Behavioral and optogenetic analyses of reinforcement processing in larval *Drosophila*

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Zusammenfassung

Eine der größten Herausforderungen der heutigen Neurowissenschaften ist es, die Komplexität der Belohnungsverarbeitung im Gehirn zu verstehen. Belohnungssysteme spielen eine kritische Rolle bei der Steigerung der biologischen *fitness*, und bei der Vermeidung schädlicher Einflüsse. Störungen dieser Systeme können neuropsychiatrische Komplikationen nach sich ziehen, wie etwa *post-traumatic stress disorder*, oder Sucht. In der vorliegenden Studie wird die *Drosophila melanogaster* Larve als Modelorganismus verwendet. Diese eignet sich aufgrund einer vorteilhaften Kombination von neuronaler Einfachheit und eines gleichzeitig erstaunlich komplexen Repertoires an Verhaltensweisen, sowie eines reichhaltigen gentechnischen Werkzeugkastens und aktuellen Erkenntnissen über ihr Konnektom. In dieser Arbeit werden klassische Verhaltensparadigmen sowie moderne optogenetische Methoden genutzt, um nähere Erkenntnisse über das Belohnungslernen dieses Tieres zu gewinnen. Zum einen beschreibe ich eine minimalistische Fähigkeit des Gehirns, das *one-trial* Lernen, sprich assoziatives Lernen ohne Wiederholung. Des Weiteren konzentriert sich meine Arbeit auf die detaillierte, parametrische Beschreibung eines identifizierten ‚Belohnungsneurons', DAN-i1, sowie auf die erstmalige Entdeckung und Charakterisierung von ‚Erleichterungslernen' in der Larve, vermittelt durch ein ‚Bestrafungsneuron', DAN-f1. Das Zentrum für Lernen und Gedächtnis in *Drosophila* ist eine höhergradige Struktur des Gehirns, die als Pilzkörper bezeichnet wird. Um assoziatives Lernen zu ermöglichen, integriert sie konditonierte und unkonditionierte Stimuli. Dopaminneurone mit unterscheidbarer Polarität signalisieren entweder appetitive oder aversive Verstärkung an intrinsische Pilzkörperneurone. Die zeitgleiche Detektion der Aktivität von Dopaminneuronen sowie einem Duftreiz führt zu einer Veränderung in der Aktivität von Pilzkörperausgangsneuronen, und führt im Verhalten entsprechend entweder zu Annäherung oder Meidung. Interessanterweise können Dopaminneurone, je nach zeitlichem Zusammenhang zwischen konditioniertem und unkonditioniertem Stimulus, Gedächtnisse von gegensätzlicher Valenz hervorbringen. Dieses Prinzip ist bekannt als ‚zeitabhängige Valenz-Umkehr' und wurde in zahlreichen Spezies beschrieben. In dieser Studie untersuche ich je ein Dopaminneuron von unterschiedlicher Polarität (DAN-i1 versus DAN-f1), welche jeweils Gedächtnisse von gegensätzlicher Valenz etablieren können, nämlich Belohnungs- versus Frustrationsgedächtnis, und Bestrafungs- versus Erleichterungsgedächtnis. Außerdem erbringe ich Beweise dafür, dass den Gedächtnistypen jeweils unterschiedliche molekulare Pfade ihrer Bildung zugrunde liegen könnten. Somit ebnet diese Studie den Weg für weitere, detaillierte Analysen von Co-Transmittern, Dopaminrezeptoren und nachgeschalteten molekularen Kaskaden. Sollten sich diese Resultate als verbreitetes Prinzip herausstellen, hat das nicht nur Implikationen für unser grundlegendes Verständnis, sondern auch für die systemische Behandlung des Dopaminsystems im Menschen.

Summary

Understanding the complexity of reward processing in the brain is one of the major challenges in the contemporary neurosciences. Reward systems play a crucial role in increasing fitness and the avoidance of adverse influcences. Disturbances in these systems may cause neuropsychatric consequences, such as post-traumatic stress disorder or addiction. The present study uses the *Drosophila melanogaster* larva as model organism. This is due to a fortunate combination of neural simplicity and a surprisingly complex reportoir of behaviors, as well as a rich genetic toolbox together with recent knowledge about their connectomics. In this work, classical behavioral paradigms as well as optogenetics are used to gain deeper insights in the reward learning of this animal. For once, I describe a minimal capacity of the brain, one-trial learning, that is associative learning without repetition. Further on, my work is centered around the detailed parametric specification of an identified 'reward neuron', DAN-i1, as well as around the first-time discovery and characterization of relief learning in the larva, which is mediated by a 'punishiment neuron', DAN-f1. The center for learning and memory in *Drosophila* is a higher-order brain structure, called the mushroom body. In order to enable associative learning, it integrates conditioned and unconditioned stimuli. Dopaminergic neurons with distinct 'polarity' signal appetitive or aversive reinforcement, receptively, to mushroom body intrinsic neurons. Coincidence detection of the activity of dopaminergic neurons and an odor cue leads to a skew in activity of mushroom body output neurons, causing a given behavior such as approach or avoidance. Interestingly, dopaminergic neurons were found to bring about memories of opposite valence, depending on the event-timing of conditioned and unconditioned stimulus. This principle, known as timing-dependent valence reversal, was reported across species. In the present study, I investigate one dopaminergic neuron of the opposite polarity each, which can establish two opposing types of memory, namely reward versus frustration and punishment versus relief memory, respectively. I further provide evidence that distinct underlying molecular pathways may contribute to the formation of the respective memory types. Thus, the present study paves the way for more detailed analyses of co-transmitters, dopamine receptors, and downstream molecular cascades. In case these results depict a common principle, this does not only have implications for our fundamental understanding, but also for the systemic treatment of the human dopamine system.

General introduction

The brain's most fundamental task is to bring about adaptive behavior. In order to do so in the face of changing environmental contingencies, memory systems allow the integration of past experience to predict future events. It is highly warranted that predictions are simple and robust to ensure that predictive behavior such as approach or avoidance can be carried out quickly and reliably. Otherwise, predictive memories need to be complex and flexible to match to changed contingencies. In this context, one-trial learning, that is learning without any repetition, and reversal-learning are essential capabilities of the brain. The paramount goal is to maximize rewards and to minimize a given risk. Rewards mostly support alimentary needs or reproduction, at least indirectly, and engage reward seeking behaviors from, for example, foraging in insects to stock markets trading in humans. Throughout the past decades, the neurosciences have tried to answer how memories of different valence are established, how specificity is conveyed within the neuronal network and what changes at the level of synapses to finally bring about a given behavior. Despite impressive advances towards this end, the circuitry of appetitive and aversive learning leading to approach and avoidance, respectively, is not yet fully understood. I suggest larval *Drosophila melanogaster* as potent study case to unravel the balance between simplicity and complexity of associative learning. With regard to this, the present study describes the mnemonic capabilities of *Drosophila* larvae, focusing on the role of single dopaminergic neurons in timing-dependent valence reversal. Before going further into detail, the capability of *Drosophila* as model organism is discussed and its merits highlighted.

***Drosophila*: small animal, great possibilities**

More than a century of research on the fruit fly *Drosophila* has decorated the little insect model organism with several Nobel Prizes. For his groundbreaking discovery of genes being arranged as chromosome structure in the salivary glands of the larva, Thomas H. Morgan was awarded the Nobel Prize for physics in 1933. Later, in 1995, Ed Lewis, Christiane Nüsslein-Volhard und Eric Wieschaus received the Nobel Prize for physiology/medicine for novel insights into developmental biology of the *Drosophila* embryo (Nüsslein-Volhard and Wieschaus 1980). Several years later, in 2017, yet another Nobel Prize for physiology/medicine was granted to Jeffrey C. Hall, Michael Rosbash und Michel W. Young for studies on the endogenous clock, this time in adult flies (Bargiello et al. 1984, Zehring et al. 1984). These honors testify to the fact that the awarded discoveries paved the way to identify principles of heredity, development, and behavior that turned out to be applicable throughout the animal kingdom, including humans. Apart from the obvious implications of these principles for biomedicine, *Drosophila* research has greatly contributed to our understanding of ion channels (Paradis et al. 2001, Dawydow et al. 2014), courtship (Villella and Hall 2008), sleep (Crocker and

Sehgal 2010), feeding (Pool and Scott 2014), neuropeptide signaling (Nässel and Zandawala 2019), aging (He and Jasper 2014), to name but a few. Due to the fact that flies and mammals share most of the classical neurotransmitters, e.g. γ-aminobutyric acid (GABA), glutamate (most likely inhibitory in *Drosophila*; Liu and Wilson 2013), acetylcholine, biogenic amines such as dopamine and serotonin and several neuropeptides (Nässel and Winther 2010), insights into the structure and function of the *Drosophila* nervous system are of relevance for research on other species also at the circuit level (Bellen et al. 2010). Moreover, at the genetic level a high degree of homology in the genes related to human genetic diseases has been identified (Reiter et al. 2001), making *Drosophila* a convenient model organism to study, for example, the neurodegenerative mechanisms linked to Alzheimer and Parkinson disease (Feany and Bender 2000, Lu and Vogel 2009, Lessing and Bonini 2009), neurological disorders like depression, epilepsy or schizophrenia (O'Kane 2011), or metabolic disorders such as obesity (Musselman and Kühnlein 2018) – at least to the extent that these diseases indeed have significant heritable components (Berkowitz 2020). With mere 100,000 neurons in adults and only 10,000 neurons in larvae (Nassif et al. 2003), *Drosophila* has a brain that is by many orders of magnitude numerically simpler to the brains of even simple mammals, and indeed is simple enough to allow elucidating basic circuit principles with relative ease. With single cell-resolution anatomical databases (Li et al. 2014, Eichler et al. 2017, Takemura et al. 2017) and with a treasure trove of genetic tools at hand (Duffy et al. 2002, Dawydow et al. 2014, Housden and Perrimon 2016) it is actually possible to study the role of single neurons within a given circuitry.

