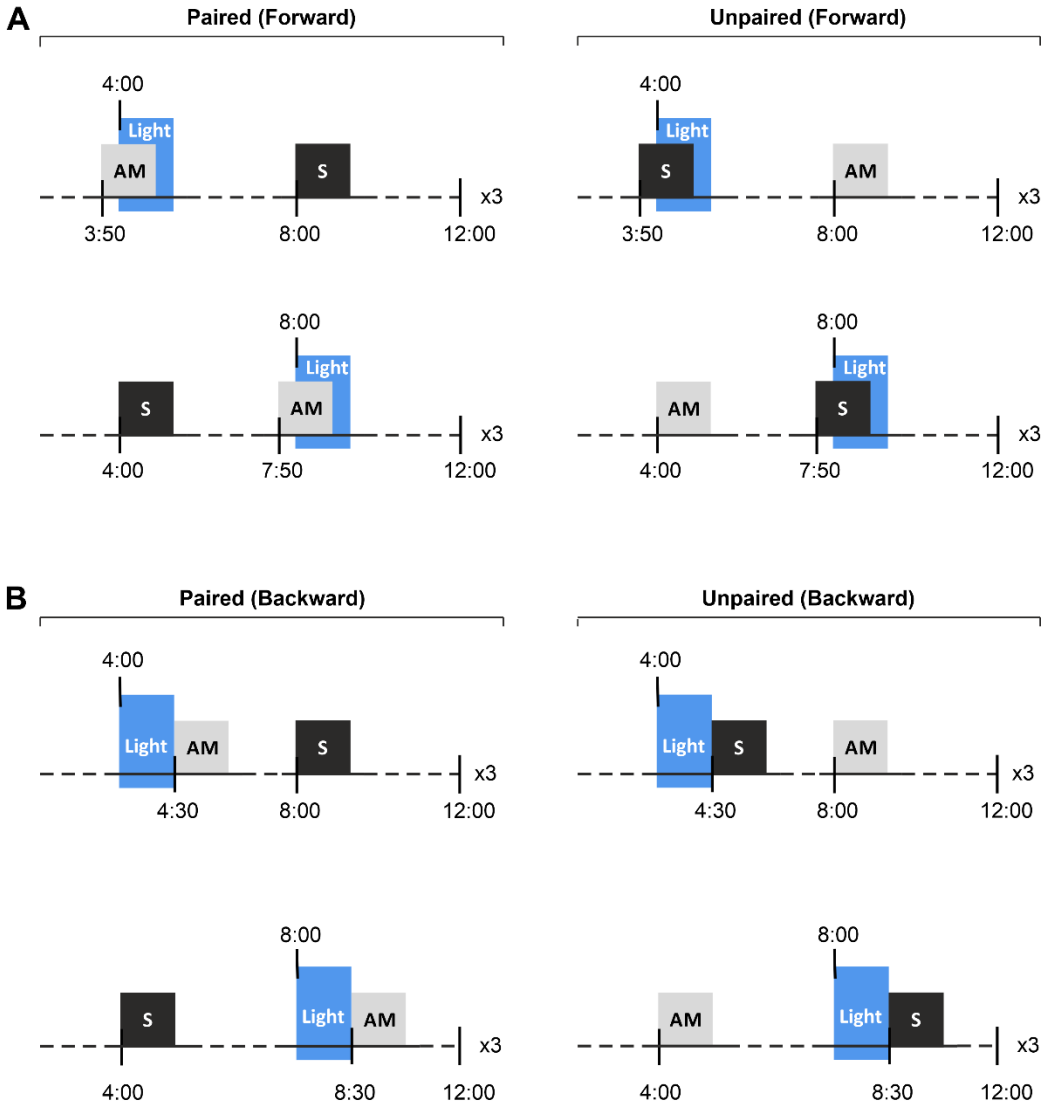

Figure S1. Detailed training procedure. Training procedure, showing that in the paired cohort, larvae were trained with timed presentation of odour (AM; grey box) together with optogenetic activation of DAN-i1^{864} by blue light (Light; blue box), followed or preceded by presentation of the solvent as an 'odour control' (S; black box). Another cohort of larvae was trained with unpaired presentation of odour and light activation of DAN-i1^{864}. Both odour presentation and DAN-i1^{864} activation by blue light lasted 30 s. In half of the cases, light activation started after 4 min, in the other half after 8 min. (A) Odour was either presented 10 s before optogenetic activation of DAN-i1^{864} (forward conditioning; ISI -10 s) or (B) 30 s after blue light activation (backward conditioning; ISI +30 s). After three training trials, the preference for the odour was tested.

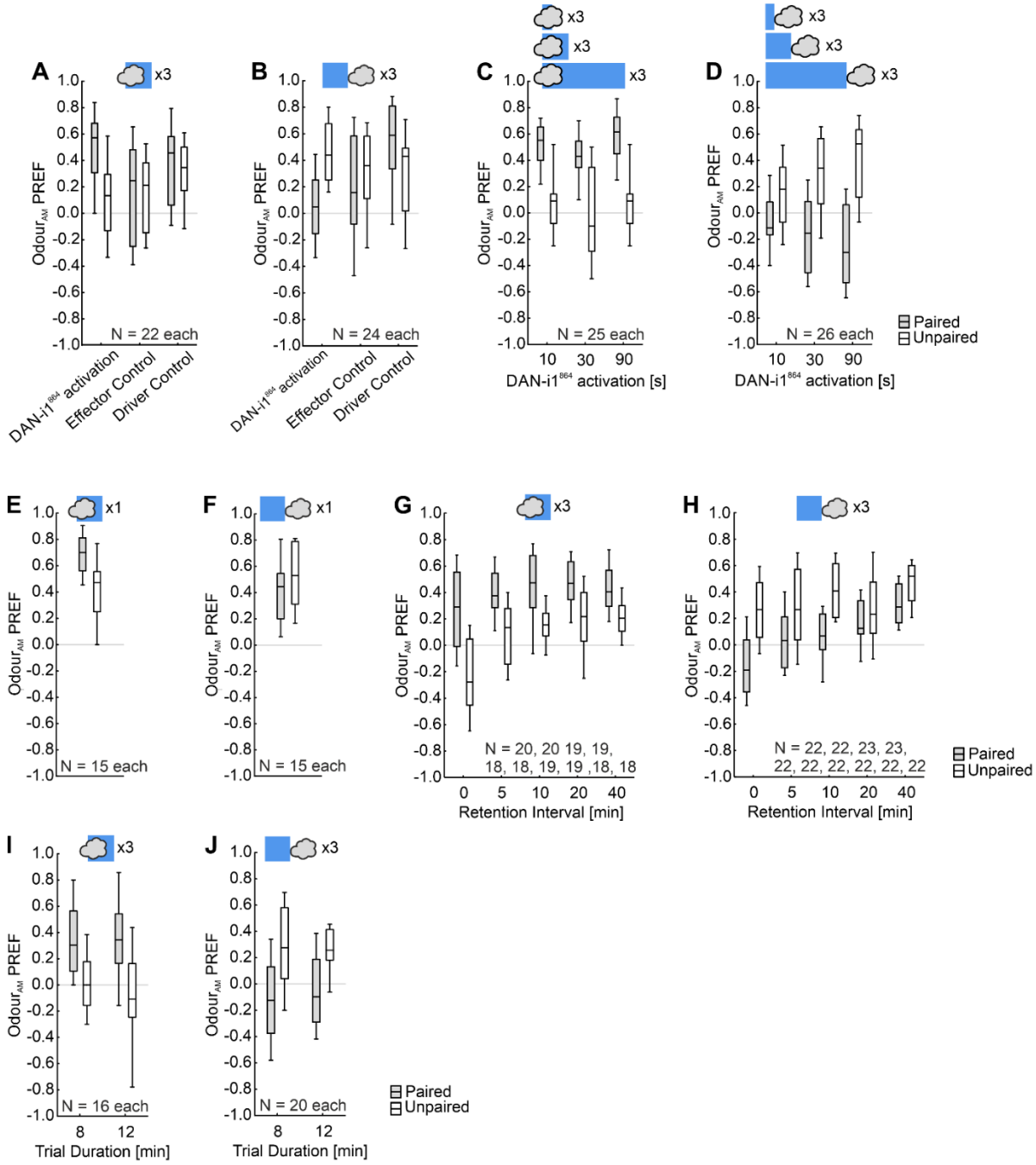

Figure S2. Preference scores for the reciprocally trained sets of larvae underlying the Memory scores from Figure 4. Shown are preferences for AM after paired (grey boxes) and unpaired (open boxes) training. Other details as in Figure 4.

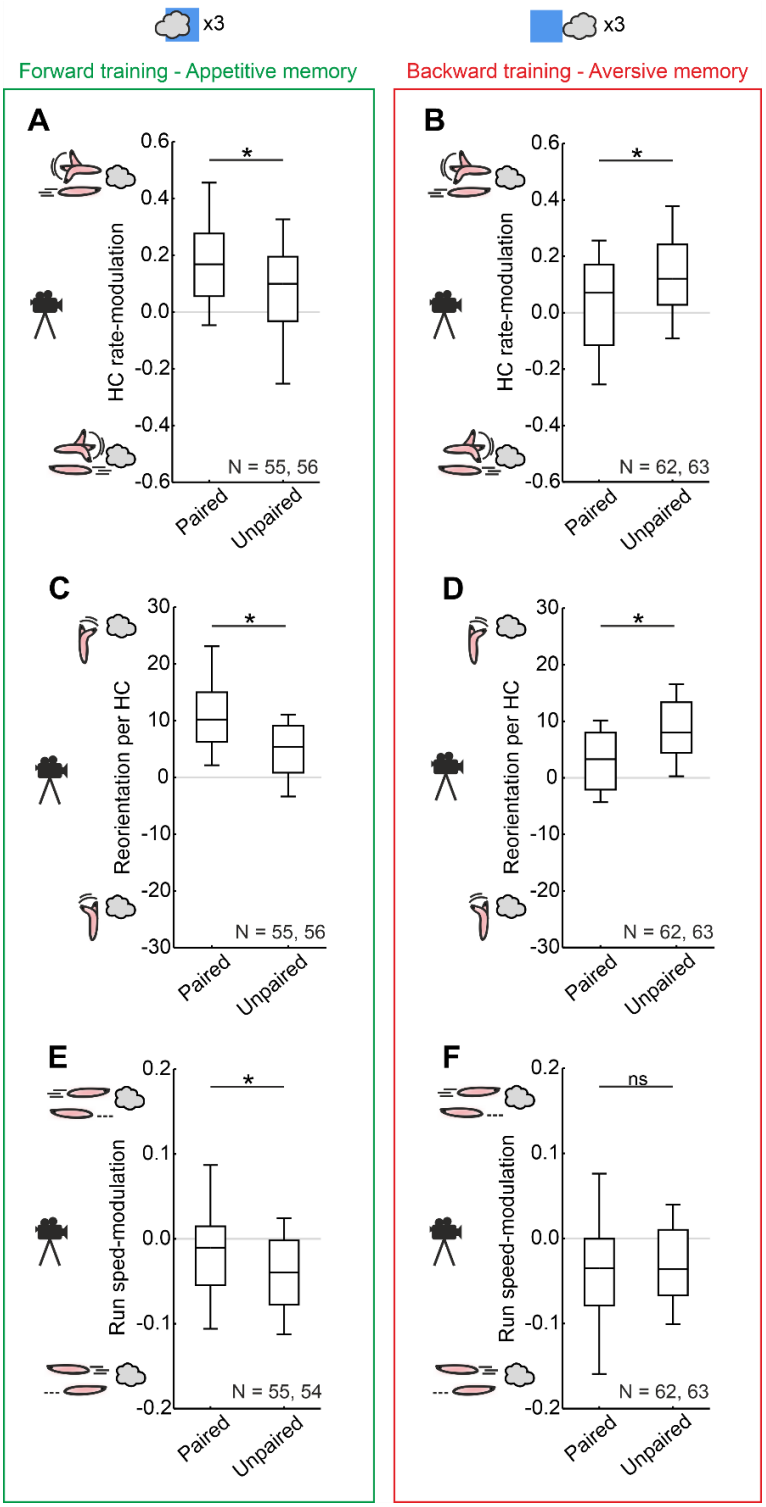

Figure S3. Results separated for paired and unpaired training scores in Figure 5. (A) Compared to larvae that were trained unpaired, after forward-paired training the larvae made fewer HCs when heading towards than heading away from the odour. (B) After backward training, the opposite was observed. (C) Larvae directed their HCs more towards the odour when they were trained forward-paired than when trained forward-unpaired. (D) After backward training, the opposite reorientation of the HCs was observed. (E) After forward-paired training, larvae run faster while heading towards the odour than while heading away, compared to larvae undergoing unpaired training. (F) No difference in run-speed was observed between backward-paired and unpaired-trained larvae. Data are combined from Figure 4B-E (30-s light duration), J and K (12-min trial duration). Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

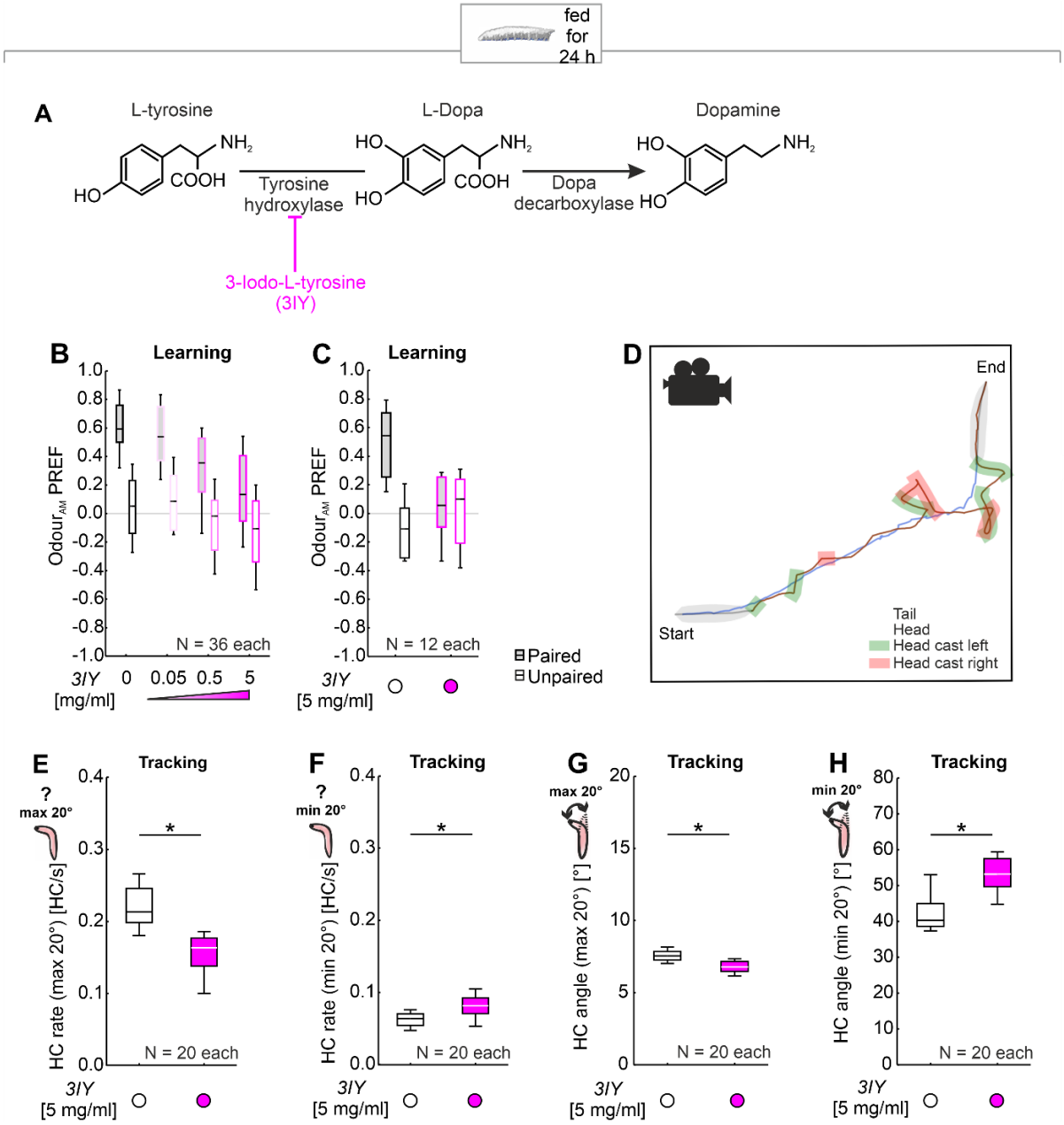

Figure S4. Preference and tracking data underlying the results shown in Figure 7. (A) Dopamine is synthesized via two enzymatic steps. In the first step the amino acid L-tyrosine is converted into L-3,4 dihydroxyphenylalanine (L-DOPA) via tyrosine hydroxylase (TH). In the second step, L-DOPA is converted to dopamine via dopa decarboxylase (DDC). In magenta, the inhibition of the TH enzyme by 3-Iodo-L-tyrosine (3IY) is shown. (B,C) Preference data refer to the Memory scores shown in Figure 7B,C, respectively. Grey boxes represent odour preference after paired training, white boxes after unpaired training. (D) Example of a track from the video-recording of a single larva showing relatively straight runs interrupted by lateral head movements (head cast, HC). (E,F) HC rate for small and large HCs, respectively, classified by a HC angle smaller or greater than 20°. This classification as well as the calculation of the HC angle is based on Paisios et al. (2017). The HC rate was decreased for small HCs (E) and increased for large HCs (F) for larvae fed with 3IY for 24 h. (G,H) HC angles classified by small and large HCs. The average HC angle was decreased for small HCs (G) and increased for large HCs (H) for larvae fed with 3IY for 24 h. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