Powerful tools enable asking targeted questions

One of the greatest beauties of *Drosophila* research lies in the availability of genetic tools to ask questions which cannot as easily be tackled in most other model organisms, thus making *Drosophila* a pioneer model in some aspects of the neurosciences. Before the dawn of fancy genetic tools gave rise to a new era in *Drosophila* neuroscience research, mutant flies were created to address genes involved in learning and memory. Classical learning mutants have a long history, starting with the characterization of *dunce* (*dnc*) flies, which are deficient in one form of cyclic adenosine monophosphate (cAMP) diphosphorase activity (Dudai et al. 1976). Following up on this, other famous learning mutants such as *rutabaga* (*rut*), deficient in Ca^{2+}/calmodulin sensitive adenylyl cyclase activity (Livingstone et al. 1984), *amnesic* (*amn*) (Quinn et al. 1979) and *radish* (*rsh*) (Folkers et al. 1993) were introduced (reviewed in Sokolowski 2001, McGuire et al. 2005, Tumkaya et al. 2018). Many doors opened to Drosophilists with the invention of the P-element mediated UAS-Gal4 system to control gene expression (Brand and Perrimon 1993, Duffy 2002). It is a binary expression system, consisting of two parts; one is a Gal4 gene encoding for the yeast transcription activator protein Gal4, and the second part is an upstream activating sequence (UAS) enhancer. The Gal4 gene

is controlled by a tissue-specific promotor ('driver gene'), such that the Gal4 is only expressed in cells with an active driver gene; resulting in the given tissue-specificity. While the presence of Gal4 determines 'where' a manipulation should happen, e.g. in a certain subset of cells, UAS controls the expression of any given downstream target gene X and thus determines the kind of manipulation, namely 'what' should happen. One parental fly strain carries the Gal4 (driver strain) and another strain carries the UAS construct (effector strain). Since the parts of the construct are not functional alone, only the progeny of the two fly strains will have gene expression in the cells of interest **(Figure 1)**.

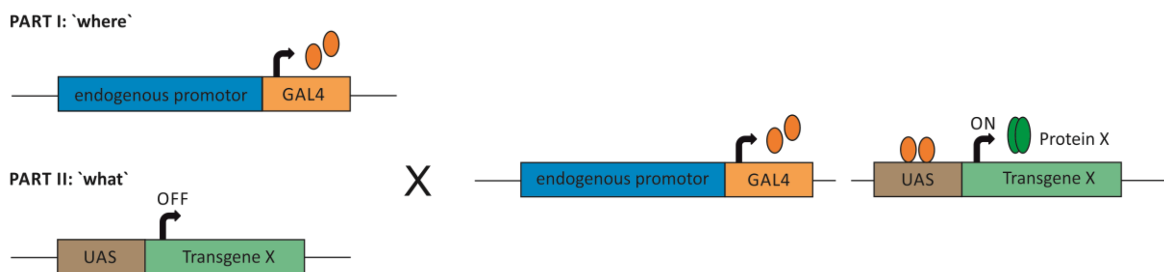


Figure 1: The binary UAS-Gal4 expression system offers targeted genetic manipulations in *Drosophila*. The UAS-Gal4 system consists of two separate parts. The Gal4 gene, which encodes for the yeast transcription activator protein Gal4, and the upstream activating sequence (UAS) enhancer. Two different constructs with either the Gal4 or the UAS are generated and transferred into a *Drosophila* embryo. Part I, the Gal4 strain, defines 'where' a manipulation should happen, such that any given subset of cells can be targeted. Part II, the UAS strain, determines 'what' kind of manipulation should happen by expressing any given transgene X downstream of UAS. Thus, one parental fly strain carries Part I, the Gal4 construct, and another one Part II, the UAS construct. Only in the progeny of these parental fly strains Gal4 specifically binds to UAS and activates transcription of the transgene X. The fly progeny will thus have gene expression in the cells of interest.

Today, with an ever-growing *Drosophila* community, thousands of different genetic fly strains are available, allowing for numerous combinations to visualize, activate, or silence any subset of cells. An open source atlas documenting the expression pattern of several driver strains within the larval brain is available online, offering users the possibility to register their own preparations into a standard brain (larvalbrain.org; Muenzing et al. 2018).

Another recent variant of the UAS-Gal4 systems exploits on the fact that Gal4 can be separated into a DNA-binding domain (DBD) and a transcription-activation domain (AD). Using two different promoters, these domains can be expressed independently of one another. In cells in which their expression patterns overlap, hetero-dimerization of the domains activates transcription (Luan et al. 2006, Pfeiffer et al. 2010). Several other binary expression systems are available, e.g. the LexA-LexAop system (Lai and Lee 2006) and the QF-QUAS system (Potter et al. 2010), which in combination with the more commonly used UAS-Gal4 system enable to perform two different manipulations in distinct subsets of cells simultaneously (Yagi et al. 2010, Venken et al. 2011a, Venken et al. 2011b). The novel genome-editing method 'clustered regularly interspaced palindromic

repeats/CRISPR associated' (CRISPR/Cas9) efficiently helps generating mutations in a chosen gene. This technique is now increasingly streamlined and could prove powerful, also in combination with the well-established UAS-Gal4 system (Basset et al. 2013, Basset and Liu 2014, Housden and Perrimon 2016).

Having the genetic tools as detailed above at hand offers unique applications, such as the manipulation of neuronal activity in vivo to study the role of specific neurons in generating a given behavioral output. There are several ways of how to interfere with neuronal activity. Very drastically, causing cell death by expressing pro-apoptotic genes such as grim, hid, or reaper is one option (Zhou et al. 1997, Wing et al. 1998). Apart from that, blocking chemical-synapse output of a neuron can be achieved by either preventing the release of neurotransmitters by expressing tetanus toxin (Sweeney et al. 1995), or by blocking chemical-synapse transmission via expression of $shibire^{ts}$, a temperature-sensitive dominant negative form of dynamin involved in vesicle recycling. The latter has the advantage that less drastic side effects have to be reckoned with, as it is not constitutively active, but only acutely blocks transmission from chemical synapses (Kitamoto 2001). Yet another approach is to manipulate ion channels. Blocking membrane depolarization can be achieved by expressing Kir2.1 which encodes a mammalian inward rectifying K^+ channel (Paradis et al. 2001), preventing action potential firing. An increase in neuronal activity can for example be accomplished via expression of the temperature sensitive cation channel dTrpA1 (Rosenzweig et al. 2005, Pulver et al. 2009).

Most importantly, the application of optogenetics in *Drosophila* greatly enriched the toolbox for genetic manipulations in the past decades. Silencing or increasing neural activity in response to light allows for acute and precisely timed control of neural activity, even on single cell level. Of note, these effects can be exerted in vivo, in the awake and freely behaving animal and do not require any surgery as it is the case for corresponding studies in mammals. By expressing channelrhodopsin (ChR2), a blue light gated (470 nm) cation channel in the neurons of interest, the respective cells can be depolarized which leads to an increase in neural activity (Boyden et al. 2005, Nagel et al. 2005, Schroll et al. 2006, Pulver et al. 2009, Fiala et al. 2010, Fenno et al. 2011, Dawydow et al. 2014). Two variants are mainly used, ChR2-XXM and ChR2-XXL which mostly differ in the kinetics of channel closing. Especially ChR2-XXL allows for a remarkable temporal and cellular precise manipulation, is even more sensitive and requires less light intensity and thus mostly obtains strong behavioral effects. However, it has a long closing state such that the neuron is still active although the light used for activation of the ChR expressed in the neuron has already been switched off (Dawydow et al. 2014). A few years ago, CsChrimson came up as yet another tool for optogenetic activation, workable at long-wavelength red light (720 nm) and thus improving cuticle penetration in adult flies (Klapoetke et al. 2014). Optogenetic tools can also be used to inhibit neural activity, for example by expressing Halorhodopsin (NpHR), a chloride pump activated by yellow light (540-580 nm) (Inada et al. 2011). A

new class of anion channelrhodopsins with a large Cl^{-} conductance has recently been engineered. Both versions of the *Gt*ACR optogenetic tool (the cyan light-gated channel *Gt*ACR1 workable at 515 nm, and the blue light-gated *Gt*ACR2 channel workable at 470 nm) require less light intensity and exhibit a higher conductance than previous inhibitory optogenetic tools (Govorunova et al. 2015, Mohammad et al. 2017). Taken together, there is a rich assortment of optogenetic tools available to remote-control neurons in *Drosophila*, and ultimately their behavior. Changes in behavioral output due to manipulation of neural activity can be measured and quantified in various learning paradigms available for adult and larval *Drosophila* (reviewed in Ehmann and Pauls 2020).

Perception and processing of olfactory and gustatory stimuli

Importantly, for the behavioral paradigms used in the present study, two sensory modalities of the *Drosophila* larva, olfaction and gustation ('taste'), play a pivotal role. Chemosensory learning enables the larva to seek and obtain food rewards and avoid harmful substrate. The role of taste appears to be relatively straight forward, informing the animal about the given behavioral meaning of the respective, already present tastant, such as a sugar or a bitter substance, and is followed by an immediate locomotor output. Olfaction, however, is essential to keep the animal updated on odors, which potentially aquire or lose a behavioral meaning. This requires a high level of discrimination (as their can be various odors and odor mixtures, and odors can drastically vary in their concentration), flexibility and dimensionality (Gerber et al. 2009). In the following, I will give a short overview of the larval olfactory and gustatory system.

The larval olfactory system is largely organized like in adult flies, and in insects in general, but at reduced cell numbers (Gerber and Stocker 2007, Vosshall and Stocker 2007, Eichler et al. 2017). Odor is detected at the dorsal organ (DO), the larval `nose` (Fishilevich et al. 2005), which is innervated by a total of 21 cholinergic olfactory receptor neurons (ORNs) typically expressing one type of odorant receptor (OR) each, which determines the detectable range of odors (Clyne et al. 1999). All 25 in the larva detected ORs are co-expressed with Orco (previously called OR83b) as co-receptor which is regulating the response threshold for odors (Vosshall and Stocker 2007). Cell bodies of the ORNs are located in the DO ganglion (DOG) from where odor information is further relayed via the antennal nerve (AN) towards the antennal lobe (AL) (Python and Stocker 2002, Ramaekers et al. 2005). The 21 AL glomeruli itself are laterally connected via 14 local interneurons (LNs) (Thum et al. 2011, Berck et al. 2016). The LNs mediate both excitatory and inhibitory synaptic connections to adaptively regulate the responses of projection neurons (PNs) (reviewed in Martin et al. 2011). LNs themselves receive excitatory input both from ORNs and PNs and maintain synapses with afferents and PNs (Vosshall and Stocker 2007). Presumably, most larval LNs express GABA and provide lateral inhibition between glomeruli, leading to a winner-take-all effect. Excitatory cholinergic LNs could do the opposite, i.e.

exerting a leveling effect (Huang et al. 2010, reviewed in Martin et al. 2011, Berck et al. 2016). The balance between inhibition and excitation of the LNs could thus determine the discriminability of the signal in downstream pathways. Interestingly, in adult flies, excitatory and inhibitory LNs are interconnected via chemical synapses, while connectivity between LNs and PNs is attained via electrical coupling. The latter might serve to increase the speed of the connections within the AL glomeruli (Yaksi and Wilson 2010). A total of 21 cholinergic PNs in the larva further connect the AL glomeruli with two higher-order olfactory learning centers, the lateral horn (LH) and the paired mushroom body (MB) structure, enabling combinatorial coding across the two ascending pathways (Python and Stocker 2002, Ramaekers et al. 2005, Masse et al. 2009, Berck et al. 2016). While processing through the LH pathway is sufficient for innate olfactory behavior (Heimbeck et al. 2001), a detour via the MB loop is necessary for learned olfactory behavior (Heisenberg et al. 1985, Heisenberg 2003, McGuire et al. 2005, Keene and Waddell 2007, reviewed in Vosshall and Stocker 2007). In mammals a corresponding segregation of pathways is observed; the cortical amygdala is thought to mediate innate behavior, functionally resembling the insect LH (Root et al. 2014), while the piriform cortex is in charge of olfactory memory storage and retrieval similar to the insect MB (Sacco and Sacchetti 2010). Within the MB, a given PN targets either one, or sometimes two of the 34 calyx glomeruli (Masuda-Nakagawa et al. 2005, Ramaekers et al. 2005). The MB calyx is formed by the dendrites of cholinergic Kenyon cells (KCs) and their parallel axons giving rise to the peduncle and, in the case of the larva, two lobes (Thum et al. 2011, Berck et al. 2016, Eichler et al. 2017, Saumweber et al. 2018). The KCs were shown to have dendritic projections in a single MB calyx glomerulus in few cases but most of them establish multiple arbors in up to six calyx glomeruli (Masuda-Nakagawa et al. 2005, Ramaekers et al. 2005, reviewed in Vosshall and Stocker 2007, Eichler et al. 2017). Thus, from the PNs to the calyx a 1:30 divergence of the odor signal takes place. Correspondingly, in adult flies approx. 1300 ORNs (Davis 2004, Keene and Waddell 2007) 62 ORs (Vosshall and Stocker 2007), 43 AL glomeruli (Lessing and Carlson 1999), approx. 180 PNs (Turner et al. 2008) and several hundred Calyx glomeruli were described **(Figure 2A, B)**.

In contrast to the olfactory system, the larval gustatory system can largely be viewed as a miniature version of the adult system but also exhibits many larval-specific elements resulting in a 'hybrid' organization (Python and Stocker 2002). It consists of three external sense organs on the head, including the dorsal (DO), the ventral (VO) and the terminal (TO) organ. Additionally, it comprises four internal sense organs, namely the dorsal (DPS), the posterior (PPS), the ventral sense organ (VPS) and the dorsal pharyngeal organ (DPO). From there, 80 gustatory receptor neurons (GRNs; Vosshall and Stocker 2007) project via four distinct nerves to the suboesophageal ganglion (SOG) from where taste information is passed on to motor control in order to elicit innate gustatory behavior as well as to the central brain, including the MB, to communicate internal reward signals

(Singh and Singh 1984, Python and Stocker 2002, Gendre et al. 2004, Colomb et al. 2007, Kwon et al. 2011, reviewed in Apostolopoulou et al. 2015, Miroschnikow et al. 2018). GRNs were shown to respond to water, sugar, as well as low and high salt (sodium chloride; NaCl) concentrations, with high-concentration salt sensing neurons also being activated by bitter tasting substances (Ebbs and Amrein 2007, reviewed in Vosshall and Stocker 2007, reviewed in Apostolopoulou et al. 2015). Four different gene families encode for proteins that determine the range of detectable qualities, namely water, sugar, salt and bitter. These are transient receptor potential (TRP) channels, *pickpocket* (PPK) sodium channels, chemosensory ionotropic receptors (IRs; iv) and transmembrane gustatory receptors (GRs) (Clyne et al. 2000, Liu et al. 2003, Thorne et al. 2004, Dahanukar et al. 2007, Benton et al. 2009, Cameron et al. 2010, Weiss et al. 2011, Miyamoto et al. 2012, Zhang et al. 2013, reviewed in Apostolopoulou et al. 2015, Freeman and Dahanukar 2015).

Interestingly, the two modalities, taste and olfaction, are not as strictly separated in larvae as they are in adult flies. One possible explanation for this discrepancy between the developmental stages is that larvae as rather stationary substrate feeder might not have the same need to distinguish between smell and taste as adult flies (Python and Stocker 2002, reviewed in Vosshall and Stocker 2007). Overall, the organization of the *Drosophila* olfactory system is strikingly similar to the one in mammals, including the expression of one or only few OR types per neuron, as well as further convergence of ORNs with the same OR in one glomerulus, up to the coding principles in the respective olfactory centers (Vosshall and Stocker 2007). In contrast, the gustatory systems are anatomically differently organized in mamals versus insects, but elicit comparable appetitive and aversive responses to tastants (Vosshall and Stocker 2007). Overall, the prallels in the chemosensory systems of mamals and *Drosophila* suggests the fly and its larva as an interesting and genetically tractable study case for associative olfactory learning about tastant reinforcers (reviewed in Vosshall and Stocker 2007).

Maggot learning

Learning is not an exclusive attribute of higher animals. Across the animal kingdom it is inevitable to learn about the occurrence of rewards and punishments. Tastant reinforcer elicit immediate reflexive behavior, for example consumption in case of a taste reward (reviewed in Schultz 2017). In early classical conditioning experiments, Pavlov presented a neutral stimulus, e.g. the sound of ringing a bell together with taste reward. In classical terminology a neutral cue would become the conditioned stimulus (CS), while the tastant reinforcer depicts the unconditioned stimulus (US). After pairing CS and US multiple times, the sound of the bell alone resulted in anticipatory learned behavior, in order to prepare the animal for the likely occurance of the US (Pavlov 1927).

Exploiting on the fact that larvae are 'feeding machines' which are always searching for food, *Drosophila* larvae can be trained to associate cues like an odor, with a given tastant. Indeed, a repertoire of associative learning paradigms has been established during the past decades. Rewards and punishments are in the present study regaraded as given US. The first associative learning experiments were, of note, conducted in adult flies. Pairing an odor as the CS with an electric shock as the US resulted in avoidance of the punished odor (Quinn et al. 1974, Tully and Quinn 1985). Likewise, adult flies were trained to associate an odor with a sugar reward, thus resulting in reward learning (Tempel et al. 1983, Schwaerzel et al. 2003). Both paradigms paved the way for many follow-up studies on associative learning in adult flies. Coresponding paradigms were developed to train larvae. A number of studies described aversive learning of odor paired with an electric shock (Khurana et al. 2009, Pauls et al. 2010a), heat shock (Khurana et al. 2012), light (von Essen et al. 2011), substrate vibration (Eschbach et al. 2011, Saumweber et al. 2014) high-concentration salt (Gerber and Hendel 2006, Niewalda et al. 2008, Widmann et al. 2016), or the bitter tastant quinine (Gerber and Hendel 2006, El-Keredy et al. 2012, Apostolopoulou et al. 2014b). Appetitive learning was demonstrated for an odor paired with various sugars (Scherer et al. 2003, Hendel et al. 2005, Michels et al. 2005, Gerber and Hendel 2006, Schipanski et al. 2008, Rohwedder et al. 2012), or amino acids (Schleyer et al. 2015a, Kudow et al. 2017, Toshima et al. 2019, reviewed in Toshima and Schleyer 2019). Indeed, larvae are capable of a number of advanced memory performances, such as one-trial learning (Weiglein et al. 2019), reversal learning (Mancini et al. 2019), learning odor specificity (Chen et al. 2011), odor intensity (Mishra et al. 2013), reinforcer specificity (Schleyer et al. 2015a), or agarose substrate concentration (Apostolopoulou et al. 2014a), and were shown to form even long-term memory (Widmann et al. 2016). For an overview of a range of training protocols for adult and larval Drosophila see Pitman et al. (2009). Since larval experiments are usually performed with animals of the third larval stage (L3), but an electron microscope-based connectome is available for animals of the first larval stage (L1) only, a joint effort was undertaken to describe also the behavioral faculties of L1 larvae. The respective study confirmed that L1 larvae are capable of the learning tasks that had previously been reported for L3 larvae (Almeida-Carvalho et al. 2017). This is in line with the finding that neural circuit connectivity is largely unchanged across postembryonic development (Gerhard et al. 2017).

<u>Taking a look inside the mushroom body – basic circuits underlying associative learning</u>

How is the association between an odor and a given reinforcer mechanistically achieved and how is an appropriate behavioral output computed within the brain? The MB depicts the center of olfactory associative learning in flies (de Belle and Heisenberg 1994, Heisenberg 2003). Four MB neuroblasts proliferate and give rise to approx. 73 mature embryonic-born γ-type KCs in the L1 larva (Technau

and Heisenberg 1982, Eichler et al. 2017). Throughout the larval stages, further proliferation brings about additional larval-born γ-type KCs as well as α'/β'-type KCs (the latter remaining largely immature during larval stages), such that a few hundered KCs in the L3 larva can be reckoned with (Eichler et al. 2017, Saumweber et al. 2018). However, all newly built KCs remain non-functional in the larva and are not required for larval associative learning (Pauls et al. 2010b). Finally, α/β-type KCs are born after puparium formation (Lee et al. 1999), such that in total approx. 2000 KCs are present in the adult fly (Technau and Heisenberg 1982, Aso et al. 2009, reviewed in Aso et al. 2020). Of note, the reported number of KCs across developmental stage varies across studies. Further investigations along these lines are warranted. The KC fiber system is tiled by the terminals of mostly either dopaminergic or octopaminergic mushroom body input neurons (DANs, OANs, respectively) as well as by the dendrites of mushroom body output neurons (MBONs) (adult flies: Aso et al. 2014a; larvae: Eichler et al. 2017, Saumweber et al. 2018), giving rise to the compartmental organization of the MB. In the L1 larva, 7 DANs (DAN-c1,-d1,-f1,-g1,-i1,-j1,-k1; in L3 larvae additionally -h1), 2 unpaired and 2 paired OANs (OAN-a1,-a2,-e1,-g1, respectively), 5 additional mushroom body input neurons of unknown neurotransmitter idendity (MBINs; -e1,-e2,-l1,-b1,-b2), and the paired giant GABAergic anterior paired lateral (APL) neuron were described (Eichler et al. 2017). The APL neuron collects input across all KCs and in turn gives negative feedback to some but not all KCs, likely regulating the sparseness of olfactory responses (resembling the GGN in locusts, see Papadopoulou et al. 2011; in *Drosophila* larvae: Masuda-Nakagawa et al. 2014). In contrast to adult flies, no dorsal paired median neuron was reported in the L1 or L3 larva (Eichler et al. 2017, Saumweber et al. 2018). The 21 MBONs were described as being either GABAergic, glutamatergic, or cholinergic (Eichler et al. 2017), paralleling the results in adult flies (Aso et al. 2014b). Of note, a remarkably small number of neurons conveys MB output in adults (34 MBONs of 21 types), compared to the input (130 DANs of 20 types) (Aso et al. 2014a) **(Figure 2B)**. All neuron type numbers are per hemisphere (exceptions are the two unpaired OANs and the APL neuron).