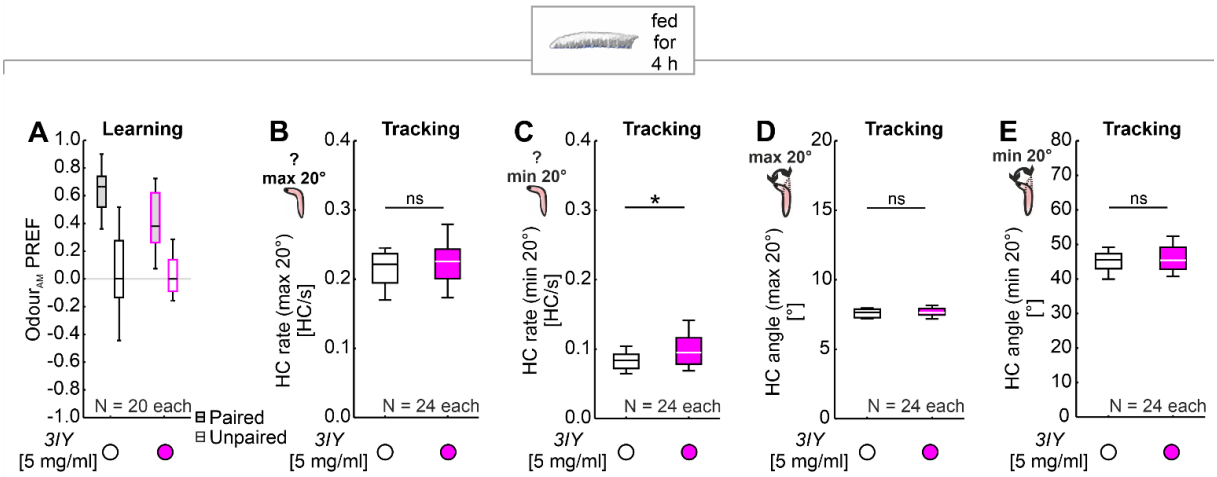

Figure S5. Preference and tracking data underlying the results shown in Figure 8. (A) Preference data refers to the Memory scores shown in Figure 8. Grey boxes represent odour preference after paired training, white boxes after unpaired training. (B,C) HC rate classified by small and large HCs. The HC rate for small HCs was unaffected by 3IY feeding (B), but slightly increased for large HCs (C). Comparing these results with those after 24 h feeding (Figure S4E-F), it seems that both 4 h and 24 h feeding of 3IY slightly increased the rate of large HCs, but only 24 h feeding decreased the rate of small HCs in addition. This resulted in a decrease in total HC rate after 24 h feeding on 3IY as seen in Figure 7F, and in an increase in total HC rate after 4 h feeding on 3IY as shown in Figure 8E. (D,E) HC angles classified by small and large HCs. No significant effect of 3IY feeding was observed. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

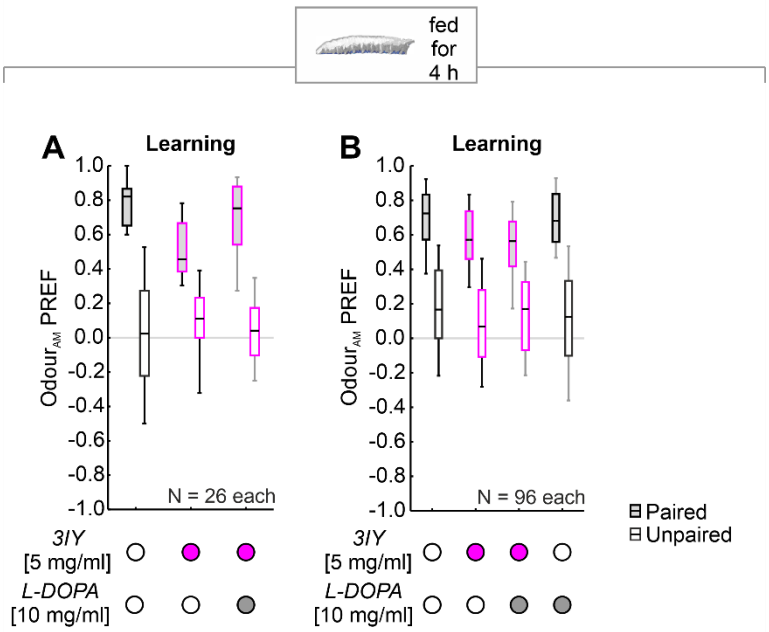

Figure S6. Preferences underlying the Memory scores in Figure 9. (A,B) Preference data refer to the Memory scores shown in Figure 9A,D, respectively. Grey boxes represent odour preference after paired training, white boxes after unpaired training. Other details as in Figure 4.

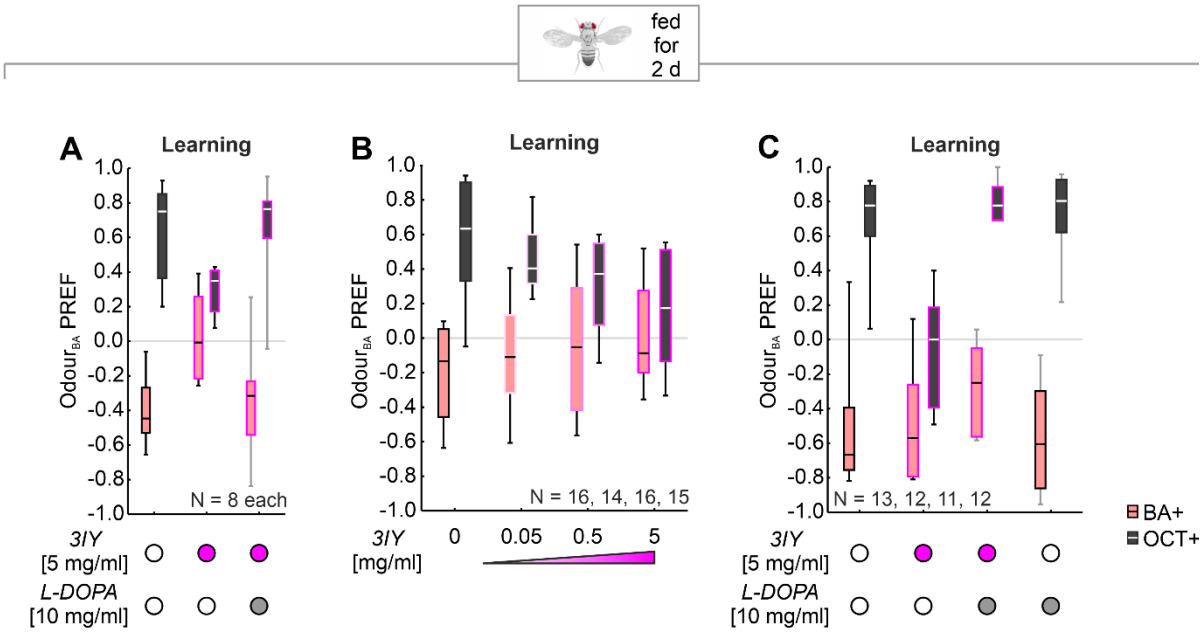

Figure S7. Preferences underlying the Memory scores in Figure 10. (A,B,C) Preference data refer to the Memory scores shown in Figure 10B,C,G, respectively. Peach boxes represent BA preference after BA-paired training (BA+), black boxes after OCT-paired training (OCT+). Other details as in Figure 4.

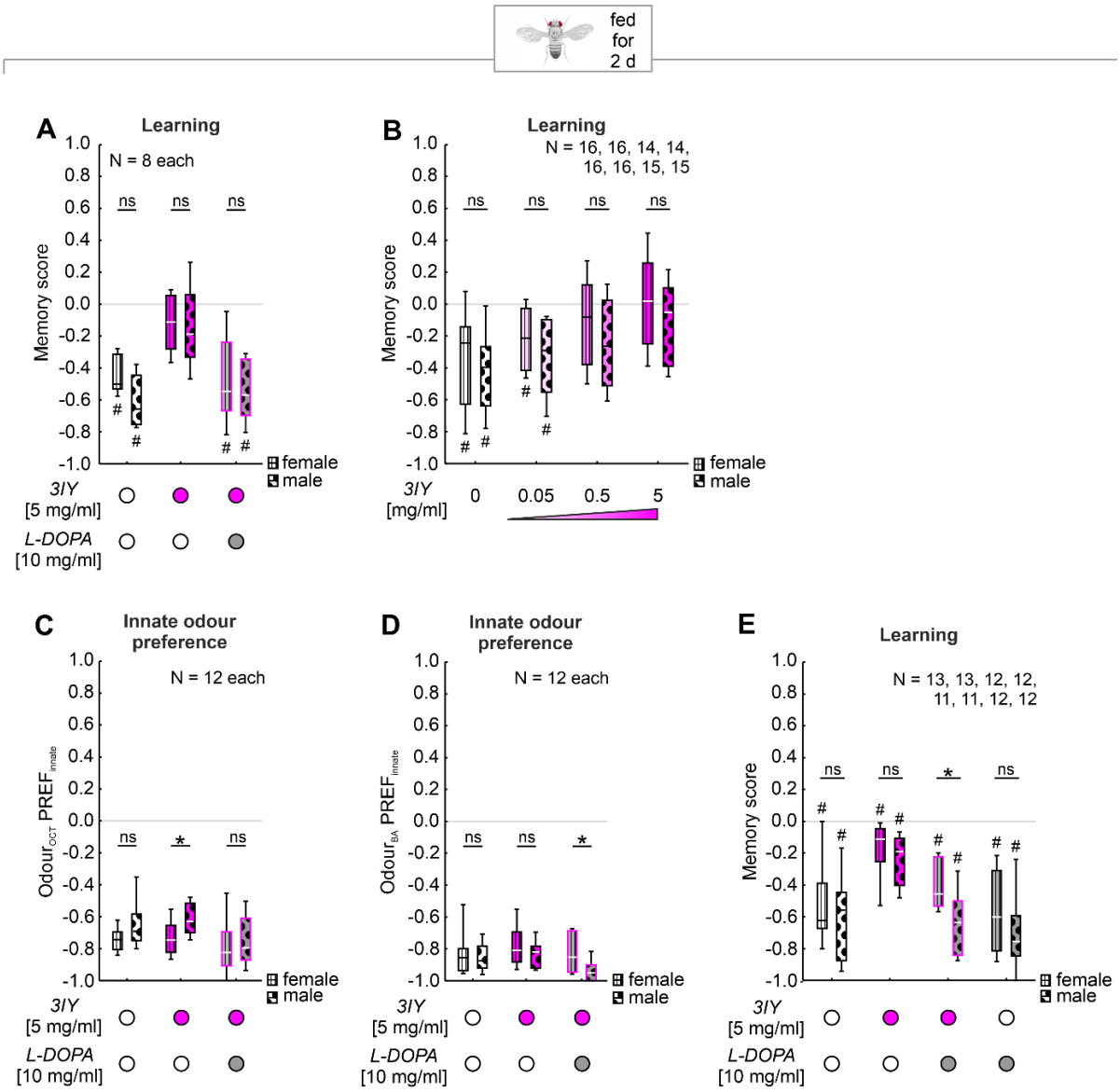

Figure S8. Analysis of gender effects of data shown in Figure 10. (A) Feeding of 3IY and/or L-DOPA had no gender specific effect. (B) No effects specific for gender with increasing concentration of 3IY feeding. (C) Only in the group fed with 3IY male flies showed slightly higher OCT preference than females. (D) Only in the group fed with 3IY and L-DOPA females showed slightly higher BA preference than males. (E) Only females fed with 3IY and L-DOPA show less punishment learning than males of the same group. Striped boxes represent females, dotted boxes males. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix.