DANs exhibit a certain 'polarity' in their valence by relaying either appetitive or aversive reinforcement signals. A group of four neurons of the primary-lineage PAM (pPAM) cluster was described to be necessary for reward learning in the *Drosophila* larva (Rohwedder et al. 2016). Two of these neurons, DAN-i1 and DAN-h1, were found to be individually reward-inducing, and one of them, DAN-h1, to be individually indispensable for odor-reward learning (Saumweber et al. 2018). By contrast, DANs of the vertical lobe and lateral appendix mediate punishment learning in larvae (Schroll et al. 2006, Selcho et al. 2009, Eschbach et al. 2020a). Correspondingly, in adult flies most PAM cluster DANs innervating the β-, β'- and γ-lobes were shown to support positive reinforcement (Burke et al. 2012, Liu et al. 2012) and most PPL DANs which innervate the vertical α- and α'- lobes relay punishment (Schwaerzel et al. 2003, Claridge-Chang 2009, Aso et al. 2012, Hige et al. 2015a,

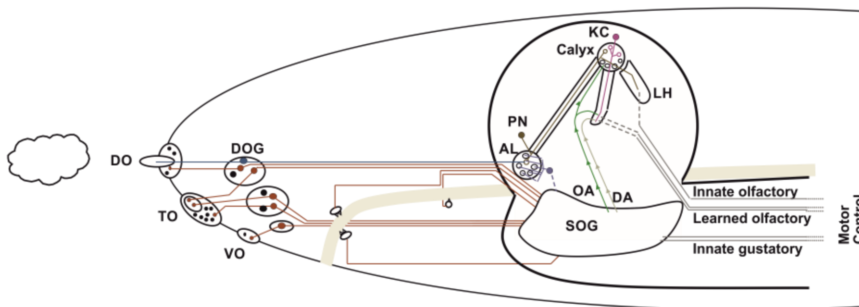
Felsenberg et al. 2017, reviewed in Boto et al. 2020). Remarkably, the increase in DAN number from larvae to adult mainly concerns the PAM cluster, suggesting a drastically improved signal-to-noise ratio regarding reward processing in adult *Drosophila* (Saumweber et al. 2018).

Four distinct dopamine receptor types have been described in *Drosophila*: dopamine 1-like receptor 1 (Dop1R1; further names: dDA1, DopR1, dD1, DUMB, DmDOP1; Gene CG9652; Kim et al. 2003), dopamine 1-like receptor 2 (Dop1R2; further names: DDR2, DAMB; Gene CG18741; Han et al. 1996), dopamine 2-like receptor (Dop2R; other name: DopR2, DD2R; Gene CG33517; Draper et al. 2007) and dopamine ecdysteroid receptor (DopEcR; other name: DmDopEcR; Gene CG18314; Srivastava et al. 2005) (also see Crocker et al. 2016, Verlinden 2018, Karam et al. 2019). Dop1R1 is coupling to the Gαs G-protein family to increase intracellular cAMP levels and was shown to be homologous to the vertebrate D1-like dopamine receptors. Contrastingly, Dop1R2, is more closely related to the invertebrate octopamine receptor family and couples to Gαq to enhance cAMP levels via phospholipase C. Dop2R shares most homologies with the vertebrate D2-like receptor family and leads to a decrease in cAMP levels via Gαi/o coupling (reviewed in Mustard et al. 2005). DopEcR can also bind to ecdysteroids and enhances cAMP levels via the PI3 kinase pathway (Srivastava et al. 2005). All four *Drosophila* dopamine receptor types were reported in KCs of adult flies (Croset et al. 2018, Kondo et al. 2020). Indeed, 24 % of all KCs were shown to express all four receptor types, whereas only 5 % of the KCs expressed none of them (Croset et al. 2018). Their involvement in associative learning will be elaborated in more detail in the *Discussion* section.

Reconstructions from connectome data confirmed the canonical circuit motive of KC-to-MBON connectivity that appears in every compartment (11 compartments in larvae: Eichler et al. 2017, Saumweber et al. 2018; 15 compartments in adults: Tanaka et al. 2008) and across developmental stages (Aso et al. 2014a, Eichler et al. 2017, Takemura et al. 2017). At this very synapse a change in synaptic weight is brought about by detection of the coincidence of a given odor and (appetitive, or aversive) DAN reinforcement (Heisenberg 2003, McGuire et al. 2005, Owald and Waddell 2015) **(Figure 2C)**. This imposes a skew on the overall drive of the output network, such that MBONs, connecting to the motor system, steer either learned approach or avoidance, respectively (Aso et al. 2014b, Owald and Waddell 2015, Saumweber et al. 2018). Notably, some MBON types in adult flies project to the lateral horn to potentially modulate innate behavioral responses. The major output, though, converges onto neuropil encompassing the MB (Aso et al. 2014a). Paralleling the *Drosophila* MB circuit, the vertebrate cerebellar circuit also consists of a three-layer network including input layer, expansion layer and output layer (flies: PNs, KCs, MBONs; vertebrates: mossy fibers, granule cells, purkinje cells) (reviewed in Modi et al. 2020).

Most remarkably, unknown connections within the MB were uncovered in the past years in both larva and adults: KC-to-DAN, DAN-to-MBON and KC-to-KC connections depict micro-circuits which potentially serve to maintain the sparse synapse specificity underlying olfactory learning and provide additional interhemispheric crosstalk (larva: Eichler et al. 2017, reviewed in Thum and Gerber 2019; adults: Takemura et al. 2017, reviewed in Cognigni et al. 2018, Boto et al. 2020, Modi et al. 2020). However, the function and interplay of these micro-circuits remains yet to be resolved. The fact that the molecular mechanisms underlying memory formation are mostly shared across insect species (Schwärzel and Müller 2006, reviewed in Schürmann 2016) depicts one of the major universal principles in memory research.

A



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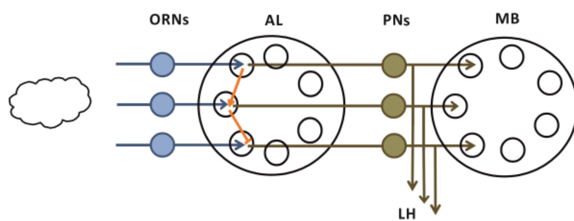

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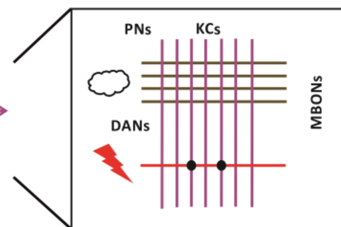

Structure/cell type	ORs	ORNs	LNs	AL glomeruli	PNs	Calyx glomeruli	KCs	DANs	MBONs
# in L3 larvae	25	21	14	21	21	34	N/A	8	21
# in adults	62	1300	N/A	43	180	N/A	2000	130	34
Neurotransmitter		ACh	ACh GABA		ACh		ACh	Dopamine	ACh GABA Glutamate

Figure 2: Schematics of the larval *Drosophila* olfactory information pipeline. (A) Schematic showing the olfactory pathway from the dorsal organ (DO), where odor is detected, up to higher-order brain centers. Odor is detected at the dorsal organ (DO), which is innervated by olfactory receptor neurons (ORNs) typically expressing one type of odorant receptor (OR) each. Odor information is then further relayed tot he antennal lobe (AL), and from there via projection neurons (PNs) to the mushroom body calyx, where simultaneous odor detection and activity of dopaminergic mushroom body input neurons (DANs) leads to changes in the Kenyon cell (KC)-to-mushroom body output neuron (MBON) synapse. The detour of odor information via the mushroom bdoy is necessary for learned olfactory behaviour, while the lateral horn (LH) is sufficient for innate olfactory behavior. The DO, the the ventral (VO) and the terminal (TO) organ are part of the gustatory system. Gustatory receptor neurons project to the suboesophageal ganglion (SOG) from where taste information is passed on to motor control to elicit innate gustatory behavior, but also to the central brain to communicate internal reward signals, also via DANs and octopaminergic mushroom body input neurons (OANs). Adapted from El-Keredy et al. 2012. **(B)** This schematic illustrates the main olfactory pathway to the mushroom body. The respective numbers of neuron type in L3

larvae and adult flies, as well as their identified neurotransmitter is indicated below. Unknown numbers of neurons are implied by N/A. Adapted from Vosshall and Stocker 2007. **(C)** Simplified schematic indicating the coincidence detection of odor information relayed by PNs and DAN activity, e.g. mediating punishment, at the level of KCs. This leads to a change of the KC-to-MBON connectivity and results in a given behavioral output, in case of negative reinforcement, aversion.

'Janus-faced' learning

While it has been studied in some detail how larvae learn to associate a given cue with the occurrence of reward or punishment, learning about the termination of a reward or punishment has been less acknowledged. However, traditional learning theories suggest that the timing of a CS relative to the US is of importance for both valence domains (Solomon and Corbit 1974, Solomon 1980). Specifically, a cue gains opposite learned valence dependent on the event-timing during training; a phenomenon referred to as timing-dependent valence reversal. In that sense, animals can be trained such that a cue comes before the reinforcer (forward conditioning), or that the cue comes after the reinforcer (backward conditioning). Regarding electric shock, for example, the cue gains negative valence upon forward conditioning and leads to punishment memory. Whereas, if the electric shock precedes the cue, it is associated with relief and gains positive valence. Of note, relief of an aversive stimulus or even pain is not just a termination of nociceptive transmission but indeed a reward (reviewed in Navratilova et al. 2015). Regarding for example sugar as tastant, a cue gains positive valence upon forward conditioning and establishes reward memory, whereas the cue gains negative valence upon backward conditioning and results in what may be called frustration memory **(Figure 3)**.

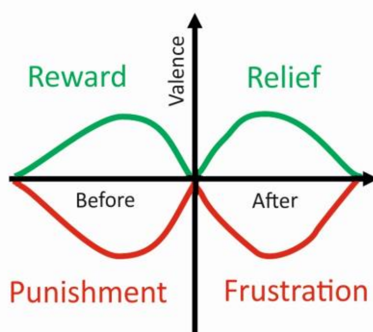

Figure 3: Four distinct memory types in *Drosophila*. Regarding forward conditioning, animals receive the odor before a given punishment (e.g. electric shock), resulting in punishment learning, whereas for backward conditioning the odor is presented after the punishment, resulting in relief learning. In case of the opposite valence domain, reward learning established by forward conditioning can turn to frustration learning when the order of events is reversed.

A handful of studies have described this dichotomy across species (reviewed in Gerber et al. 2014, Gerber et al. 2019). After some preliminary attempts to elicit backward learning, the introduction of the startle response as a measure of conditioned fear in rodents and humans enabled a novel experimental handle on valence reversal. The particular feature of the modulation of the startle response is that it can be viewed as a bivalent measure of memory. That is, startle is decreased in case of positively valenced memory, whereas startle is increased in case of negatively valenced memory (rodents: Koch and Schnitzler 1979, Koch 1999, Fendt and Fanselow 1999, Fendt and Koch 2013; humans: Norrholm et al. 2006, van Well et al. 2012). If a cue predicted something bad to happen, the startle response was found to be increased, whereas it was decreased by cues following an

unpleasant event (Andreatta et al. 2010, Andreatta et al. 2012, Mohammadi et al. 2014, Andreatta et al. 2016; Luck and Lipp 2017).

The same principle of timing-dependent valence reversal applies for simpler organisms such as the fruit fly. Training adult *Drosophila* in an olfactory associative learning paradigm with electric shock as punishment led to punishment versus relief learning dependent on the event-timing (Tanimoto et al. 2004, Yarali et al. 2008, Yarali et al. 2009, Yarali and Gerber 2010). These opposing types of memory could also be established by forward or backward pairing of the optogenetic activation of a single DAN, PPL1-01, with an odor (Aso and Rubin 2016, König et al. 2018). Data on the larva suggest that punishment memory can be observed after odor-shock training, a trend for relief learning after shock-odor training, however, did not reach significance (Khurana et al. 2009, loc. cit. Figure 3). In the honey bee, however, punishment versus frustration memory could already be accertained early on (Hellstern et al. 1998). Regarding the appetitive valence domain and in the *Drosophila* larva, optogenetic activation of the rewarding DAN-i1 resulted in two opposing memory types, reward and frustration learning, respectively (Saumweber et al. 2018). Whether the same principle applies for the aversive domain, that is, if an individual punishing DAN can bring about oppositely-valenced memories was so far not known. Likewise, it remains to be investigated if valence reversal is a common principle of individual DANs in general, and also research about the molecular mechanisms underlying timing-dependent valence reversal is still at its infancies. This includes the intrusive question if indeed both memory types of opposite valence are dopamine-dependent. In the following section I will introduce dopamine as neurotransmitter and its importance for associative learning across species.

Is dopamine doing the trick?

Dopamine depicts an evolutionary conserved catecholamine neurotransmitter (Yamamoto and Vernier 2011). In *Drosophila,* dopamine was shown to have a key role in development (Neckameyer 1996), pigmentation (Sugumaran 2002), locomotion (Silva et al. 2020), sleep and arousal (van Swinderen and Andretic 2011), learning (Aso et al. 2010, Berry et al. 2012, Liu et al. 2012), response to drugs of abuse (Bainton et al. 2000, Li et al. 2000), and many more aspects. In contrast to mammals where ingested dopamine cannot cross the blood-brain barrier, it can have direct effects on the insect central nervous system (Budnik et al. 1989). Dopamine biosynthesis proceeds from tyrosine to L-Dopa, mediated by tyrosine hydroxylase (TH) and further on from L-Dopa to dopamine, mediated by dopa decarboxylase (reviewed in Yamamoto and Seto 2014). After being synthesized in the cytoplasm, dopamine is stored in synaptic dense core vesicles. A vesicular monoamine transporter (VMAT) transfers dopamine across the membrane (Greer et al. 2005). Clearance of dopamine from the synaptic cleft involves the dopamine active transporter (DAT) that takes

dopamine back into the presynaptic neuron (Porzgen et al. 2001). Apart from that, dopamine can also be recycled by glia cells or metabolized into inactive compounds (reviewed in Yamamoto and Seto 2014). In order to investigate whether learning processes are dopamine dependent, two different approaches are commonly used to interfere with dopamine synthesis. On the one hand, pharmacological drug administration of for example α-methyl-p-tyrosine, or 3-Iodo-L-tyrosine inhibits TH synthesis. This method can be applied acutely, however, it affects dopamine systemically in the whole body and can potentially lead to unwanted developmental- or locomotor phenotypes. More spatially specific yet with less temporal specificity, TH synthesis can be blocked using TH-RNAi. The fact that this manipulation can be genetically restricted to only the neurons of interest can be advantageous (reviewed in Yamamoto and Seto 2014).

While dopamine was initially thought to rather signal punishment in *Drosophila*, octopamine (the analog to norepinephrine in vertebrates) was believed to signal reward (Heisenberg 2003, Schwaerzel et al. 2003). Later studies that made use of optogenetic tools to activate neurons covered by the TH-GAL4 strain encouraged the prevailing hypothesis that DANs relay aversive signals (Schroll et al. 2006, Claridge-Chang 2009, Aso et al. 2010, Aso et al. 2012). In the honey bee, both electrical stimulation of a single OAN, VUMmx1, as well as octopamine injection in one MB calyx, was shown to be reward-inducing (Hammer 1993, Hammer and Menzel 1998). The larval MB calyx receives OA input from two non-overlapping OANs, sVUMmd1 und sVUMmx1, originating in the suboesophagial zone (Selcho et al. 2014; termed OAN-a1 and OAN-a2 in L1 larvae, Eichler et al. 2017). In flies, octopamine was reported to mainly mediate the transient reinforcing properties of sweetness. Nutrient value was found to provide additional reinforcement, independent of octopamine (Burke et al. 2012). Along the same lines, dopamine receptor mutant flies showed impaired reward learning which could not be restored by OAN stimulation (Kim et al. 2007, Burke et al. 2012, Liu et al. 2012). A final confirmation of the rewarding faculty of DANs was delivered by two studies using Gal4 driverstrains (DDC-Gal4; HL9-Gal4; R58E02-Gal4) which, different from TH-Gal4, also covered some of the PAM cluster DANs. Activation of the neurons covered by these driver strains resulted in appetitive memory, also in flies lacking octopamine (Burke et al. 2012, Liu et al. 2012). Similarly, in larval *Drosophila* four pPAM cluster neurons were described to mediate appetitive but not aversive memory (Rohwedder et al. 2016). Novel studies claim a role for octopamine in odor sensing and odor discrimination (McLachlan et al. 2018).

Thus, anatomically distinct DANs mediate either aversive or appetitive reinforcement, including appetitive input from octopamine to the PAM cluster DANs (Burke et al. 2012, Liu et al. 2012 reviewed in Waddell 2013). These findings are in line with insights from vertebrate research, where different subsets of DANs respond to rewarding or punishing stimuli or to both rewarding and punishing stimuli (Matsumoto et al. 2009, Bromberg-Martin et al. 2010, reviewed in Schultz 2010,

Zweifel et al. 2011). Aversive DANs of the ventral tegmental area (VTA) signal to the medial prefrontal cortex, or project onto GABAergic neurons of the rostromedial tegmental area. Rewarding DANs of the VTA project to the nucleus accumbens (NAC) (Lammel et al. 2011, Lammel et al. 2012, reviewed in Waddell 2013). Indeed, dopamine has been attributed a dual role in invertebrates; in vertebrates, respective findings are not fully acknowledged until now. The anatomically segregated organization of appetitive and aversive DANs, however, seems to be a conserved feature across species (reviewed in Waddell 2013).

Overview of Thesis structure

The present study is comprised of three parts which address the behavioral and optogenetic analyses of reinforcement learning, using the *Drosophila* larva as model system.

In **(I)** I describe the capability of *Drosophila* larvae to perform 'small-data' predictive learning tasks. While repetition is usually required to consolidate memory, there are cases in which this is not necessary. In humans, this would apply for very positive events, such as for example the first kiss, or for rather negative, traumatic events, like a car accident. Indeed, I found that, when training *Drosophila* larvae in an olfactory associative learning task without repetition, they establish a very robust appetitive memory for fructose as well as for other sugar types of different qualities, e.g. which are only sweet, or only nutritious to the animals. Similarly, appetitive memory is formed if the activation of the rewarding DAN-i1 is paired with an odor in the one-trial paradigm. Contrastingly, aversive reinforcer, e.g. high-concentration salt or quinine, were more difficult to learn for the animals, warranting higher sample sizes and longer training trial durations or generally more-trial training. Further parametric investigations revealed that one-trial fructose memory was less strong than after more-trial training, however, both decayed similarly quickly. This study provides novel insights about the memory capacity of *Drosophila* larvae. The fact that biological systems are capable of small-data predictive learning is of interest with regard to the design of artificial intelligence and intelligent algorithms which are, contrastingly, dependent on a notorious large amount of data.