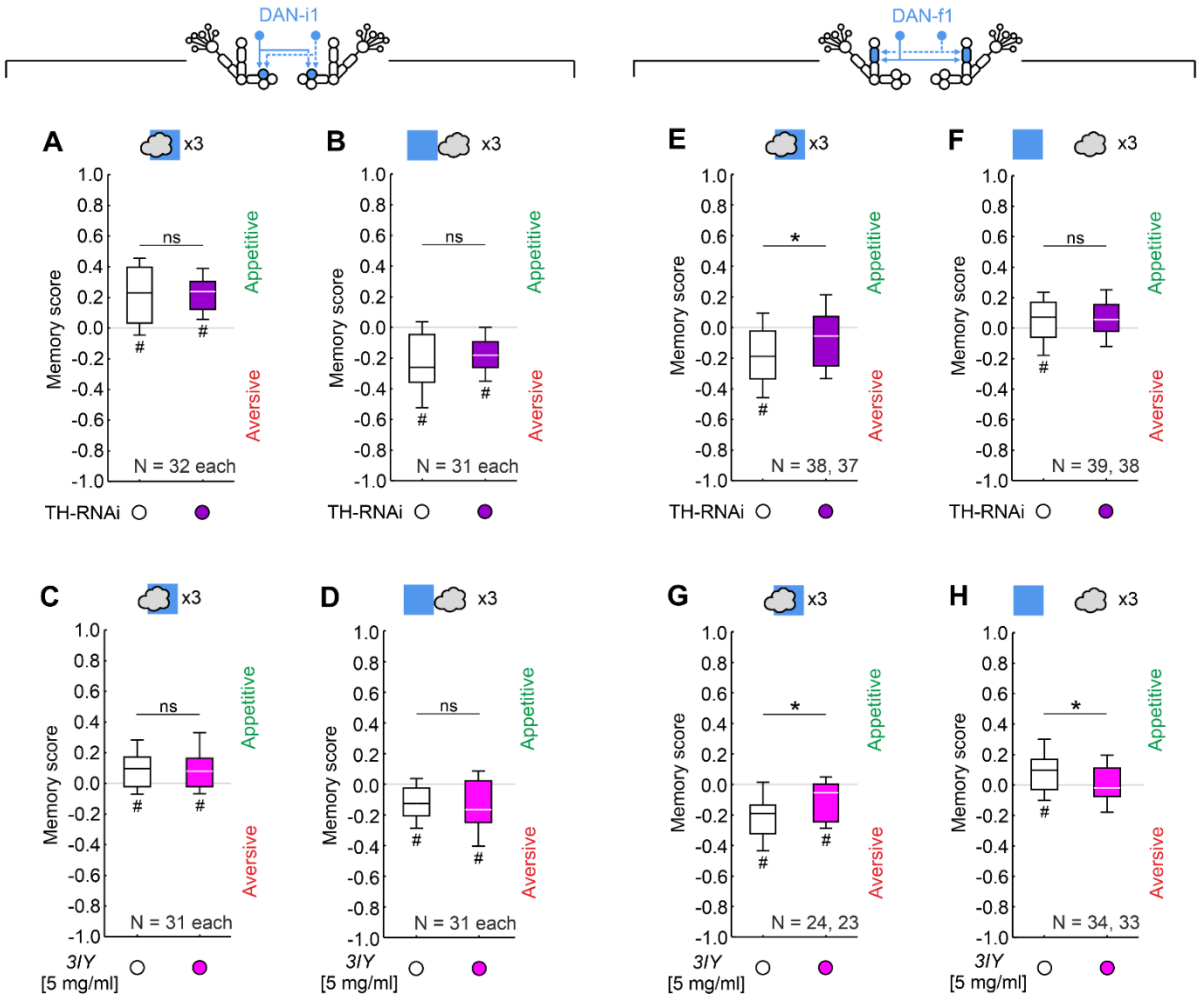

Figure S9. Influence of dopamine synthesis in memories brought by DAN-i1^{864} and DAN-f1^{2180} activation. Larvae were trained with repetitive forward or backward pairing of odour presentation (cloud) and optogenetic activation of DAN-i1^{864} or DAN-f1^{2180} by blue light (blue box) and subsequently tested for their odour preference. Positive and negative Memory scores reflect appetitive or aversive associative memory, respectively. (A-D) Odour was either presented 10 sec before (forward paired) or right after the end of the DAN-i1^{864} activation (backward paired). (A, B) Testing larvae of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL;TH-RNAi revealed neither an effect on (A) reward memory after forward training nor (B) on frustration memory after backward training. (C, D) Also, blocking dopamine synthesis by feeding 5 mg/ml 3IY for 4h in the larvae of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL had no effect on (C) reward memory nor (D) frustration memory. (E-H) Odour presentation started either 10 sec before (forward paired) or 60 sec after activation of DAN-f1^{2180} (backward paired). (E, F) Larvae of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL;TH-RNAi showed (E) no punishment memory after forward training compared to the control group which does not express TH-RNAi. (F) After backward training, however, no difference was observed between the groups but only the control group shows relief memory. (G, H) Larvae of the driver strain covering DAN-f1^{2180} crossed to UAS-ChR2-XXL fed with 5mg/ml 3IY for 4h showed (G) reduced punishment memory after forward training and (H) no relief memory after backward training. See Figure S10 for Odour preferences underlying the Memory scores. Other details as in Figure 4. Statistical results are reported in Table S1 in the Appendix. Data in panel A, B from Aliće Weiglein.

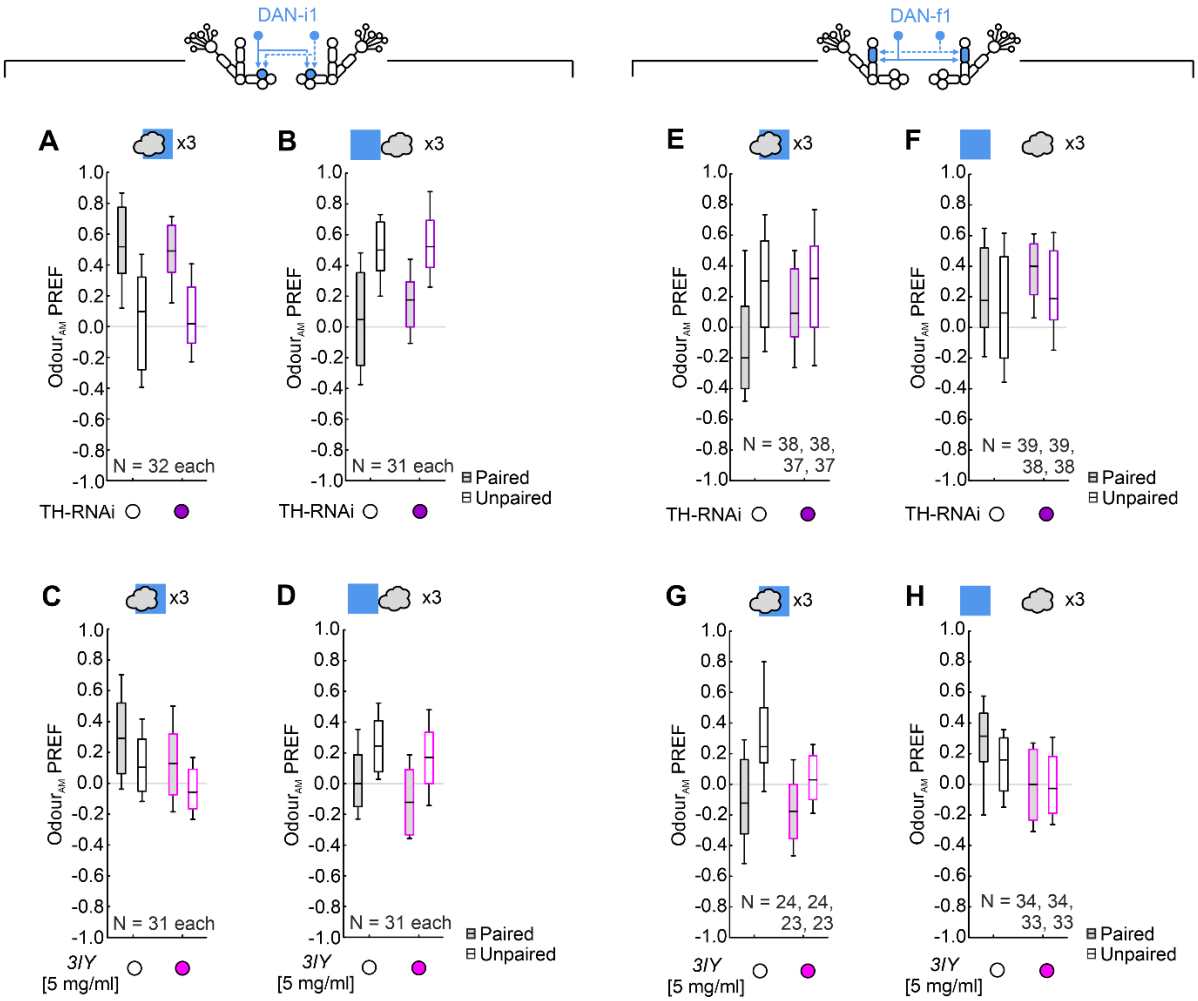

Figure S10. Odour preferences for the reciprocally trained sets of larvae underlying the Memory scores from Figure S10. Grey boxes represent odour preference after paired training, white boxes after unpaired training. (A-H) Preference data refer to the Memory scores shown in Figure S9 A-H, respectively. Other details as in Figure 4. Data in panel A, B from Aliće Weiglein.

Table S1. Statistical results

Figure	Test	Result	
4B	**KW test**	H (2) = 7.70, p = 0.0212	
	MWU tests	DAN-i1^{864} activation vs Effector control	U = 143.00, p = 0.0208
		DAN-i1^{864} activation vs Driver control	U = 137.00, p = 0.0142
	OSS tests	DAN-i1^{864} activation	p = 0.0009, N = 22
		Effector control	p = 0.8318, N = 22
		Driver control	p = 0.5235, N = 22
4C	**KW test**	H (2) = 21.76, p < 0.0001	
	MWU tests	DAN-i1^{864} activation vs Effector control	U = 15.00, p = 0.0081
		DAN-i1^{864} activation vs Driver control	U = 73.00, p < 0.0001
	OSS tests	DAN-i1^{864} activation	p < 0.0001, N = 24
		Effector control	p = 0.5413, N = 24
		Driver control	p = 0.0066, N = 24
4D	**KW test**	H (2) = 4.16, p = 0.1249	
	OSS tests	10s	p = 0.0003, N = 25
		30s	p = 0.0002, N = 25
		90s	p < 0.0001, N = 25
4E	**KW test**	H (2) = 11.25, p = 0.0036	
	MWU tests	10s vs 30s	U = 227.00, p = 0.0431
		10s vs 90s	U = 158.00, p = 0.0010
		30s vs 90s	U = 265.00, p = 0.1846
	OSS tests	10s	p = 0.0290, N = 26
		30s	p = 0.0005, N = 26
		90s	p < 0.0001, N = 26
4F	**OSS test**	p = 0.0352, N = 15	
4G	**OSS test**	p = 0.7905, N = 15	
4H	**KW test**	H (4) = 6.38, p = 0.1727	
	OSS tests	0 min	p = 0.0004, N = 20
		5 min	p < 0.0001, N = 19
		10 min	p = 0.0001, N = 18
		20 min	p = 0.0075, N = 19
		40 min	p = 0.0309, N = 18
4I	**KW test**	H (4) = 14.01, p = 0.0073	
	MWU tests	0 min vs 5 min	U = 184.00, p = 0.1199
		0 min vs 10 min	U = 218.00, p = 0.5812
		0 min vs 20 min	U = 117.00, p = 0.0035
		0 min vs 40 min	U = 133.50, p = 0.0112
	OSS tests	0 min	p = 0.0001, N = 22
		5 min	p = 0.0106, N = 23
		10 min	p = 0.0009, N = 22
		20 min	p = 0.1338, N = 22

		40 min	$p = 0.0266$, N = 22
4J	**MWU test**	$U = 120.00$, $p = 0.7774$	
	OSS tests	8 min	$p = 0.0213$, N = 16
		12 min	$p = 0.0213$, N = 16
4K	**MWU test**	$U = 186.00$, $p = 0.7150$	
	OSS tests	8 min	$p = 0.0414$, N = 20
		12 min	$p = 0.0118$, N = 20
5A	**MWU test**	$U = 1111.00$, $p = 0.0012$	
	OSS tests	forward	$p = 0.0581$, N = 55
		backward	$p = 0.0300$, N = 62
5B	**MWU test**	$U = 570.00$, $p < 0.0001$	
	OSS tests	forward	$p = 0.0001$, N = 55
		backward	$p = 0.0013$, N = 62
5C	**MWU test**	$U = 1300.00$, $p = 0.0272$	
	OSS tests	forward	$p = 0.0027$, N = 55
		backward	$p = 0.7035$, N = 62
7B	**KW test**	$H(3) = 8.44$, $p = 0.3780$	
	MWU tests	No drug vs. 0.05 mg/ml 3IY	$U = 533.50$, $p = 0.1992$
		No drug vs. 0.5 mg/ml 3IY	$U = 518.00$, $p = 0.1447$
		No drug vs. 5 mg/ml 3IY	$U = 405.00$, $p = 0.0063$
	OSS tests	No drug	$p < 0.0001$, N = 36
		0.05 mg/ml 3IY	$p < 0.0001$, N = 36
		0.5 mg/ml 3IY	$p = 0.0019$, N = 36
		5 mg/ml 3IY	$p = 0.0039$, N = 36
7C	**MWU test**	$U = 19.00$, $p = 0.0024$	
	OSS tests	No drug	$p = 0.0005$, N = 12
		3IY alone	$p = 0.7744$, N = 12
7D	**MWU test**	$U = 852.00$, $p = 0.0061$	
	OSS tests	No drug	$p < 0.0001$, N = 50
		3IY alone	$p = 0.0066$, N = 50
7E	**MWU test**	$U = 192.00$, $p = 0.8392$, N = 20 each	
7F	**MWU test**	$U = 76.00$, $p = 0.0008$, N = 20 each	
7G	**MWU test**	$U = 28.00$, $p < 0.0001$, N = 20 each	
8A	**MWU test**	$U = 125.00$, $p = 0.0439$	
	OSS tests	No drug	$p < 0.0001$, N = 20
		3IY alone	$p = 0.0004$, N = 20
8B	**MWU test**	$U = 230.50$, $p = 0.7963$	
	OSS tests	No drug	$p < 0.0001$, N = 22
		3IY alone	$p < 0.0001$, N = 22
8C	**MWU test**	$U = 344.00$, $p = 0.1188$	
	OSS tests	No drug	$p < 0.0001$, N = 30
		3IY alone	$p < 0.0001$, N = 30
8D	**MWU test**	$U = 254.00$, $p = 0.4897$, N = 24 each	

8E	**MWU test**	U = 174.00, p = 0.0193, N = 24 each	
8F	**MWU test**	U = 247.00, p = 0.4037; N = 24 each	
9A	**KW test**	H (2) = 10.69, p = 0.0048	
	MWU tests	No drug vs. 3IY alone	U = 165.50, p = 0.0016
		3IY alone vs. 3IY+L-DOPA	U = 215.00, p = 0.0250
		No drug vs. 3IY+L-DOPA	U = 286.00, p = 0.3459
	OSS tests	No drug	p < 0.0001, N = 26
		3IY alone	p = 0.0005, N = 26
		3IY + L-DOPA	p < 0.0001, N = 26
9B	**KW test**	H (2) = 2.02, p = 0.3650	
	OSS tests	No drug	p < 0.0001, N = 28
		3IY alone	p < 0.0001, N = 28
		3IY + L-DOPA	p < 0.0001, N = 28
9C	**KW test**	H (2) = 2.42, p = 0.2977	
	OSS tests	No drug	p < 0.0001, N = 24
		3IY alone	p < 0.0001, N = 24
		3IY + L-DOPA	p < 0.0001, N = 24
9D	**KW test**	H (3) = 14.06, p = 0.0028	
	MWU tests	No drug vs. 3IY alone	U = 3262.50, p = 0.0005
		No drug vs. 3IY+L-DOPA	U = 4084.50, p = 0.1743
		3IY alone vs. 3IY+L-DOPA	U = 3673.00, p = 0.0152
		No drug vs. L-DOPA alone	U = 4246.50, p = 0.3484
	OSS tests	No drug	p < 0.0001, N = 96
		3IY alone	p < 0.0001, N = 96
		3IY + L-DOPA	p < 0.0001, N = 96
		L-DOPA alone	p < 0.0001, N = 96
10B	**KW test**	H (2) = 11.89, p = 0.0026	
	MWU tests	No drug vs. 3IY alone	U = 2.00, p = 0.0019
		3IY alone vs. 3IY+L-DOPA	U = 6.00, p = 0.0074
		No drug vs. 3IY+L-DOPA	U = 29.00, p = 0.7929
	OSS tests	No drug	p = 0.0078, N = 8
		3IY alone	p = 0.2891, N = 8
		3IY + L-DOPA	p = 0.0078, N = 8
10C	**KW test**	H (3) = 11.08, p = 0.0113	
	MWU tests	No drug vs. 0.05 mg/ml 3IY	U = 83.00, p = 0.2361
		No drug vs. 0.5 mg/ml 3IY	U = 72.00, p = 0.0365
		No drug vs. 5 mg/ml 3IY	U = 47.00, p = 0.0042
	OSS tests	No drug	p = 0.0005, N = 16
		0.05 mg/ml 3IY	p = 0.0018, N = 14
		0.5 mg/ml 3IY	p = 0.2101, N = 16
		5 mg/ml 3IY	p = 1.0000, N = 15
10D	**KW test**	H (3) = 43.40, p = 0.0001	
	MWU tests	No drug: females vs. males	U = 577.50, p = 0.2496
		3IY alone: females vs. males	U = 526.00, p = 0.3124

	OSS tests	No drug females	$p < 0.001$, N = 37
		No drug males	$p < 0.001$, N = 37
		3IY alone females	$p = 0.0895$, N = 35
		3IY alone males	$p = 0.0090$, N = 35
10E	**KW test**	H (2) = 4.42, $p = 0.1097$	
	OSS tests	No drug	$p < 0.0001$, N = 12
		3IY alone	$p < 0.0001$, N = 12
		3IY + L-DOPA	$p < 0.0001$, N = 12
10F	**KW test**	H (2) = 2.71, $p = 0.2575$	
	OSS tests	No drug	$p < 0.0001$, N = 12
		3IY alone	$p < 0.0001$, N = 12
		3IY + L-DOPA	$p < 0.0001$, N = 12
10G	**KW test**	H (3) = 14.68, $p = 0.0021$	
	MWU tests	No drug vs. 3IY alone	$U = 29.00$, $p = 0.0083$
		No drug vs. 3IY+L-DOPA	$U = 59.00$, $p = 0.4869$
		3IY alone vs. 3IY+L-DOPA	$U = 12.00$, $p = 0.0009$
		No drug vs. L-DOPA alone	$U = 68.00$, $p = 0.6053$
	OSS tests	No drug	$p < 0.0001$, N = 13
		3IY alone	$p < 0.0001$, N = 12
		3IY + L-DOPA	$p < 0.0001$, N = 11
		L-DOPA alone	$p < 0.0001$, N = 12
S3A	**MWU test**	$U = 1099.50$, $p = 0.0095$, N = 55, 56	
S3B	**MWU test**	U = 1395.00, p = 0.0059, N = 62, 63	
S3C	**MWU test**	U = 830.00, p < 0.001, N = 55, 56	
S3D	**MWU test**	U = 1072.00, p < 0.001, N = 62, 63	
S3E	**MWU test**	U = 1127.00, p = 0.0303, N = 55, 54	
S3F	**MWU test**	U = 1822.00, p = 0.5193, N = 62, 63	
S4E	**MWU test**	$U = 29.00$, $p < 0.0001$, N = 20 each	
S4F	**MWU test**	$U = 85.00$, $p = 0.0019$, N = 20 each	
S4G	**MWU test**	$U = 47.00$, $p < 0.0001$, N = 20 each	
S4H	**MWU test**	$U = 46.00$, $p < 0.0001$, N = 20 each	
S5B	**MWU test**	$U = 231.00$, $p = 0.2440$, N = 24 each	
S5C	**MWU test**	$U = 186.00$, $p = 0.0364$, N = 24 each	
S5D	**MWU test**	$U = 256.00$, $p = 0.5160$, N = 24 each	
S5E	**MWU test**	$U = 268.00$, $p = 0.6876$, N = 24 each	
S8A	**KW test**	H (5) = 23.40, $p = 0.0003$	
	MWU tests	No drug: females vs. males	$U = 16.00$, $p = 0.1036$
		3IY alone: females vs. males	$U = 31.00$, $p = 0.9581$
		3IY + L-DOPA: females vs. males	$U = 27.00$, $p = 0.6365$
	OSS tests	No drug females	$p = 0.0078$, N = 8
		No drug males	$p = 0.0078$, N = 8
		3IY alone females	$p = 0.7266$, N = 8
		3IY alone males	$p = 0.4531$, N = 8
		3IY + L-DOPA females	$p = 0.0078$, N = 8
		3IY + L-DOPA males	$p = 0.0078$, N = 8

S8B	**KW test**	H (7) = 20.53, p = 0.0045	
	MWU tests	No drug: females vs. males	U = 99.00, p = 0.2828
		0.05 mg/ml 3IY: females vs. males	U = 77.00, p = 0.3462
		0.5 mg/ml 3IY: females vs. males	U = 100.00, p = 0.2999
		5 mg/ml 3IY	U = 94.00, p = 0.4553
	OSS tests	No drug females	p = 0.0042, N = 16
		No drug males	p = 0.0005, N = 16
		0.05 mg/ml 3IY females	p = 0.0225, N = 14
		0.05 mg/ml 3IY males	p = 0.0018, N = 14
		0.5 mg/ml 3IY females	p = 0.4545, N = 16
		0.5 mg/ml 3IY males	p = 0.4545, N = 16
		5 mg/ml 3IY females	p = 1.0000, N = 15
		5 mg/ml 3IY males	p = 1.0000, N = 15
S8C	**KW test**	H (5) = 12.01, p = 0.0346	
	MWU tests	No drug: females vs. males	U = 48.50, p = 0.1842
		3IY alone: females vs. males	U = 36.00, p = 0.0404
		3IY + L-DOPA: females vs. males	U = 63.00, p = 0.6236
	OSS tests	No drug females	p = 0.0005, N = 12
		No drug males	p = 0.0010, N = 12
		3IY alone females	p = 0.0005, N = 12
		3IY alone males	p = 0.0005, N = 12
		3IY + L-DOPA females	p = 0.0005, N = 12
		3IY + L-DOPA males	p = 0.0005, N = 12
S8D	**KW test**	H (5) = 11.81, p = 0.0375	
	MWU tests	No drug: females vs. males	U = 71.50, p = 1.0000
		3IY alone: females vs. males	U = 62.00, p = 0.5834
		3IY + L-DOPA: females vs. males	U = 29.00, p = 0.0141
	OSS tests	No drug females	p = 0.0005, N = 12
		No drug males	p = 0.0005, N = 12
		3IY alone females	p = 0.0005, N = 12
		3IY alone males	p = 0.0005, N = 12
		3IY + L-DOPA females	p = 0.0005, N = 12
		3IY + L-DOPA males	p = 0.0005, N = 12
S8E	**KW test**	H (7) = 30.58, p = 0.0001	
	MWU tests	No drug: females vs. males	U = 84.00, p = 1.0000
		3IY alone: females vs. males	U = 55.00, p = 0.9526
		3IY + L-DOPA: females vs. males	U = 23.00, p = 0.0151
		L-DOPA alone: females vs. males	U = 56.50, p = 0.3865
	OSS tests	No drug females	p = 0.0063, N = 13
		No drug males	p = 0.0034, N = 13
		3IY alone females	p = 0.0063, N = 12
		3IY alone males	p = 0.0005, N = 12
		3IY + L-DOPA females	p = 0.0010, N = 11
		3IY + L-DOPA males	p = 0.0010, N = 11
		L-DOPA alone females	p = 0.0005, N = 12
		L-DOPA alone males	p = 0.0063, N = 12

S9A	**MWU test**	$U = 464.00$, $p = 0.6650$	
	OSS tests	No TH-RNAi	$p < 0.0001$, N = 32
		TH-RNAi	$p < 0.0001$, N = 32
S9B	**MWU test**	$U = 411.50$, $p = 0.2482$	
	OSS tests	No TH-RNAi	$p = 0.0002$, N = 31
		TH-RNAi	$p = 0.0003$, N = 31
S9C	**MWU test**	$U = 478.00$, $p = 0.9775$	
	OSS tests	0 mg/ml 3IY	$p = 0.0161$, N = 31
		5 mg/ml 3IY	$p = 0.0294$, N = 31
S9D	**MWU test**	$U = 436.50$, $p = 0.5403$	
	OSS tests	0 mg/ml 3IY	$p = 0.0014$, N = 31
		5 mg/ml 3IY	$p = 0.0428$, N = 31
S9E	**MWU test**	$U = 486.50$, $p = 0.0221$	
	OSS tests	No TH-RNAi	$p = 0.0001$, N = 38
		TH-RNAi	$p = 0.2430$, N = 37
S9F	**MWU test**	$U = 726.00$, $p = 0.8826$	
	OSS tests	No TH-RNAi	$p = 0.0095$, N = 39
		TH-RNAi	$p = 0.0730$, N = 38
S9G	**MWU test**	$U = 172.00$, $p = 0.0276$	
	OSS tests	0 mg/ml 3IY	$p = 0.0015$, N = 24
		5 mg/ml 3IY	$p = 0.0347$, N = 23
S9H	**MWU test**	$U = 389.00$, $p = 0.0.0315$	
	OSS tests	0 mg/ml 3IY	$p = 0.0090$, N = 34
		5 mg/ml 3IY	$p = 0.7283$, N = 33

Table S2. Key resources

Reagent or Resource	Source or Reference	Identifiers
Fly strains		
attP40/attP2	Pfeiffer et al. 2010 Kindly provided by HHMI Janelia Research Campus, USA	
CS_{gpu}		DGGR_105666
MB320C-Gal4 (covering the PPL1-γ1pedc neurons (also known as PPL1-01 and MB-MP1))	Aso et al., 2014	
SS00864-Gal4 (covering DAN-i1)	Saumweber et al. 2018 Kindly provided by HHMI Janelia Research Campus, USA	
UAS-ChR2-XXL (optogenetic effector)	Dawydow et al. 2014	BDSC no. 58374
w1118		BDSC no. 3605, 5905, 6326
Chemicals		
3-octanol (OCT)	Fluka, Steinheim, Germany	CAS 589-98-0
Agarose (electrophoresis grade)	Roth, Karlsruhe, Germany	CAS: 9012-36-6
Benzaldehyde (BA)	Fluka, Steinheim, Germany	CAS 100-52-7
D-fructose (FRU)		
D-Sucrose	Roth, Karlsruhe, Germany Hartenstein, Würzburg, Germany	CAS: 57-50- 1
n-amylacetate (AM)	Merck, Darmstadt, Germany	CAS: 628-63-7
Paraffin oil	AppliChem, Darmstadt, Germany	CAS: 8042-47-5
Drugs		
3,4-dihydroxyphenylalanine (L-DOPA)	Sigma-Aldrich, Steinheim, Germany	CAS: 59-92-7
3-Iodo-L-tyrosine (3IY)	Sigma-Aldrich, Steinheim, Germany	CAS: 70-78-0
Software		
Corel Draw 2019	Corel Corporation	SCR_013674
Statistika 13	StatSoft Inc	SCR_014213

Technical summary of higher-order forms of learning in larval *Drosophila*

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Investigations of *Drosophila melanogaster* led to the discovery of evolutionarily conserved genetic and molecular networks underlying for example early development, the function of ion channels and synapses, and circadian rhythms (reviewed in Foster and Helfrich-Förster, 2001; Johnston and Nüsslein-Volhard, 1992; Troy Littleton and Barry Ganetzky, 2000). These findings defined *Drosophila* as a classic model system for biomedical research. Surprisingly to many, corresponding commonalities were discovered when researchers began to apply concepts of early experimental psychology to their work in *Drosophila*, yielding insight into evolutionarily conserved genetic and molecular determinants of simple forms of associative learning and memory (Dudai et al., 1976; Heisenberg et al., 1985; Tully and Quinn, 1985). These studies gained momentum by their combination with methods for the induced expression of transgenes selectively in specific groups of neurons (Brand and Perrimon, 1993), defining *Drosophila* as a model to study the genetic and molecular processes underlying synaptic plasticity and simple forms of cognition (Zars et al., 2000a, 2000b) (reviewed in Boto et al., 2020; Cognigni et al., 2018; Gerber and Aso, 2017; Guven-Ozkan and Davis, 2014).

The peculiar potential of larval *Drosophila* for the neurogenetic analysis of learning was realized early on (Aceves-Piña and Quinn, 1979) with renewed interest when robust paradigms for the association of odours and taste reward (Neuser et al., 2005; Scherer et al., 2003) and between odours and optogenetically activated internal reward neurons (Schroll et al., 2006) were established. Larvae possess about 10 times fewer neurons than adult flies, but feature adult-like circuit motifs for example in the olfactory pathways underlying associative learning (Diegelmann et al., 2013; Eschbach and Zlatic, 2020; Gerber and Stocker, 2007; Thum and Gerber, 2019; Vosshall and Stocker, 2007). The larva´s nervous system has been partially mapped into a light-microscopical atlas at single-cell resolution (Li et al., 2014), and transgenic

driver strains can be generated to experimentally manipulate these neurons, in small groups or even one at a time, in the context of learning experiments (Rohwedder et al., 2016; Saumweber et al., 2018). Furthermore, the ongoing electron microscopic reconstruction of the chemical-synapse connectome of the larval brain and ventral nerve cord revealed a stunning circuit complexity in the mushroom body, a key central brain structure of associative learning in larvae and in insects in general (Eichler et al., 2017). Indeed, more than half of the classes of synaptic connection had apparently escaped earlier attention! The functional analysis and computational interpretation of these newly discovered circuit motifs, subsequently confirmed in adult *Drosophila* (Li et al., 2020; Takemura et al., 2017; Zheng et al., 2018) and likely shared across insects, largely define the current research agenda of the field. One key element to this enterprise is to probe for the cognitive abilities and, as we argue more importantly, the cognitive limits conferred by a mushroom body that is numerically simplified yet maintains the full complexity in its circuit organization as is the case of larval *Drosophila*. In this context we probe larval *Drosophila* in three tasks that are well established in contemporary experimental psychology (Rescorla, 1988a, 1988b) and that are characteristically more complex than the established, simple learning paradigms mostly applied in *Drosophila* to date. Specifically, we investigate the larvae for conditioned inhibition, sensory preconditioning, and second-order conditioning.

Conditioned inhibition

In a typical associative learning task, a cue (A) is presented together with for example a reward (+). Such 'paired' training (A+) endows the cue with the capacity to elicit learned behaviour in a later test. This is often called 'conditioned excitation' because the cue is said to *excite* the expectation of the reward to occur, and to prompt behaviour *in anticipation of receiving* it. In contrast, 'conditioned inhibition' refers to the opposing process, establishing the cue with the capacity to *inhibit* the expectation of the reward to occur, and to prompt behaviour *in anticipation of not receiving* it (Rescorla, 1969). Conditioned inhibition can be established, for example, by training the subjects such that whenever the reward is presented the cue is precisely not presented, and whenever the cue is presented the reward is not ('unpaired' training: +/A). In other words, both of these forms of learning refer to the reward, but while paired training establishes A as a predictor of reward occurrence (conditioned excitation), unpaired training establishes A as a predictor for the reward´s non-occurrence (conditioned

inhibition). Learning through unpaired training is characteristically complex because although it is about the reward it takes place at a moment when the reward itself is not physically present.

Behaviourally opposing effects of paired versus unpaired training have been reported in larval *Drosophila* (Saumweber et al., 2011; Schleyer et al., 2018, 2020; adult *Drosophila*: Jacob and Waddell, 2020; honeybees *Apis mellifera*: Bitterman et al., 1983; discussion in Schleyer et al., 2018). Here we confirm and extend these earlier results with respect to hallmark features of conditioned inhibition, using presentations of a sugar taste reward unpaired from an odour cue.