(II) focuses on timing-dependent valence reversal in the appetitive domain. More specifically, the role of the rewarding DAN-i1 in timing-dependent valence reversal, as for the first time shown in Saumweber et al. (2018) is under investigation. Indeed, I confirm that dependent on the event-timing of odor and DAN activation reward and frustration memory is established, respectively. I further investigated parametric features of the opposing memory types. Interestingly, a tripled duration of DAN-activation did not lead to an increase in reward or frustration memory scores. However, one-trial memory was only detectable for forward, not for backward conditioning which is in line with the findings from (I) suggesting that aversive memory requires more-trial training, or prolonged trial durations. The most striking difference between the two memory types was that reward memory

stayed detectable for up to 40 min, while frustration memory decayed rapidly. This hints to different mechanisms underlying the two opposing types of memories, warranting further investigations of forward versus backward learning.

In **(III)** I describe timing-dependent valence reversal in the aversive domain. Here, I characterize two DANs, DAN-f1 and DAN-d1, which had previously been described to relay aversive signals (Eschbach et al. 2020a). Of note, DAN-f1 was found to mediate both punishment and relief memory. To my best knowledge, this is the first time that relief learning could be confirmed in the *Drosophila* larva. I further on focused on the comparison of parametric features of punishment versus relief learning. A prolonged DAN activation is in this case especially interesting according to the logic of classical learning theories, which predict that relief memory should be stronger after a longer presentation of the aversive stimulus (here activation of the punishing DAN). Yet, neither punishment, nor for relief memory scores were increased. Interestingly, punishment memory was stable for up to 10 min, while relief memory was only detectable directly after training. Of note, these findings resemble the results regarding the appetitive domain in (II), suggesting that the molecular properties of forward and backward memories could be similar across valence domains. Interestingly, for the second neuron, under investigation, DAN-d1, only punishment memory could be revealed. Thus, some but not all DANs mediate timing-dependent valence reversal. Identifying the molecular basis of DAN signaling after forward and backward training is a prerequisite for understanding reinforcement learning in the larva and in general.

I - Single-trial learning with appetitive and aversive tastant reinforcement in larval *Drosophila*

Based on: Weiglein A, Gerstner F, Mancini N, Schleyer M, Gerber B. 2019. One-trial learning in larval *Drosophila*. *Learn Mem*.

For all animals, it is beneficial to learn predictors of where and when rewards such as nutrients can be found, or under which conditions threats such as predators, injury, or toxic food need to be reckoned with. Such predictive learning is a prerequisite for the anticipatory control of behavior (Hoffmann et al. 2007). In this context, one-trial associative learning is an interesting study case, since a single co-occurrence of events is actually of little predictive value. Nevertheless, in biological learning systems, one-trial associative learning has been observed in a number of species and across valence domains (e.g. zebrafish: Blank et al. 2009; chicken: Cherkin 1969; quail: Hilliard et al. 1997; mice: Abt et al. 1961; rats: Cammarota et al. 2005, Wood et al. 2004; humans: Haesen et al. 2017), notably including insects. For example, honey bees show one-trial learning of odors as predictors of sugar water (Takeda 1961, reviewed in Giurfa and Sandoz 2012) or of electroshock punishment (Vergoz et al. 2007, reviewed in Tedjakumala and Giurfa 2013). In adult *Drosophila*, a single trial of odor preceding electroshock punishment or sugar reward can establish aversive or appetitive associative memory, respectively (Tully and Quinn 1985, Beck et al. 2000, Colomb et al. 2009, Scheunemann et al. 2013). In addition, one-trial learning can be demonstrated upon pairing odor with the optogenetic activation of subsets of dopaminergic neurons innervating the MB in adult flies (Aso and Rubin 2016, König et al. 2018).

Larval *Drosophila* has recently emerged as an analytically potent study case for understanding the neurogenetics of associative learning (reviewed in Gerber and Stocker 2007, Diegelmann et al. 2013a, Thum and Gerber 2019). This is due to a fortunate combination of learning ability, neural simplicity in terms of cell numbers, tractability for synapse-resolution connectomics, and the availability of both a light-microscopy atlas of its neurons and the genetic toolbox available for manipulating them one at a time (Duffy 2002, Venken et al. 2011b, Li et al. 2014, Housden and Perrimon 2016). Two kinds of paradigm for odor-taste associative learning in the larva are commonly used, namely absolute and differential conditioning. In absolute conditioning, separate experimental groups of larvae are trained to associate an odor with either the presence or the absence of a reward such as fructose, for example, and are then tested for their odor preference. In differential conditioning, one odor is paired with a reward whereas a second odor is presented without reward (the chemical identity of the odors is alternated between experimental groups). In a subsequent test, the animals are given the choice between the two odors. In neither case, however, have systematic analyses of one-trial associative learning been reported. So far analyses of associative learning in the

larva have rather focused almost exclusively on three-trial conditioning, whether for various sugars (Scherer et al. 2003, Schipanski et al. 2008, Rohwedder et al. 2012) and amino acids as taste rewards (Schleyer et al. 2015a), for optogenetically induced reward learning through the activation of subsets of dopaminergic neurons (Saumweber et al. 2018), for punishment by substrate vibration (Eschbach et al. 2011, Saumweber et al. 2014) or electric shocks (Pauls et al. 2010a), or for quinine as taste punishment (Gerber and Hendel 2006, El-Keredy et al. 2012, Apostolopoulou et al. 2014b). Only for high concentrations of salt (sodium chloride), have analyses of three-trial learning (Gerber and Hendel 2006, Niewalda et al. 2008) recently been complemented by an experiment reporting associative memory after just one training trial (Widmann et al. 2016; loc. cit. Figure 7A).

The present study systematically tests for one-trial associative learning using fructose, arabinose, sorbitol and aspartic acid as taste rewards, for optogenetic activation of the dopaminergic DAN-i1 neuron as a reward signal, and for high-concentration salt and quinine as taste punishment. Focusing on fructose, further key parametric features of one-trial learning are provided.

Results

One-trial memory is detectable for all sugar types, but not for aspartic acid as reward

Larvae were trained in the continuous absolute conditioning paradigm such that for one experimental group the animals received the odor *n*-amylacetate together with a taste reward (paired training group), whereas in a second experimental group the larvae received the odor and the reward unpaired from one another (unpaired training group). The difference in odor preference between paired-trained and unpaired-trained animals thus reflects associative memory and is quantified as the performance index (PI). Positive and negative PI scores indicate appetitive and aversive associative memory scores, respectively. We observed appetitive memory after only one such training trial with fructose **(Figure 4A)**, arabinose **(Figure 4B)** and sorbitol **(Figure 4C)**, but not with aspartic acid **(Figure 4D)**. Specifically, for fructose, arabinose and sorbitol memory scores increased with more prolonged training trial durations, reaching asymptotic levels with training trial durations of approx. 2-4 min; for aspartic acid, memory scores were uniformly low given a non-significant Kruskal-Wallis test **(Figure 4E)**. Using three training trials, and mostly two-odor differential conditioning, appetitive memory has previously been observed for all these tastant rewards (fructose: Scherer et al. 2003, Saumweber et al. 2011; arabinose and sorbitol: Rohwedder et al. 2012; aspartic acid: Schleyer et al. 2015a).