Sensory preconditioning

Sensory preconditioning (Brogden, 1939) refers to the learning that results from two 'neutral' sensory cues A and B, say the visual appearance and the song of a bird, occurring together and thus establishing their combination as a novel psychological object. In other words, it refers to the chunking of inputs into mnemonic objects according to the statistics of their past co-occurrence. The characteristic complexity of such sensory preconditioning is that it takes place in the absence of reinforcement, and that it allows for pattern completion if one of the cues is not physically present, for example when the song of a robin immediately calls up its visual appearance. Experimentally, sensory preconditioning can be revealed in a two-phase experiment of the logical form of i) presenting AB, then ii) training A+, followed by the test of B revealing a response. Of note, responding to B during the test requires chained processing during phase ii) such that by virtue of the AB association A is calling up B, which then is associated with +, and/or chained processing during the test such that by virtue of the AB association B is calling up A which then calls up the A+ association.

Sensory preconditioning has been shown in bees and adult *Drosophila* (Brembs and Heisenberg, 2001; Müller et al., 2000; Phan et al., 2021 preprint), including for binary odour compounds as cues. We sought to establish sensory preconditioning in larvae, likewise between the elements of binary odour compounds.

Second-order conditioning

Second-order conditioning (Pavlov, 1927; Rescorla, 1980) refers to the observation that when a cue A is firmly associated with for example a reward it can itself be rewarding when presented together with another stimulus B – even if the reward is not physically present at

that moment. Such a two-phase experiment would have the logical form of i) training A+, ii) presenting AB, and then the test of B revealing a response. In humans, money would be an example of a second-order reward. The conceptual significance of second-order conditioning is that it allows to pursue temporally distant goals in a chained manner, like working for money for buying food.

Second-order conditioning has been observed in bees and adult *Drosophila* (Bitterman et al., 1983; Brembs and Heisenberg, 2001; Hussaini et al., 2007; Tabone and De Belle, 2011; Takeda, 1961; Yamada et al., 2022 preprint), again including for binary odour compounds as cues. We sought to establish second-order conditioning for larvae, using binary odour compounds as well.

With a focus on cognition in the larva, the present paper thus is part of a more general quest to explore the limits of what can be studied in *Drosophila* as a model system. As limits can only be determined by failing in transgressing them, in such a quest the 'failure' in a task as we report it for sensory preconditioning and for second-order conditioning arguably is more informative than the 'success' that we report for conditioned inhibition.

Materials & Methods

Animals, materials and chemicals

Drosophila melanogaster were raised in mass culture on standard cornmeal-molasses food and maintained at 25°C, 60–70% relative humidity, and a 12:12 h light/dark cycle. For behavioural experiments 5-days-old, 3rd instar, feeding-stage wild-type Canton Special larvae of either sex were used. Cohorts of approximately 30 larvae were collected from the food vials, rinsed in water, collected in a water droplet and then used for experiments.

For behavioural experiments Petri dishes of 9 cm diameter (Nr. 82.1472 Sarstedt, Nümbrecht, Germany) were filled with 1 % agarose solution as the substrate (PUR; electrophoresis grade; CAS: 9012-36-6, Roth, Karlsruhe, Germany) or with fructose as the sugar taste reward (+) added to that agarose solution (2 M; purity 99 %; CAS: 57-48-7 Roth, Karlsruhe, Germany). As will be specified in the Results section, for odour presentation either custom-made Teflon containers or Petri dish lids equipped with filter papers were used. The Teflon containers were of 5 mm diameter with perforated lids with 5-10 holes, each of approximately 0.5 mm diameter. These were filled with 10 µl of odour solution before the

experiment and used for one day. The mentioned Petri dish lids were equipped with four filter papers onto which 5 µl of odour solution were pipetted shortly before each experiment; these filter papers were renewed after each experiment. As odour substances, either n-amylacetate, (AM; CAS: 628-63-7, Merck, Darmstadt, Germany; diluted 1:20 in paraffin oil; CAS: 8042-47-5, AppliChem, Darmstadt, Germany) or 1-octanol were used (1-OCT; CAS: 111-87-5; Merck, Darmstadt, Germany; undiluted, unless mentioned otherwise). Paraffin is without behavioural significance in larval *Drosophila* (Saumweber et al., 2011).

Behavioural experiments

Effects of PAIRED versus UNPAIRED training with one training trial

Experiments followed standard procedures using a one-odour, single-training-trial protocol (Saumweber et al., 2011; Weiglein et al., 2019; for a manual see Michels et al., 2017) (Fig. 1A). Larvae underwent either paired or unpaired presentations of AM as the odour and the sugar taste reward (+), followed by a preference test for the odour.

For paired training (PAIRED) two containers with AM were located on opposite sides of a Petri dish filled with sugar-supplemented agarose. Cohorts of approximately 30 larvae were placed to the middle of the Petri dish and left undisturbed for 2.5 min to migrate the Petri dish (AM+). Subsequently the larvae were collected with a brush and transferred to a second 'blank' Petri dish containing neither the sugar reward nor the odour and left free to move about the Petri dish for further 2.5 min; in this case two empty odour containers (EM) were placed on the Petri dish. In half of the cases this sequence (AM+/EM) was as mentioned, while in the other half of the cases it was the reverse (EM/AM+). Subsequently the larvae were transferred to a test Petri dish with agarose but no sugar added (unless mentioned otherwise); in this case one odour container with AM was placed on one side and an EM container on the opposite side of the Petri dish. After 3 min the number of larvae on each side (@AM and @EM, respectively) and on a 10-mm wide middle zone were counted, including the larvae crawling up the sidewalls of the Petri dish; larvae crawling up the lid of the Petri dish were excluded (< 5%). From these numbers the preference for the odour was calculated as:

(1) $$Odour\ preference = \frac{@\mathrm{AM} - @\mathrm{EM}}{\mathrm{Total}}$$

Thus, Odour preference values may range from +1 to -1, with positive values indicating approach to the odour whereas negative values indicate avoidance.

For unpaired training (UNPAIRED), an independent cohort of larvae was likewise placed to Petri dishes, in this case, however, featuring only agarose with the sugar reward added but no odour (EM+), or the odour but no sugar reward added to the agarose (AM). Again, either the indicated sequence (EM+/AM), or the reverse sequence (AM/EM+) was used, followed by the test for odour preference as described above.

To quantify associative memory, a Memory score was calculated as the difference in Odour preference after PAIRED versus after UNPAIRED training:

$$(2)\quad Memory\ score = \frac{\text{Odour preference PAIRED} - \text{Odour preference UNPAIRED}}{2}$$

Thus, values for the Memory score may range from +1 to -1 with positive values indicating appetitive associative memory whereas negative values would indicate aversive associative memory.

To quantify the effect of using different sequences of odour and sugar reward presentation, we calculated a Difference index separately for the PAIRED and UNPAIRED groups as follows:

$$(3)\quad Difference\ index_{PAIRED} = \frac{\text{EM/AM+} - \text{AM+/EM}}{2}$$

$$(4)\quad Difference\ index_{UNPAIRED} = \frac{\text{EM+/AM} - \text{AM/EM+}}{2}$$

Thus, values of the Difference score may range from +1 to -1. In the PAIRED group positive values indicate higher preferences when the training sequence was EM/AM+ as compared to the reverse sequence, whereas for the UNPAIRED group positive values indicate higher preferences when the training sequence was EM+/AM than for the reverse sequence.

In turn, in the PAIRED group negative Difference scores indicate lower preferences when the training sequence was EM/AM+ as compared to the reverse sequence, whereas for the UNPAIRED group negative values indicate lower preferences when the training sequence was EM+/AM than for the reverse sequence.

While the signs of the Difference score are arbitrary in a technical sense, we note that one of the theoretical concepts we seek to test suggests that the Difference scores should be zero

for the PAIRED group but, because of conditioned inhibition, should be negative for the UNPAIRED group.

Effects of PAIRED versus UNPAIRED training with three training trials

All procedures were the same as in the preceding section, except that two additional training trials were performed.

Sensory preconditioning

To test for sensory preconditioning in larval *Drosophila*, we adapted the paradigm described above by adding an additional training phase. Thus, larvae underwent first a preconditioning training to establish an association between two odours, followed by a conditioning training for one of the odours as described above and a test of odour preference for the non-rewarded odour, using a 2x4x1-group experimental design (Fig. 4).

More detailed, in the first training phase, the Preconditioning phase, larvae received the two odours AM and 1-OCT either presented together as a compound (AM&1-OCT/EM or EM/AM&1-OCT; COMPOUND PRECONDITIONED) or separated from each other (AM/1-OCT or 1-OCT/AM; SEPARATED PRECONDITIONED) on Petri dishes with agarose but no added sugar. The respective sequence was repeated for three times and followed by a second training phase, the Conditioning phase, in which larvae of the PAIRED group received a single paired presentation of AM and the sugar reward (AM+/EM or EM/AM+), larvae of the HANDLING group were presented with neither the odour nor the sugar reward (EM/EM), of the SUGAR EXPOSURE group received only the sugar reward (EM+/EM or EM/EM+) and of the ODOUR EXPOSURE group only the odour (AM/EM or EM/AM). In the following test, larvae were tested for their preference of the non-conditioned odour, 1-OCT, determined with due adjustment according to Equation 1.

To quantify the difference between SEPARATED PRECONDITIONED and COMPOUND PRECONDITIONED animals, a Difference score was calculated as:

$$(5)\quad Difference\ index = \frac{\text{Odour preference COMPOUND} - \text{Odour preference SEPARATED}}{2}$$

Thus, positive scores indicate a higher odour preference in COMPOUND PRECONDITIONED larvae compared to SEPARATED PRECONDITIONED larvae, whereas negative values indicate the opposite.

Sensory preconditioning is indicated by higher Difference scores in larvae that received PAIRED training during the Conditioning phase compared to larvae of the HANDLING, SUGAR EXPOSURE and ODOUR EXPOSURE groups.

Instead of odour containers, throughout the experiment lids equipped with four (during training) or two (during test) sticky filter papers were used. 1 min before the lid was placed on the Petri dish, each of the filter papers was loaded with 5 µl of AM and/or 5µl of 1-OCT. Other details were as described above. Variations of the paradigm are mentioned within the results section.

Second-order conditioning

To probe for second-order conditioning in larval *Drosophila*, first, larvae underwent a 1st-order conditioning training to establish an odour-reward association, followed by a 2nd-order conditioning training in which the previously rewarded odour and another odour are presented. Subsequently larvae are tested for their odour preference, resulting in a 2x4x1-group experimental design (Fig. 6).

In the 1st Conditioning phase, larvae of the PAIRED group received a single paired presentation of AM and the sugar reward (AM+/EM or EM/AM+), of the HANDLING group were presented with neither the odour nor the sugar reward (EM/EM), of the SUGAR EXPOSURE group received only the sugar reward (EM+/EM or EM/EM+) and of the ODOUR EXPOSURE group only the odour (AM/EM or EM/AM). This was followed by a 2nd Conditioning phase, in which the previously rewarded odour AM was either presented as a compound together with a second odour (AM&1-OCT/EM or EM/AM&1-OCT; COMPOUND CONDITIONED) or separated from each other on different Petri dishes with agarose (AM/1-OCT or 1-OCT/AM; SEPARATED CONDITIONED). Subsequently larvae were tested for their preference for the odour that was not trained in the 1st-order conditioning phase, 1-OCT, and the Odour preference was determined with due adjustment according to Equation 1.

For quantification of the difference between SEPARATED CONDITIONED and COMPOUND CONDITIONED animals, a Difference score was calculated according to Equation 5, with positive scores indicating higher odour preference in larvae COMPOUND CONDITIONED compared to larvae SEPARATED CONDITIONED, whereas negative values indicate the opposite.

Second-order conditioning is indicated by higher Differences scores in larvae of the PAIRED trained group compared to larvae of the HANDLING, SUGAR EXPOSURE and ODOUR EXPOSURE groups.

Other details were as described in the previous section. Variations of the paradigm are mentioned within the results section.

Statistics

Non-parametric statistics were performed throughout (Statistica 13, RRID:SCR_014213, StatSoft Inc., Tulsa, USA). To test if values are significant relative to chance level (zero), one-sample sign tests (OSS) were used. When compared across multiple independent groups Kruskal-Wallis tests (KW) with subsequent pair-wise comparisons by Mann-Whitney U-tests (MWU) were performed. To ensure a within-experiment error rate below 5% Bonferroni-Holm correction (Holm, 1979) was applied. Data is shown as Box plots with the median as the middle line, the 25 and 75 % quantiles as box boundaries, and the 10, 90 % quantiles as whiskers. Results of the statistical tests are documented in Table S1 in the supplement.

Results

PAIRED and UNPAIRED training modulate odour preferences in an opposing manner

Odour preferences after one trial of PAIRED odour-reward training are higher than after UNPAIRED training, that is after presentations of odour temporally separated from the reward (Fig. 1A). Such a difference indicates appetitive associative memory and is reflected in a positive Memory score (Fig. 1B). Corresponding results were obtained after three training trials (Fig. 1C, D), confirming earlier reports (for example El-Keredy et al., 2012; Saumweber et al., 2011; Schleyer et al., 2015; Weiglein et al., 2019). Such results do not allow for a conclusion whether learning has taken place through PAIRED training, through UNPAIRED training, or both, however. This is because through PAIRED training the larvae may learn that the odour predicts the occurrence of the reward, leading to an increase in Odour preference relative to baseline. In turn, through UNPAIRED learning they may learn the opposite, namely that the odour predicts the non-occurrence of the reward, leading to a decrease in Odour preference

relative to baseline. This begs the question what the baseline level of Odour preference is, cleared of associative effects of the training experience.

In larval *Drosophila* such a baseline Odour preference can be conveniently determined by testing the animals in the presence of the reward. That is, the difference in Odour preference after PAIRED versus UNPAIRED training that is observed under conventional testing conditions is abolished when the testing is carried out in the presence of the reward (Fig. 2A), leading to Memory scores indistinguishable from chance level (Fig. 2B). This effect has been reported before, including controls showing that 'innate' Odour preferences in experimentally naïve animals are not altered by the presence of the reward (for example Paisios et al., 2017; Schleyer et al., 2011, 2015, 2020), a result that we replicate here (Fig. S1). These findings can be grasped best by the notion of appetitive associative memory supporting a learned search for the reward, which is adaptively abolished when the sought-for reward is indeed present (for more detailed discussions see Gerber and Hendel, 2006; Schleyer et al., 2011). For the current context, the important point is that the residual Odour preference that are observed when the reward is present during testing thus reflects the Odour preference cleared of the influence of associative memories and therefore can be used as baseline against which associative effects of PAIRED and UNPAIRED training can be measured (stippled line in Fig. 2A). This shows that Odour preferences after PAIRED training are increased relative to baseline, while after UNPAIRED training they are decreased as compared to baseline (Fig. 2A). The same is observed after three training trials (Fig. 2C, D), confirming earlier results reports (Paisios et al., 2017; Saumweber et al., 2011; Schleyer et al., 2011, 2015b, 2020; Weiglein et al., 2019). We conclude that PAIRED versus UNPAIRED training have parametrically opposite effects on Odour preference.

Does UNPAIRED training establish conditioned inhibition or learned inattention?

The opposing behavioural effects of PAIRED versus UNPAIRED training conform to the experimental psychology constructs of conditioned excitation versus inhibition, proposing that these opposite behavioural tendencies reflect the prediction of reward occurrence versus reward non-occurrence as implemented in prediction-error learning rules (for example Malaka et al., 1995; Rescorla and Wagner, 1972). However, in an alternative scenario it was suggested that the respective training protocols result in increases versus decreases in how

effectively conditioned stimuli are processed. For the current case, the opposing effects of PAIRED versus UNPAIRED training would thus be suggested to come about by increased versus decreased learned attention to the odour. However, our results show that UNPAIRED training can not only establish decreases in Odour preference relative to baseline, but indeed establishes avoidance (Fig. 2C). This confirms earlier results (Schleyer et al., 2018) and is incompatible with learned inattention as an explanation for the effects of UNPAIRED training because a lack of attention may reduce odour preference to zero, but cannot establish avoidance. This makes it superfluous to run separate experiments to check for learned inattention as an alternative scenario to explain learning through UNPAIRED training in what are called retardation of acquisition and summation tests (see section 'The measurement of inhibition' in Rescorla, 1969 for more details).

The prediction-error learning rules mentioned in the preceding paragraph have it that conditioned excitation ensues when there is a positive prediction error, that is when for example a reward is received although it was not predicted. For the current case, this is straightforward for PAIRED training when the odour is presented together with the reward. The prediction-error is strongly positive in the first trial ('pleasant surprise'), and becomes less and less as training progresses - because the reward is predicted better and better. This results in strong learning already in the first trial and an asymptotic learning curve as training progresses.

In turn, conditioned inhibition ensues when there is a negative prediction error, that is when for example a predicted reward is not actually received ('frustrating surprise'). For UNPAIRED training, this is not straightforward, however. Considering the current case, this is because one wonders what the source of a reward-prediction would be at the moment of odour presentation. Prediction-error learning rules typically make the assumption that presenting the reward alone can establish associative memories for the context in which this happens (Bouton and Moody, 2004; Bouton and Nelson, 1998; Dweck and Wagner, 1970; Grau and Rescorla, 1984; Nadel and Willner, 1980; Rescorla, 1972). When the odour is subsequently presented in that same context, these context-reward associations provide an expectation of reward which, however, frustratingly is not received. The ensuing negative prediction-error then is the basis for conditioned inhibition to accrue to the odour.

While it was admitted already by its proponents that this assumption has an *ad hoc* character, it does not generally seem implausible and provides an interesting experimental test

because such negative prediction errors would only arise when during UNPAIRED training the reward-only presentation comes first, establishing context-reward associations, and the odour-only presentation comes second. For the reverse sequence of the odour being presented first, there would not yet be any context-reward association that could be frustrated. Such sequence dependence of Odour preference values after UNPAIRED training should be particularly prominent when only one training trial is used, because for multiple training trials contextual memories established in trial #1 would need to be reckoned with from trial #2 on, 'ironing out' sequence-effects as training proceeds. Of note, the learned inattention scenario to account for learning through UNPAIRED training would not suggest sequence-effects in either case. With these considerations in mind, we analysed two large dataset that we accumulated over the years with course students, interns, scientific guest and apprentice staff using our standard one-trial training procedure (Fig. 3A-D; total sample size N= 898) as well as corresponding experiments with three training trials (Fig. 3E-H; total sample size N= 379).

Considering these combined datasets confirms that Odour preference values after one trial PAIRED training are higher than after UNPAIRED training (Fig. 3A), yielding positive Memory scores indicative of appetitive associative memory (Fig. 3B); corresponding results were obtained for the dataset using three training trials (Fig. 3E, F). When Odour preference values are separated by the sequence of odour and sugar reward presentation during training it turns out that for the PAIRED case of one-trial training, Odour preference values are slightly and, given the large sample size, significantly lower when the blank presentation (EM) comes first and the odour-reward pairing (AM+) comes second as compared to the reverse AM+/EM sequence (Fig. 3C left two box plots). Evidence is more clear-cut for the UNPAIRED case, with Odour preference values obviously lower for the EM+/AM sequence than for the AM/EM+ sequence (Fig. 3C right two box plots). Indeed, compared to the PAIRED case the sequence of training events has more impact in the UNPAIRED case, as quantified by the Difference index (Fig. 3D). No differences of Odour preference values between the training sequences were observed for three trial training (Fig. 3G, H). We conclude that sequence-effects in one-trial PAIRED training are weaker than after UNPAIRED training, and absent for multiple training trials. This pattern of results conforms to the suggestions of prediction-error learning rules outlined in the preceding paragraph, and cannot be accounted for by scenarios invoking changes in learned attention to the odour as the psychological mechanisms of learning through UNPAIRED training.

No evidence of sensory preconditioning

To test for sensory preconditioning, larvae received first two odours (AM & 1-OCT) either as a COMPOUND or SEPARATED from each other (Preconditioning phase) followed by PAIRED odour (AM)-sugar reward training (Conditioning phase) and subsequently testing for their preference for the non-rewarded odour (1-OCT) (see also sketches Fig. 4A). The rationale of the experiment was, that if larvae learn that these two odours 'belong' together larvae of the COMPOUND group will show a higher preference for the non-rewarded odour (1-OCT) in the test than larvae of the SEPARATED group. After three trials of Preconditioning and a single trial of Conditioning, COMPOUND training indeed yielded into higher preference for the non-rewarded odour (1-OCT) than SEPARATED training (Fig. 4A, left two box plots).

To confirm that the difference in preferences is due to the association between AM and the reward during the Conditioning phase we tested different control groups in which the Conditioning phase was varied in a way that no association between odour and sugar reward should take place while keeping the Preconditioning phase unchanged. Thus, additional to the previous mentioned PAIRED training, larvae were either put on only agarose Petri dishes without any odour (HANDLING), were exposed to only the sugar reward without any odour (FRU EXPOSURE) or were presented with only the odour AM on an agarose Petri dish (ODOUR EXPOSURE). Surprisingly, in all the tested control groups COMPOUND training resulted in a higher preference for the non-rewarded odour (1-OCT) than SEPARATED training (Fig. 4A). Also the quantification of the difference between a given COMPOUND and SEPARATED group displayed by the Difference index revealed no difference between PAIRED conditioning and the control groups (Fig. 4B), thus no sensory preconditioning was observed. However, the type of preconditioning influences the tested odour preference, meaning COMPOUND preconditioning overall resulted in higher odour preference than SEPARATED preconditioning (Fig. 4B, most-right box plot).

Varying the number of training trials of the Preconditioning and Conditioning phase confirmed these results. After a single trial of Preconditioning and three trials of Conditioning (Fig. 4C-D) COMPOUND preconditioning and SEPARATED preconditioning led to indistinguishable odour preferences (Fig. 4C) resulting in equal Difference indices (Fig. 4D). Only a single trial of Preconditioning and a single trial of Conditioning yielded in higher odour preferences after COMPOUND preconditioning only in the PAIRED and ODOUR EXPOSURE

trained groups (Fig. 4E), again resulting in equal Difference indices (Fig. 4F). But COMPOUND preconditioning led to higher odour preference than SEPARATED preconditioning (Fig. 4F, most-right box plot).

Overall, our experiments do not find evidence of sensory preconditioning in larval *Drosophila.* To rule out the possibility that larvae are not able to build an association between the odour AM and the sugar reward during the Conditioning phase after Preconditioning, we conducted another control experiment (Fig. 5). Larvae first underwent three trials of Preconditioning with the COMPOUND or SEPARATED presentation of two odours (AM and 1-OCT) followed by a single PAIRED or UNPAIRED training of AM and a sugar reward and subsequently were tested for their preference for the rewarded odour AM (see sketches Fig. 5). Odour preferences after PAIRED training were higher than after UNPAIRED training (Fig. 5A) resulting in appetitive associative memory reflected by a positive Memory score (Fig. 5B). Interestingly, the memories were not different between COMPOUND and SEPARATED preconditioned animals, thus the type of odour presentation during the Preconditioning phase had no influence on the Memory score.

Altogether, we conclude that larvae do not prefer a non-rewarded odour more when it was previously presented in a compound together with a later rewarded odour than when the two odours were previously presented separately. However, larvae tend to like an odour more when it has previously been presented as a compound along with another odour, and they still learn the association between one of the odours and a sugar reward.

No evidence of second-order conditioning

Next, we were asking for second-order conditioning in larvae, meaning if larvae will show a higher preference to an odour (1-OCT) when it itself was not rewarded but when it was presented together as a compound with another previously rewarded odour (AM) (COMPOUND) than when both odours were presented separately (SEPARATED) (see also sketches Fig. 6A). When performing three trials of PAIRED 1st-order conditioning and a single trial of 2nd-order conditioning in an either COMPOUND or SEPARATED manner, COMPOUND training yielded to higher odour preference scores than SEPARATED training (Fig. 6A, left two box plots). However, when we varied the 1st-order conditioning phase in a way that no odour-sugar reward association should take place, also COMPOUND training led to higher preferences than SEPARATED training (Fig. 6A). The quantification of these

differences reflected by the Difference index revealed no difference between the groups with respect to the 1st-order conditioning phase (Fig. 6B), suggesting no second-order conditioning in larval *Drosophila*. However, regardless of the type of training during 1st-order conditioning, COMPOUND presentation of two odours resulted in higher odour preferences than SPEARATED presentation of these odours.

To test for the possibility that larvae do not show second-order conditioning because they are not capable of maintaining an odour-sugar reward association when they are afterwards presented with two odours, we conducted another control experiment. Larvae were either trained PAIRED or UNPAIRED with one odour (AM) and a sugar reward followed by presenting the previously trained odour either together in a compound (COMPOUND) or separated from another second odour (1-OCT) (SEPARATED) and subsequently were asked for their preference of the previously rewarded odour (AM) (see also sketches Fig. 7A). Paired training turned out to yield in higher odour preferences than separated training (Fig. 7A) resulting in appetitive associative memory reflected by a positive Memory score (Fig. 7B). However, COMPOUND training led to stronger memory than SEPARATED training.

Overall, we found no evidence that larvae prefer an odour that was itself not rewarded but that was paired together with a previously rewarded odour. However, we demonstrated that larvae are able to maintain an association between an odour and a sugar-reward regardless of whether that odour was subsequently presented together with another odour, and that larvae show a higher preference for an odour when it was presented together with another odour in a compound than when the two odours were presented separately.

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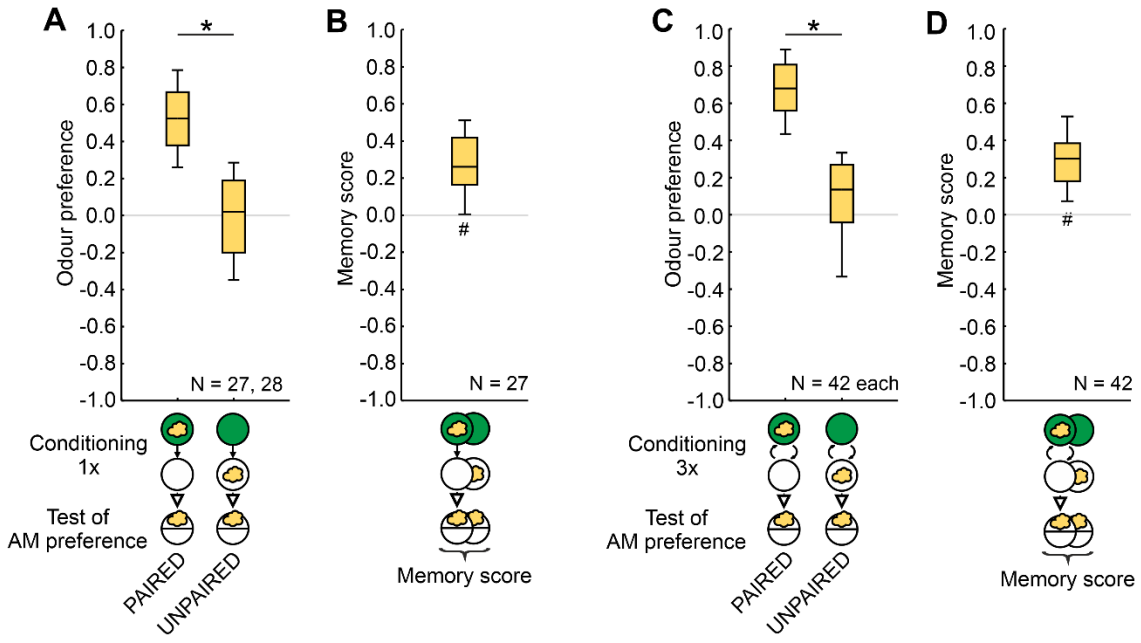

Fig. 1. Odour-sugar associative memory in larval *Drosophila*. (A-D) *Drosophila melanogaster* larvae received either PAIRED training of the odour (yellow cloud, n-amylacetate) and a fructose sugar reward (green circle) presented on the same Petri dish, or they received UNPAIRED training, that is the sugar reward and the odour were presented on separate Petri dishes. To equate PAIRED and UNPAIRED training for total training duration and for the transfer procedure between Petri dishes, blank periods with a Petri dish featuring neither the odour nor the sugar reward (open circle) were added for the PAIRED case. The sequence of events during training was as depicted in half of the cases and the reverse in the other half of the cases (not shown). After training, larvae were tested for their Odour preference (Equation 1) and a Memory score was calculated as the difference between the Odour preferences of the PAIRED versus the UNPAIRED trained animals, divided by two (Equation 2). Positive and negative Memory scores therefore represent appetitive and aversive associative memories, respectively. After one-trial PAIRED training the larvae show higher Odour preferences values than after UNPAIRED training (A). This difference is quantified as a positive Memory score, indicating appetitive associative memory (B). The same pattern of results is observed after three training trials (C, D). Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns indicate significance and non-significance, respectively, in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement.

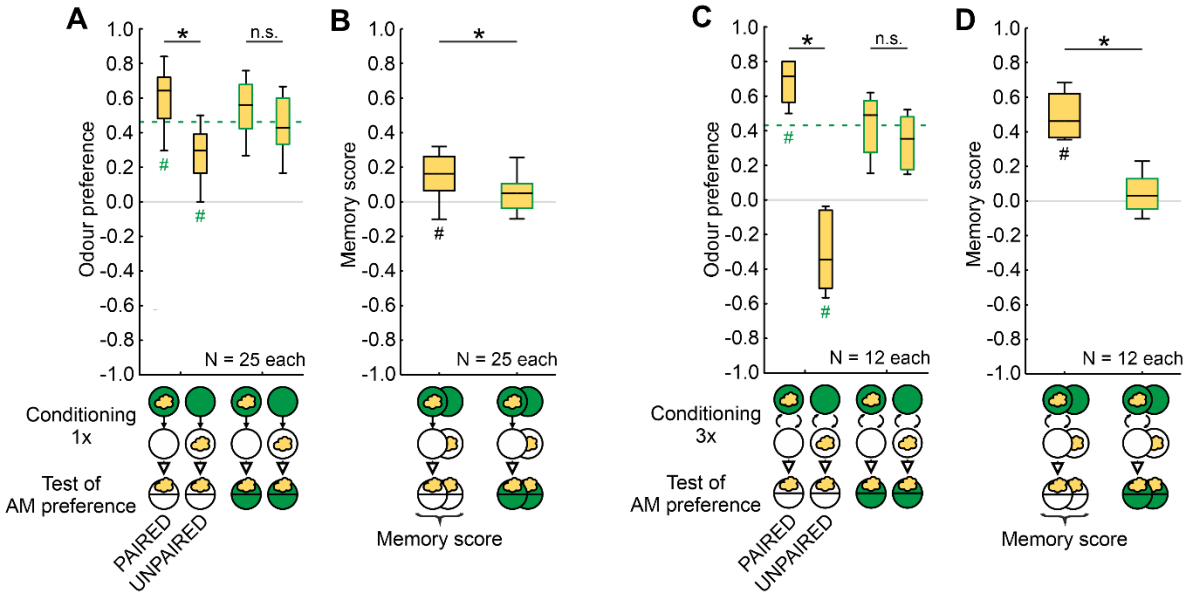

Fig. 2. PAIRED and UNPAIRED training modulate odour preferences in an opposing manner. (A-D) Larvae were trained with PAIRED or UNPAIRED presentations of odour and the sugar reward and were subsequently tested for their Odour preference, either in the absence or in the presence of the sugar reward. After one-trial PAIRED training and when tested in the absence of the reward, the larvae show higher Odour preference values than larvae trained in an UNPAIRED manner (A) (two box-plots to the left). However, when the larvae were tested in the presence of the reward, this difference in Odour preference is abolished (A) (two box-plots to the right); their respective Odour preference values can thus be pooled to represent baseline levels of Odour preference, cleared of the influence of associative memories (green stippled line). Comparisons against this baseline demonstrate increased Odour preferences after PAIRED training and decreased Odour preferences after UNPAIRED training (green #-symbol). Fittingly, Memory scores are higher when the animals are tested in the absence than in the presence of the reward (B) (in contrast, innate Odour preferences, that is Odour preferences of experimentally naïve animals, are unaffected by the presence of the reward: Fig. S1). The same pattern of results is observed after three training trials (C, D); as mentioned in the body text, it is of conceptual significance that after UNPAIRED training odour avoidance is observed when the test is carried out in the absence of the reward (C) (second box-plot from the left). Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement. All other details as in Fig. 1.