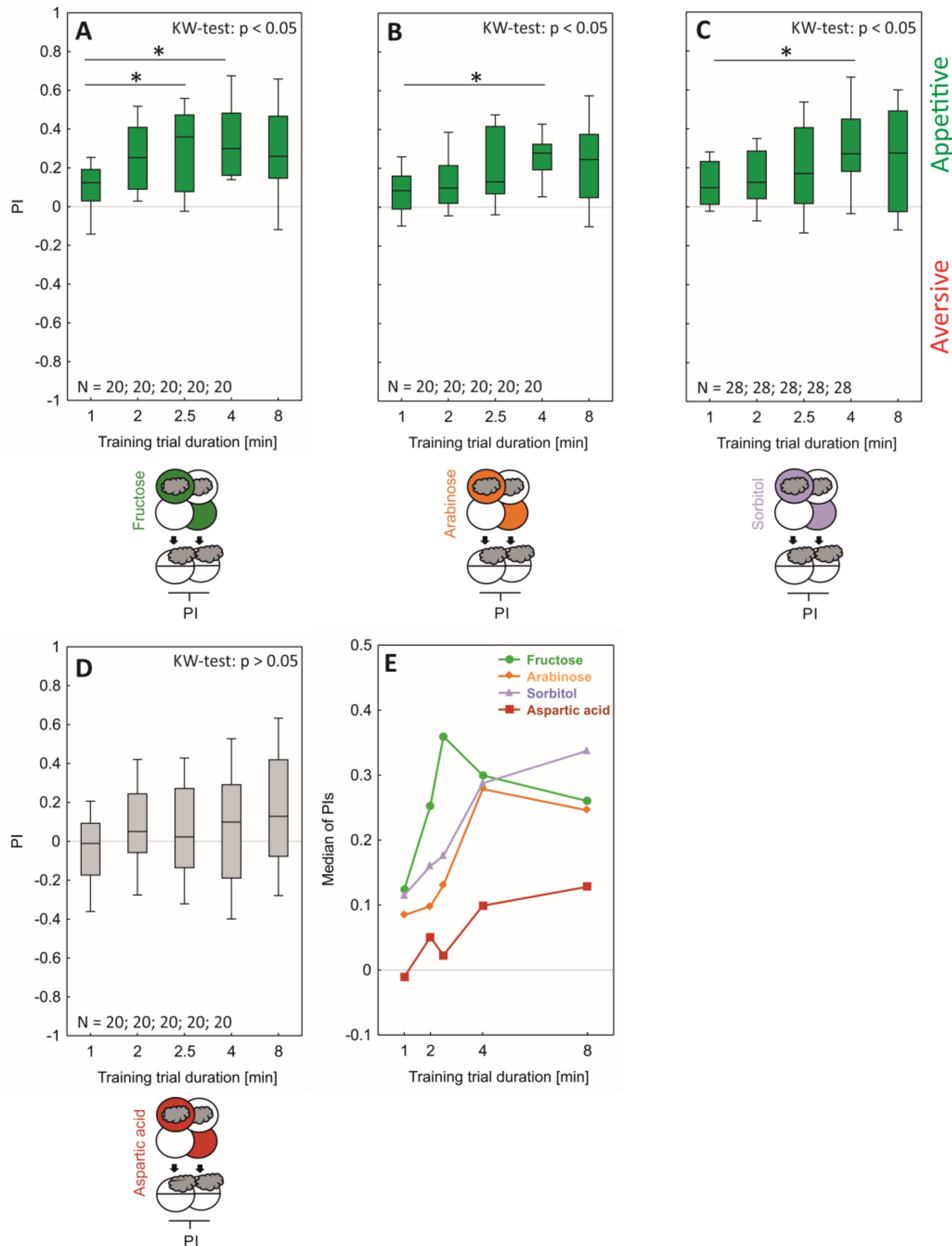

Figure 4: One-trial associative memory is detectable for sugars, but not for aspartic acid reward. Larvae underwent single training trials of 1, 2, 2.5, 4, or 8 min duration, with odor and the respective taste reward either together (paired) or separate (unpaired). Three different kinds of sugar or aspartic acid were used as a reward, as shown towards the bottom of the figures in the sketches of the training and testing procedures. Green, orange, light purple and red Petri dishes represent fructose, arabinose, sorbitol and aspartic acid, respectively; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Throughout this study, the sequence of events during training was as depicted in half of the cases, whereas for the other half it was reversed (not shown). Differences in odor preference after paired versus unpaired training are quantified by the performance index (PI) and thus reflect associative memory. PIs > 0 indicate appetitive memory, PIs < 0 aversive memory. PIs were positive for all training trial durations and increased with longer training trial durations for **(A)** fructose, **(B)** arabinose and **(C)** sorbitol. For **(D)** aspartic acid, no appetitive memory was detectable. Green fill of box plots reflects significance appetitive memory, red fill of box plots reflects significant aversive memory at p < 0.05 in OSS-tests with Bonferroni-Holm correction. A KW test across all experimental conditions is significant at p < 0.05 for all sugar types but

not for aspartic acid. Asterisks above horizontal lines reflect significance at p < 0.05 in MWU-tests with Bonferroni-Holm correction. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure. Preference scores underlying the PIs are documented in Figure S1. **(E)** The medians of the respective PIs from (A-D) are shown across training trial duration.

One-trial memory is detectable for high-concentration salt but not for quinine as punishment

Using three training trials, and mostly two-odor differential conditioning, aversive memory has previously been observed for both high-concentration salt and for quinine (high-concentration salt: Gerber and Hendel 2006, Niewalda et al. 2008, Widmann et al. 2016; quinine: Gerber and Hendel 2006, Schleyer et al. 2011, El-Keredy et al. 2012). In both cases, the behavioral expression of aversive memory is best grasped as a form of escape because it requires the presence of the punishment during the test to motivate learned avoidance (innate olfactory behavior is not affected by the presence of either tastant: Gerber and Hendel 2006, Schleyer et al. 2011). Here we show that high-concentration salt (1.5 M sodium chloride) is also effective as a punishment in the one-trial paradigm. Aversive memory scores were significantly negative after one training trial of 4 min and 8 min duration **(Figure 5A);** a trend towards appetitive memory after 1 min training did not reach significance (OSS: p = 0.02, which is above the Bonferroni-Holm-corrected significance threshold of 0.0167). For quinine, using only one training trial in our paradigm aversive memory could only be detected after 8 min training trial duration. The Kruskal-Wallis test across training trial durations was, however, not significant (p = 0.2) **(Figure 5B, C)**.

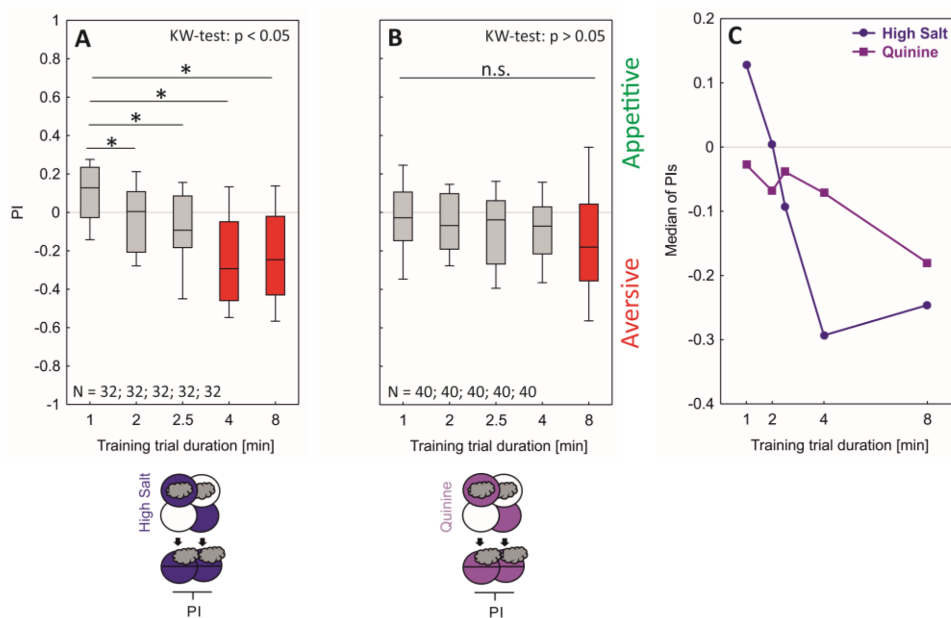

Figure 5: One-trial associative memory is detectable for high-concentration salt but not for quinine as punishment. Larvae underwent single training trials of 1, 2, 2.5, 4, or 8 min duration, with odor and the respective taste punishment either together (paired) or separate (unpaired). Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. Two different taste punishments were used, as shown towards the bottom of the figures in the sketches of the training and testing procedures. Blue and magenta Petri dishes indicate high-concentration salt and quinine, respectively; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Aversive memory scores were observed when testing in the presence of **(A)** high-concentration salt for the longer training trial durations. When testing in the presence of **(B)** quinine aversive memory for the longest trial duration was found. Of Note, a KW test between the groups was significant for (A) ($p < 0.05$) but not for (B). Other details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S2. **(C)** The medians of the respective PIs from (A, B) are shown across training trial duration.

Optogenetic activation of DAN-i1 has a rewarding effect in the one-trial paradigm

I further investigated whether optogenetic activation of DAN-i1, as covered by the SS00864-DAN driver strain, would have a rewarding effect in our one-trial paradigm (for three training trials see Saumweber et al. 2018). Indeed, activation of DAN-i1 at the times when otherwise a tastant reinforcer would have been presented resulted in appetitive memory scores in the experimental genotype; memory scores were significantly different from zero and from both genetic controls **(Figure 6A)**. The expression pattern of the DAN-i1 driver strain crossed to the ChR2-XXL effector strain was confirmed by immunohistochemistry against ChR2-XXL as the effector protein **(Figure 6B)**. The hemispherically unique DAN-i1 neurons each innervate the upper toe of the medial lobe of the mushroom body of each brain hemisphere **(Figure 6C)** (Saumweber et al. 2018).
On the basis of the above results, I decided to further investigate one-trial fructose memory. I chose fructose since it yielded the highest scores, in particular for relatively short training trial durations **(Figure 4E)**, and because fructose has been used the most in previous multiple-trial studies on the mechanisms of learning in the larva.

One-trial fructose memory is behaviorally expressed in the absence but not in the presence of fructose

For fructose memory after three training trials, it has been reported that memory is not behaviorally expressed if the test is carried out in the presence of fructose (Gerber and Hendel 2006, Schleyer et al. 2011, Schleyer et al. 2015a). Arguably, after odor-fructose associative learning the larvae track down the odor in search of fructose, a behavior that is no longer adaptive as soon as the sought-for fructose reward is found (innate olfactory behavior is not affected by the presence of fructose: Gerber and Hendel 2006, Schleyer et al. 2011, Schleyer et al. 2015a). Indeed, I observed the same effect for one-trial fructose memory: larvae behaviorally expressed fructose memory when tested in

the absence, but not when tested in the presence of fructose **(Figure 7).** Next, I sought to ascertain whether, using the present paradigm, memory scores further increased when more training trials were used.

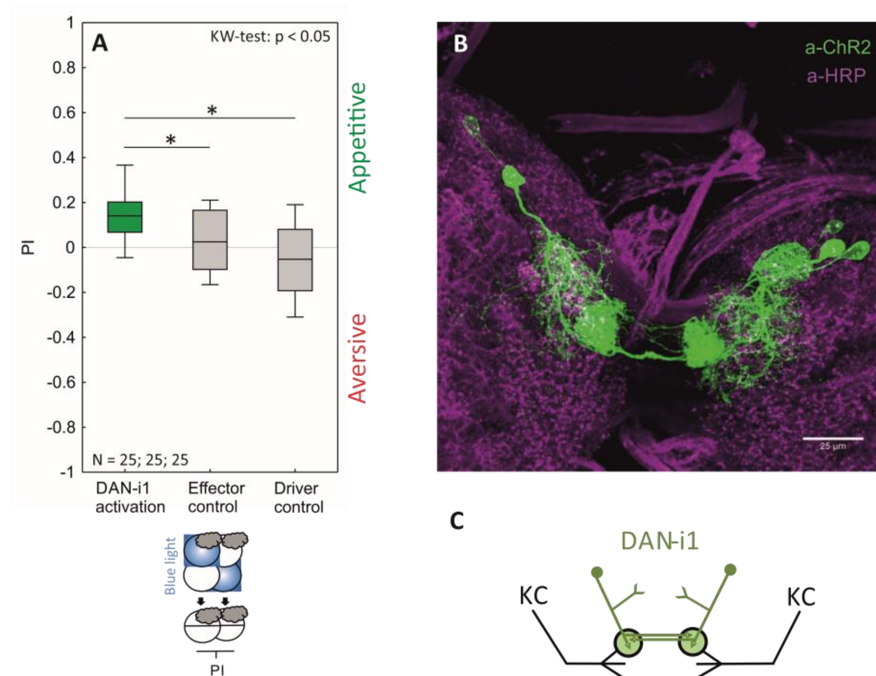

Figure 6: One-trial memory for the association of odor and optogenetic activation of DAN-i1. (A) Larvae were trained by presenting odor and blue light for the optogenetic activation of DAN-i1 either together (paired) or separately (unpaired), with 2.5 min trial duration. Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. The sketches towards the bottom of the figures depict training and testing procedures. Radiating blue Petri dishes indicate light activation during training; Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added were used throughout. The grey cloud indicates the odor *n*-amylacetate. PIs are positive for the experimental genotype (DAN-i1 activation: DAN-i1>UAS-ChR2-XXL) but not for the genetic controls (Effector control: attP40/attP2>UAS-ChR2-XXL; Driver control: DAN-i1>w^{1118}), implying that optogenetic activation of DAN-i1 covered by the SS00864 driver strain leads to reward memory after one training trial. Further details as in Figure 4. Preference scores underlying the PIs are documented in Figure S3. **(B)** Immunohistochemical preparation of the mushroom body region of the experimental genotype. ChR2-XXL is visualized by a primary mouse anti-ChR2 antibody and a secondary Cy3 donkey anti-mouse antibody (green). Confirming an earlier report (Saumweber et al. 2018), this reveals strong and reliable expression in the DAN-i1 neuron of both hemispheres, plus a few additional cell bodies that vary across specimens; Alexa 488 anti-HRP staining yields staining of neuronal membranes for reference (magenta). Data were acquired with a 63x glycerol objective. **(C)** Schematic drawing of the left and right mushroom body and the innervation of the upper toe of the medial lobe of the mushroom body by the DAN-i1 neurons of both hemispheres (shown in green).