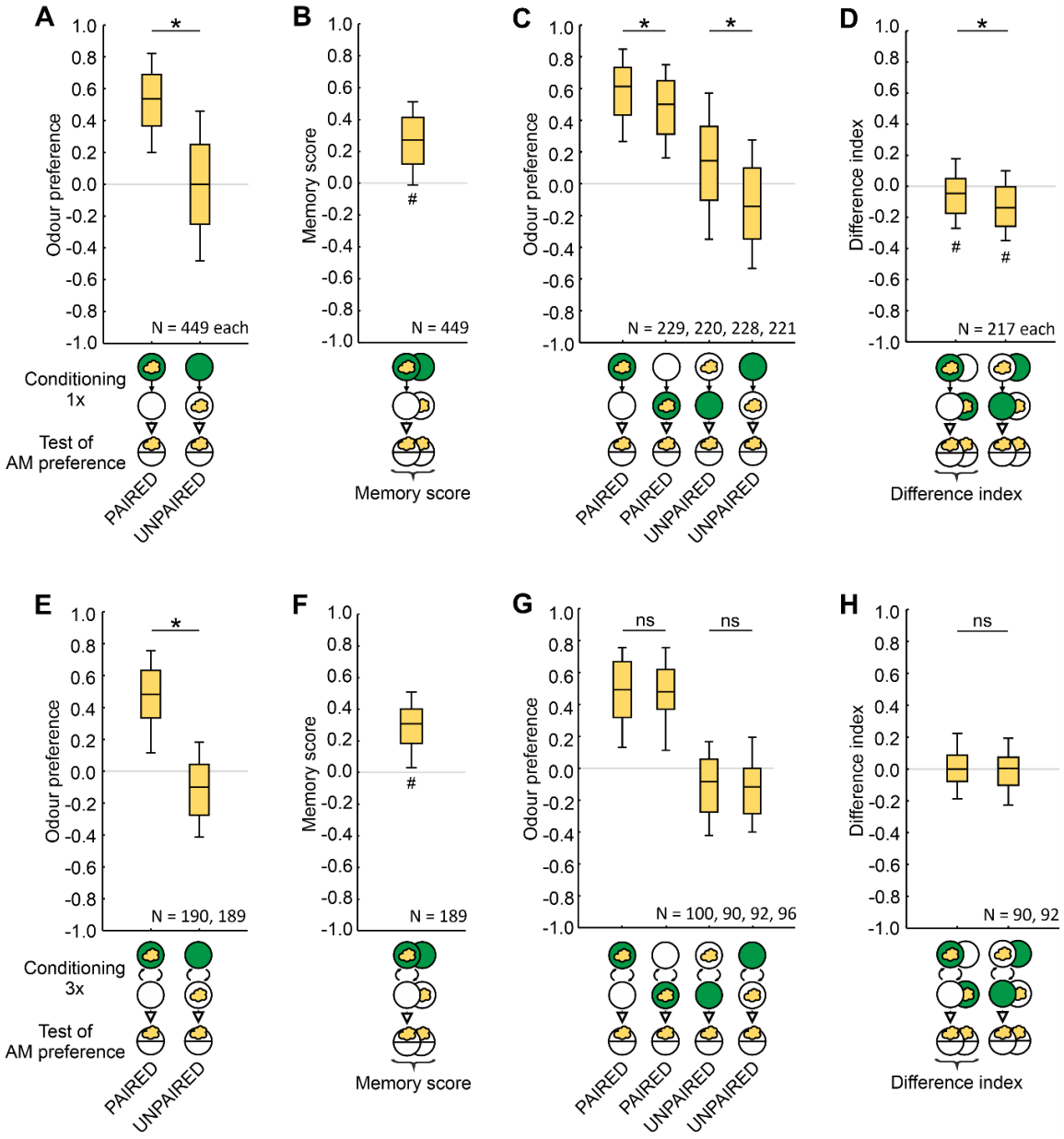

Fig. 3. Stronger effect of the sequence of training events for UNPAIRED than for PAIRED training. (A, B) Larvae received one trial of either PAIRED or UNPAIRED training of odour and the sugar reward. The sequence of events during training was as depicted in half of the cases and the reverse in the other half of the cases (not shown here, but see C, D). In all cases, training was followed by a test of Odour preference, which turned out to be higher after PAIRED training than after UNPAIRED training (A), resulting in positive Memory scores indicative of appetitive associative memory (B). (C, D) Data from (A), separated by the sequence of events during training. Upon PAIRED training, Odour preference values are slightly lower when the blank period (open circle) comes first and the odour-reward pairing comes second, as compared to the reverse sequence (C) (two box-plots to the left). The same effect is observed upon UNPAIRED training (C) (two box-plots to the right). A quantification of these differences by the Difference index (Equation 3, Equation 4) reveals stronger effects of the sequence of training events during UNPAIRED training (D). (E-H) As in (A-D), but for three training trials, providing evidence for associative memory (A, B), but no evidence of an effect of the sequence of events during training (G, H). Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement. All other details as in Fig. 1.

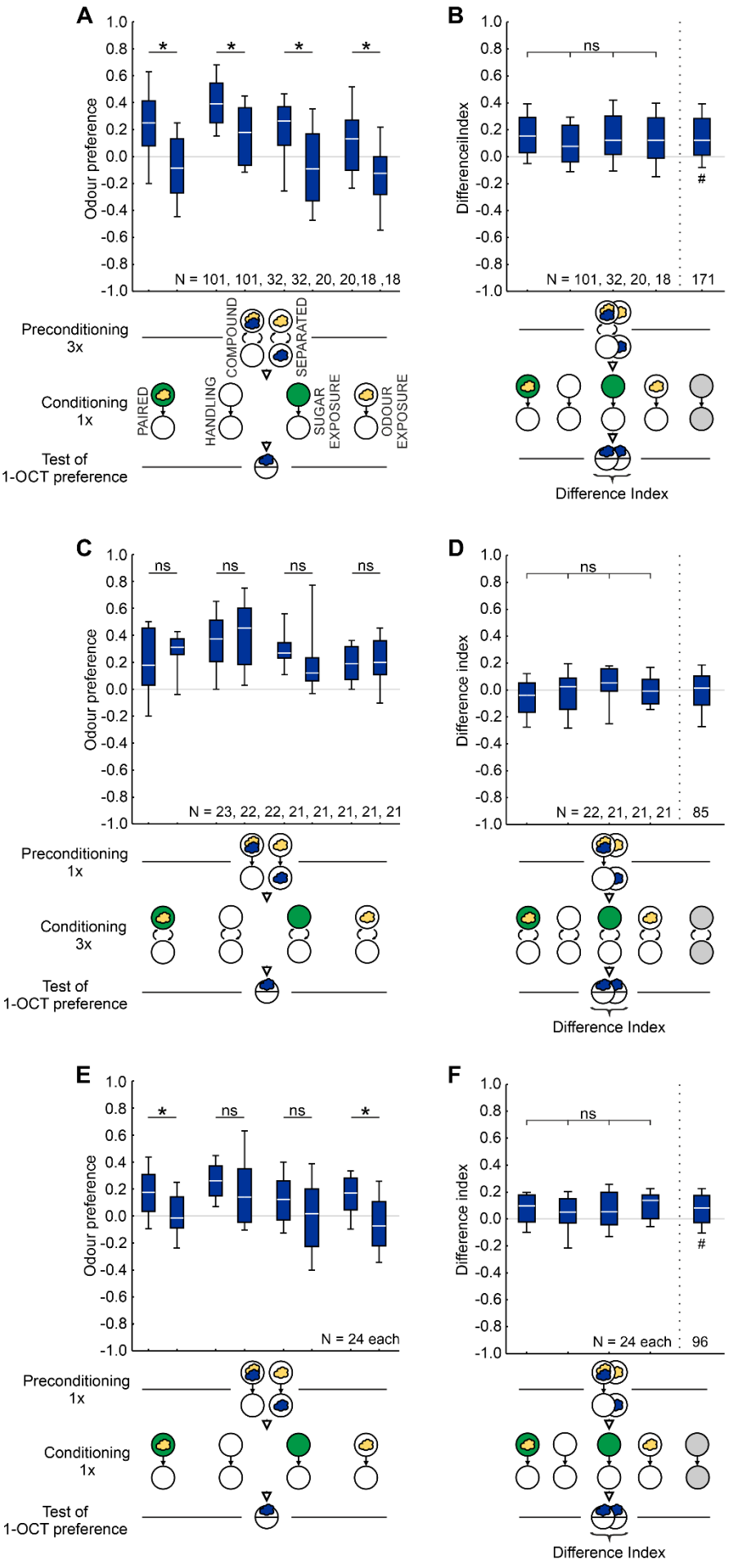
A
B
C
D
E
F
Odour preference
Difference index
DifferenceIIndex
N = 101, 101, 32, 32, 20, 20,18 ,18
N = 101, 32, 20, 18
171
N = 23, 22, 22, 21, 21, 21, 21, 21
N = 22, 21, 21, 21
85
N = 24 each
96
ns
*
#
Preconditioning 3x
Conditioning 1x
Test of 1-OCT preference
PAIRED
HANDLING
COMPOUND
SEPARATED
SUGAR EXPOSURE
ODOUR EXPOSURE
Preconditioning 1x
Conditioning 3x
Difference Index

Fig. 4. No evidence of sensory preconditioning in larval *Drosophila*. Larvae underwent a training consisting of two phases. During the first phase, the Preconditioning phase, larvae were presented with two odours, AM (yellow cloud) and 1-OCT (blue cloud), either together on the same agarose Petri dish (white circle; COMPOUND) or separated on different Petri dishes (SEPARATED). This training phase was followed by the Conditioning phase in which larvae received either PAIRED training of the indicated odour and sugar reward (green circle), no odour and no sugar reward (HANDLING), only the sugar reward (SUGAR EXPOSURE) or only the odour (ODOUR EXPOSURE). Subsequently larvae were tested for their preference for the odour not used during the conditioning phase. Positive and negative preferences reflect approach or avoidance towards the odour in the test, respectively (Equation 1). (A-B) When larvae received three training trials of Preconditioning and one training trial of Conditioning, (A) COMPOUND Preconditioning led to higher odour preferences than SEPARATED Preconditioning. (B) The quantification of this difference (Equation 5) revealed no effect of the type of Conditioning in the second phase. Independent of what happened during the Conditioning phase, overall COMPOUND Preconditioning led to higher odour preferences than SEPARATED Preconditioning displayed by a positive Difference index (box most right). (C-D) After one training trial of Preconditioning and three trials of Conditioning, the same effects were observed as in A-B with the exception that independent of the Conditioning phase, there was no difference of how the odours were presented during the Preconditioning phase (box most right). (E-F) Training only a single trial of Preconditioning and Conditioning each led to the same effects as in A-B. Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test or KW-test. Results of the statistical tests are documented in Table S1 in the supplement.

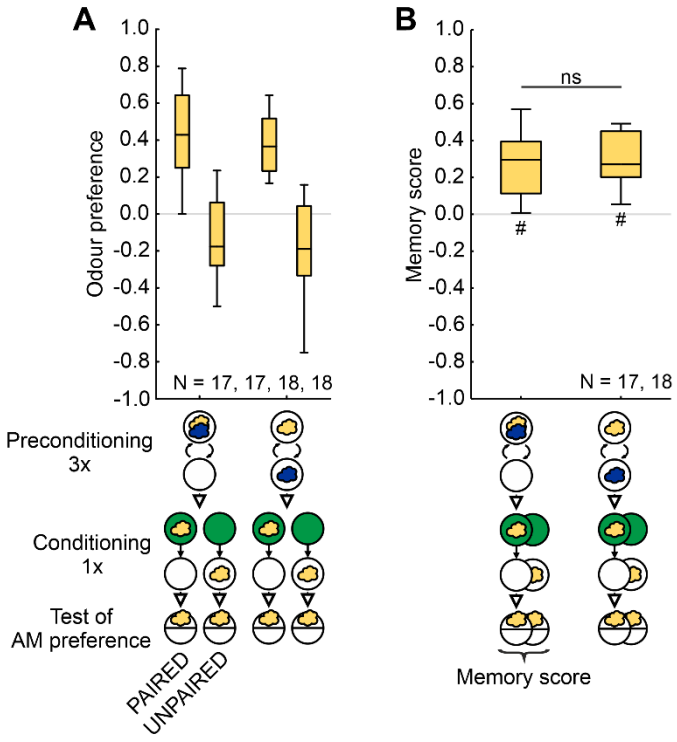

Fig. 5. Memory formation is not disturbed by Preconditioning with odours. Larvae underwent a Preconditioning phase of three trials in which they received two odours either together on the same agarose Petri dish (white circle; COMPOUND) or separated on different Petri dishes (SEPARATED) followed by a Conditioning phase in which larvae received one trial of either PAIRED or UNPAIRED training of one of the odours and the sugar reward. Subsequently larvae were tested for their preference the odour used during the Conditioning phase. Positive and negative preferences reflect approach or avoidance towards AM in the test, respectively (see Equation 1). (A) Regardless of the type of Preconditioning, PAIRED training led to higher odour preferences than UNPAIRED training, (B) resulting in positive Memory scores in both groups that do not differ. Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement. All other details as in Fig. 1.

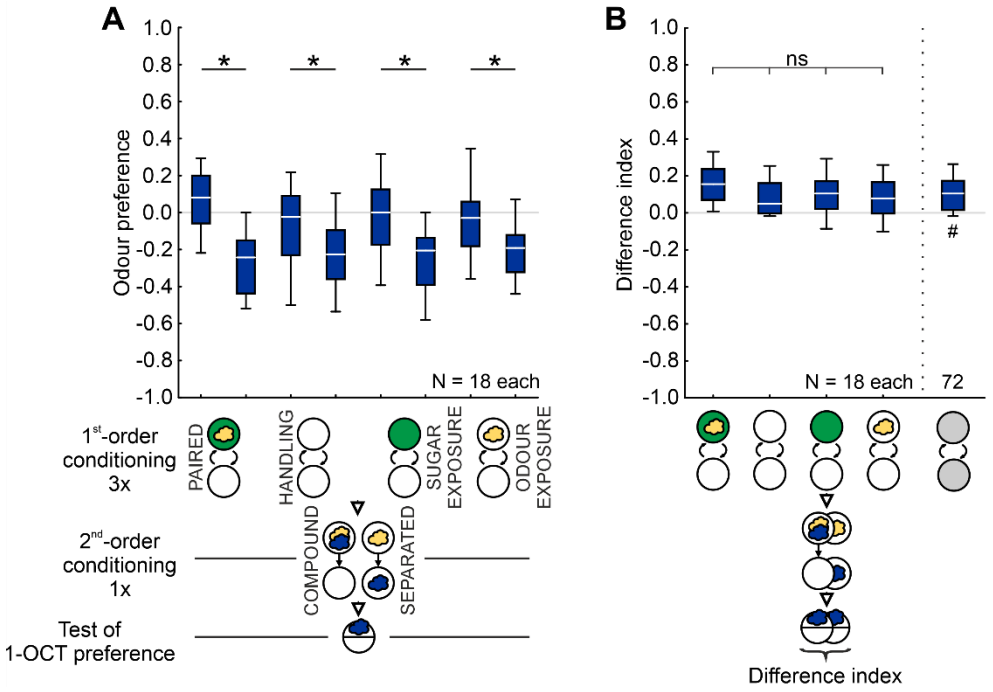

Fig. 6. No evidence of second-order conditioning in larval *Drosophila*. (A-B) The second-order conditioning paradigm consisted of two phases. During the first Phase, 1st-order conditioning, larvae received three trials of either PAIRED training of AM as the odour and a sugar reward (green circle), no odour and no sugar reward (HANDLING), only the sugar reward (SUGAR EXPOSURE) or only the odour (ODOUR EXPOSURE). This phase was followed by a 2nd-order conditioning phase in which larvae were presented for a single time with two odours, AM and 1-OCT, either together on the same agarose Petri dish (COMPOUND) or separated on different Petri dishes (SEPARATED). Subsequently larvae were tested for their preference for the odour not used in the 1st-order conditioning phase. (A) COMPOUND trained animals show higher odour preferences than SEPARATED trained animals. (B) The quantification of this difference revealed no effect of the type of training during the 1st-order conditioning phase. Overall, COMPOUND 2nd-order conditioning led to higher odour preferences than SEPARATED 2nd-order conditioning displayed by a positive Difference index (box most right). Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test or KW-test. Results of the statistical tests are documented in Table S1 in the supplement. All other details as in Fig. 4.

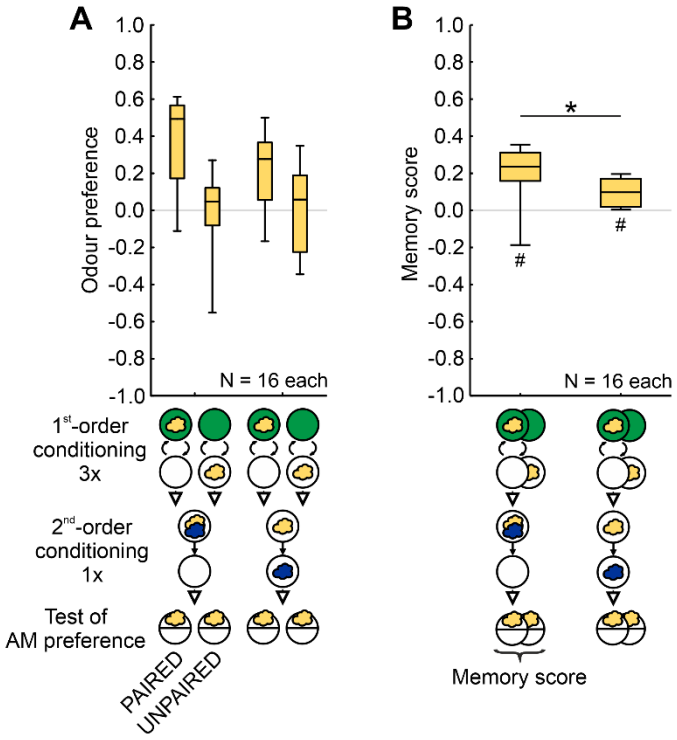

Fig. 7. Memory formation is not disturbed by odour exposure after odour-sugar reward conditioning. (A-B) In a 1st-order conditioning phase, larvae received three trials of either PAIRED or UNPAIRED training of AM and a sugar reward. This was followed by a 2nd-order conditioning phase in which the larvae were presented once with AM and 1-OCT either together in a compound (COMPOUND) or separately on different Petri dishes (SEPARATED). Subsequently larvae were tested for their preference for AM. (A) Regardless of what happened during the 2nd-order conditioning phase, PAIRED training led to higher odour preferences than UNPAIRED training, (B) resulting in positive Memory scores in both groups. Larvae that received COMPOUND training, however, showed a stronger memory the SEPARATED trained larvae. Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. # indicates significance to chance level, * and ns significance and non-significance, respectively, in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement. All other details as in Fig. 5.

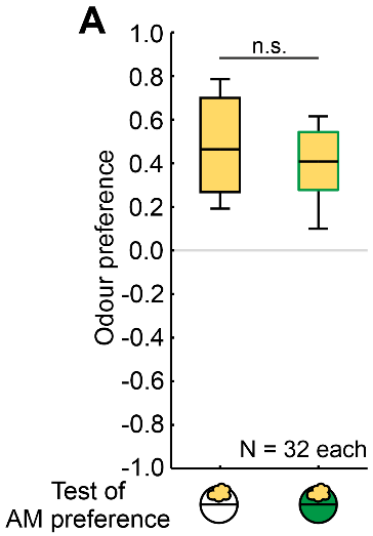

Fig. S1. Naïve odour preference is unchanged in presence of a sugar reward. Larvae were placed on a Petri dish filled with agarose (open circle) or sugar-supplemented agarose (green circle) together with one odour cup of AM (yellow cloud) on one side and an empty odour cup on the other side of the Petri dish. After 3min, the number of larvae on each side and in a middle zone were counted and an odour preference was calculated according to Equation 1. Larvae showed approach to the odour independent of sugar was present or not. Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure. Ns indicates non-significance in MWU-test. Results of the statistical tests are documented in Table S1 in the supplement.

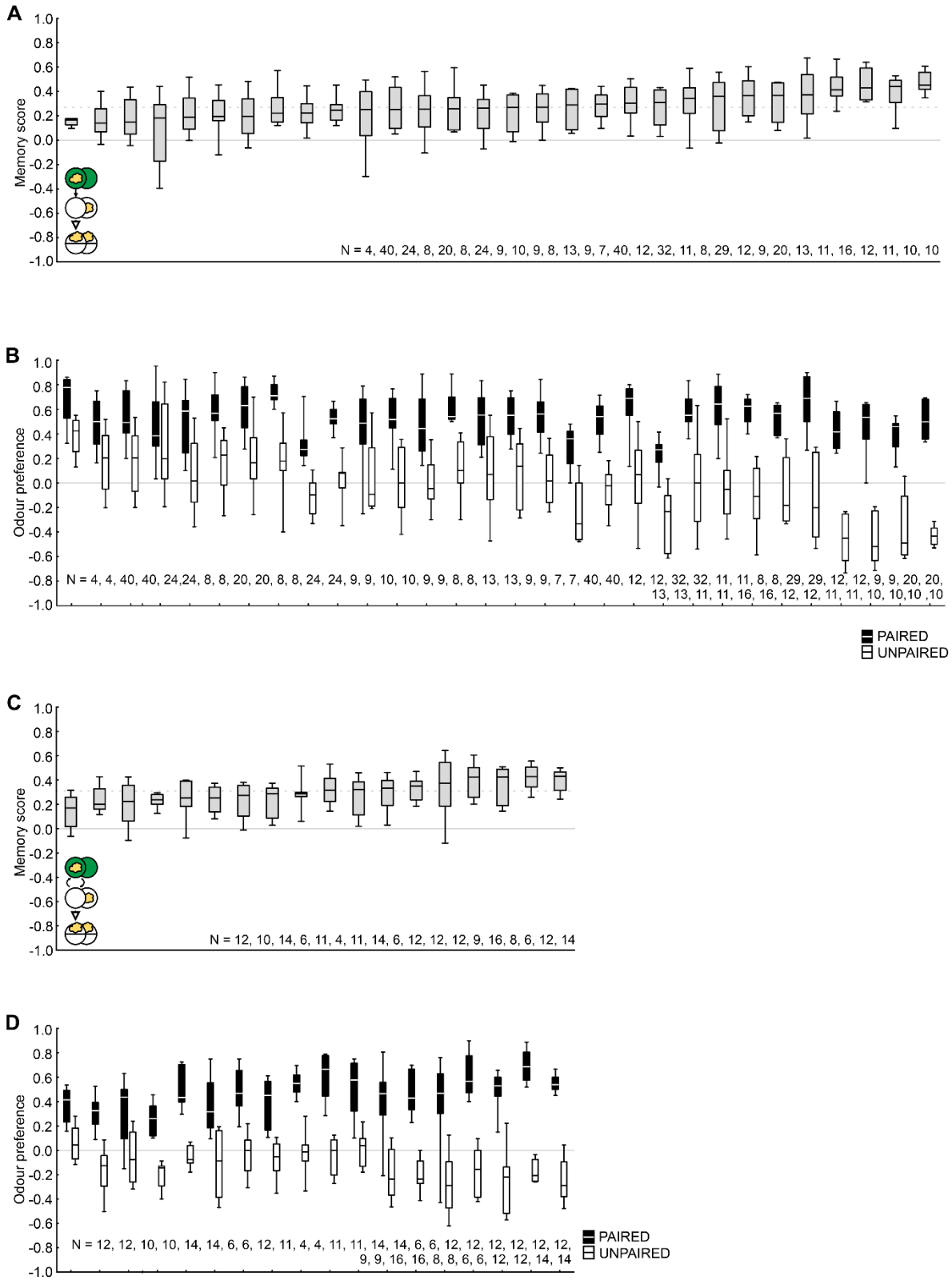

Fig. S2. **Separated data for the pooled data depicted in Fig. 3.** (A) Single datasets underlying Fig. 3B. (B) Single preferences for the paired (black boxes) and unpaired (white boxes) trained groups underlying Fig. 3A. (C) Single datasets underlying Fig. 3F. (D) Single preferences for the paired (black boxes) and unpaired (white boxes) trained groups underlying Fig. 3E. Box plots represent the median as the midline, the 25/75% quantiles as box boundaries and 10/90% quantiles as whiskers. Sample sizes are indicated within the figure.

Table S1. Statistical results

1A	**MWU test**	U = 35.00, p < 0.0001, N = 27, 28	
1B	**OSS test**	$p < 0.0001$, N = 27	
1C	**MWU test**	U = 50.00, p < 0.0001, N = 42 each	
1D	**OSS test**	p < 0.0001, N = 42	
2A	**KW test**	H (3, N = 100) = 27.99, p < 0.0001	
	MWU tests	Test@PUR	U = 84.00, p < 0.0001, N = 25 each
		Test@FRU	U = 214.50, p = 0.0585, N = 25 each
	OSS tests	PAIRED	p = 0.0009, N = 25
		UNPAIRED	p = 0.0002, N = 25
2B	**MWU test**	U = 178.00, p = 0.0093, N = 25 each	
	OSS tests	Test@PUR	p = 0.0041, N = 25
		Test@FRU	p = 0.6900, N = 25
2C	**KW test**	H (3, N = 48) = 34.71, p > 0.0001	
	MWU tests	Test@PUR	U = 0.00, p < 0.0001, N = 12 each
		Test@FRU	U = 51.00, p = 0.2362, N = 12 each
	OSS tests	PAIRED	p = 0.0010, N = 12
		UNPAIRED	p = 0.0005, N = 12
2D	**MWU test**	U = 1.00, p < 0.0001, N = 12 each	
	OSS tests	Test@PUR	p = 0.0005, N = 12
		Test@FRU	p = 0.3877, N = 12
3A	**MWU test**	U = 23500.50, p < 0.0001, N = 449 each	
3B	**OSS test**	p < 0.0001, N = 449	
3C	**KW test**	H (3, N = 898) = 440.13, p < 0.0001	
	MWU tests	PAIRED	U = 18638.50, p < 0.0001, N = 229, 220
		UNPAIRED	U = 14784.00, p < 0.0001, N = 228, 221
3D	**MWU test**	U = 18965.50, p < 0.0001, N = 220, 221	
	OSS tests	PAIRED	p < 0.0001, N = 220
		UNPAIRED	p < 0.0001, N = 221
3E	**MWU test**	U = 2277.50, p < 0.0001, N = 190, 189	
3F	**OSS test**	p < 0.0001, N = 189	
3G	**KW test**	H (3, N = 378) = 215.68, p < 0.0001	
	MWU tests	PAIRED	U = 4397.00, p = 0.7870, N = 100, 90
		UNPAIRED	U = 4116.00, p = 0.4220, N = 92, 96
3H	**MWU test**	U = 3849.00, p = 0.4136, N = 90, 92	
	OSS tests	PAIRED	p = 1.0000, N = 90
		UNPAIRED	p = 0.9170, N = 92
4A	**KW test**	H (7) = 92.60, p < 0.0001	
	MWU tests	PAIRED	U = 2200.50, p < 0.0001, N = 101 each
		HANDLING	U = 284.00, p = 0.0023, N = 32 each
		SUGAR EXPOSURE	U = 104.50, p = 0.0102, N = 20 each
		ODOUR EXPOSURE	U = 102.00, p = 0.0235, N = 18 each

4B	**KW test**	H (3, N = 171) = 2.43, p = 0.4880	
	OSS test	p < 0.0001, N = 171	
4C	**KW test**	H (7) = 15.38, p = 0.0314	
	MWU tests	PAIRED	U = 223.50, p = 0.5102, N = 23, 22
		HANDLING	U = 219.50, p = 0.7893 N = 22, 21
		SUGAR EXPOSURE	U = 137.00, p = 0.0368, N = 21 each
		ODOUR EXPOSURE	U = 211.00, p = 0.8209 N = 21 each
4D	**KW test**	H (3, N = 85) = 3.83, p = 0.2802	
	OSS test	p = 0.3857, N = 85	
4E	**KW test**	H (7) = 30.70, p = 0.0001	
	MWU tests	PAIRED	U = 167.50, p = 0.0133, N = 24 each
		HANDLING	U = 219.50, p = 0.1609, N = 24 each
		SUGAR EXPOSURE	U = 224.00, p = 0.1904, N = 24 each
		ODOUR EXPOSURE	U = 139.00, p = 0.0022, N = 24 each
4F	**KW test**	H (3, N = 96) = 1.99, p = 0.5756	
	OSS test	p = 0.0001, N = 96	
5B	**MWU**	U = 152.00, p = 0.9868	
	OSS tests	COMPOUND	p = 0.0003, N = 17
		SEPARATED	p < 0.0001, N = 18
6A	**KW test**	H (7) = 35.14, p < 0.0001	
	MWU tests	PAIRED	U = 31.50, p < 0.0001, N = 18 each
		HANDLING	U = 94.50, p = 0.0340, N = 18 each
		SUGAR EXPOSURE	U = 77.00, p = 0.0075, N = 18 each
		ODOUR EXPOSURE	U = 90.50, p = 0.0247, N = 18 each
6B	**KW test**	H (3) = 4.92, p = 0.1779	
	OSS test	p < 0.0001, N = 72	
7B	**MWU test**	U = 69.00, p = 0.0275	
	OSS tests	COMPOUND	p = 0.0213, N = 16
		SEPARATED	p = 0.0005, N = 16

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Ehrenerklärung

Ich versichere hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; verwendete fremde und eigene Quellen sind als solche kenntlich gemacht.

Ich habe insbesondere nicht wissentlich:

- Ergebnisse erfunden oder widersprüchliche Ergebnisse verschwiegen,
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Mir ist bekannt, dass Verstöße gegen das Urheberrecht Unterlassungs- und Schadensersatzansprüche des Urhebers sowie eine strafrechtliche Androhung durch die Strafverfolgungsbehörden begründen kann.

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Juliane Thöner