Fructose memory scores increase with training trial number

For two-odor differential conditioning, using 1-min training trials and scoring individual larvae across the complete testing period including the early phases of orientation and presumably indecisiveness, Neuser et al. (2005) found no evidence for fructose memory after one or two training trials, whereas for three, four and eight training trials these scores were uniformly positive. In the present paradigm, using 2.5-min training trials and end-point counting at the end of the 3-min testing period, even one training trial was shown to be enough to establish fructose memory **(Figure 4A, Figure 7)**.

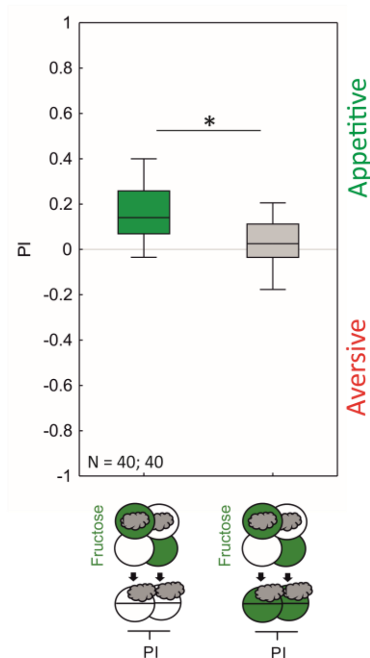

Figure 7: **One-trial associative memory is not behaviorally expressed in the presence of fructose during the test.** Larvae underwent single training trials of 2.5 min duration, with odor and fructose reward either together (paired) or separate (unpaired). Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. The sketches towards the bottom of the figures depict the training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Larvae were tested either on pure agarose substrate or in the presence of fructose. If tested on the pure agarose substrate they behaviorally expressed appetitive associative memory (left), whereas no behavioral memory expression was observed in the presence of fructose (right). This is arguably because appetitive associative memory for the odor is expressed in a search for the reward, which ceases if the sought-for reward is present. Further details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S4.

However, memory scores were higher if two or three training trials were used rather than only one training trial **(Figure 8A)**, confirming the higher memory scores for the higher number of training trials reported in Neuser et al. (2005). The increase in memory strength from one to more trials was more pronounced than what Thane et al. (2019) had found as not significant trend. In general, this finding is consistent both with common sense and widely accepted prediction-error learning theories (Rescorla and Wagner 1972). Regardless of the number of training trials, memory was not behaviorally expressed in the presence of fructose **(Figure 8B)**, confirming the data from **Figure 7** as well as previous reports (Gerber and Hendel 2006, Schleyer et al. 2011, Schleyer et al. 2015a).

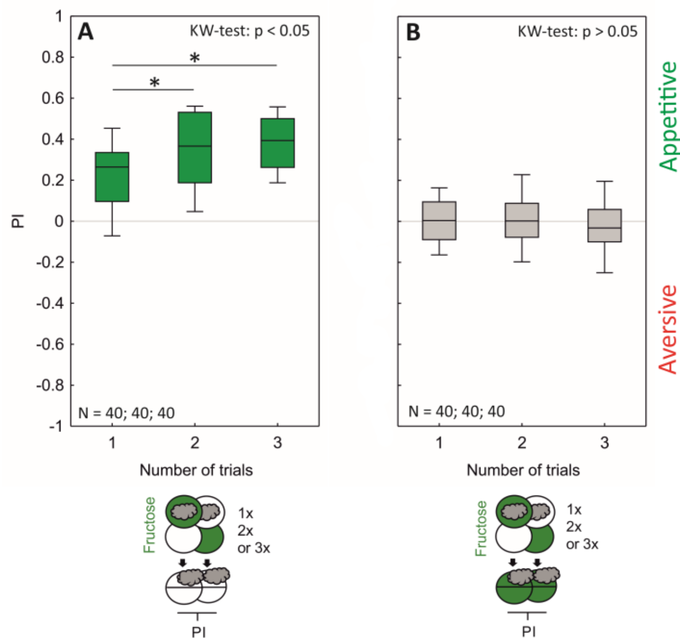

Figure 8: Comparison of associative memory after one, two and three training trials. Larvae underwent training trials of 2.5 min duration, with odor and fructose reward either together (paired) or separate (unpaired). Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. The sketches towards the bottom of the figures depict the training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Animals were trained either once, twice or three times. **(A)** If tested on a pure agarose substrate, the larvae showed appetitive memory after one, two and three training trials; memory scores increased with trial number. **(B)** If tested in the presence of fructose, appetitive associative memory is not expressed, regardless of the number of training trials. Further details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S5.

Notably, the data from **Figure 7** and **Figure 8** allow an interesting re-analysis. As specified in equation (2) of the *Materials & Methods* section, the associative memory scores (i.e. the PI scores) reflect the difference in odor preference between paired-trained and unpaired-trained larvae. The PI scores therefore do not allow a conclusion to be drawn as to whether the animals in the paired group have learned that reward can be found <u>where the odor is</u>, or whether the animals in the unpaired group have learned that reward can be found precisely <u>where the odor is not</u>, or whether both paired-memory and unpaired-memory are established (see Schleyer et al. 2018 for discussion). This can be revealed, however, by separately considering the Preference scores after paired or unpaired training relative to the 'baseline' Preference scores that are observed when the animals are tested in the presence of fructose (Saumweber et al. 2011). Under such test conditions the olfactory behavior of the larvae is cleared of any influence of associative memory **(Figure 7, Figure 8B)**. Analysis of the Preference scores using this baseline approach reveals surprisingly weak yet statistically significant paired-memory, and relatively robust unpaired-memory (**Figure 9A,** combining the data underlying **Figure 7**, **Figure 8** and **Figure S6**, total sample size of N = 120). The same is observed after three training trials, although in this case paired memory appears more robust (**Figure 9B,** N = 40) (also see Schleyer et al. 2018).

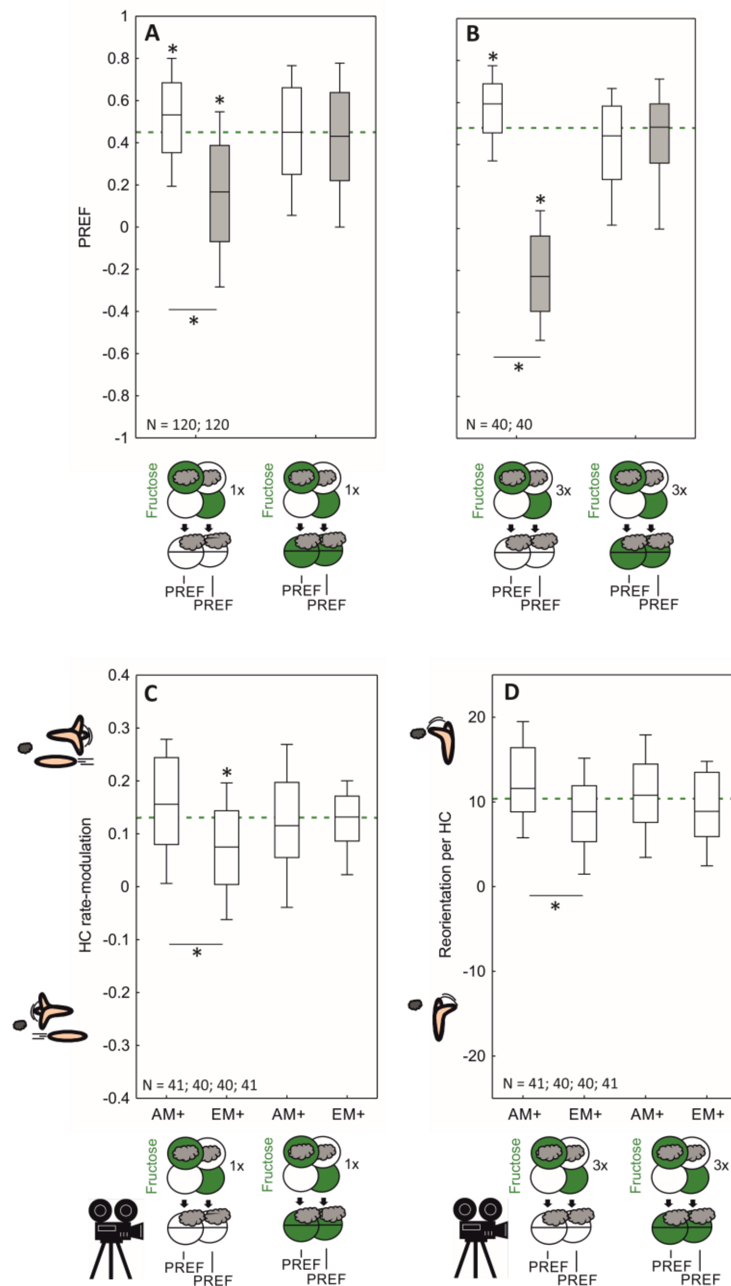

Figure 9: Post-hoc analyses of Preference scores (PREF) and the modulations of locomotion underlying them, after paired and unpaired training. The sketches towards the bottom of the figures depict the training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Further details as described in Figure 4. **(A)** Analysis of the PREF scores underlying the associative Performance indices (PI) combined from Figure 7, Figure 8 and Figure S6. If tested in the presence of fructose, PREF scores are equal after paired and unpaired training, so their data were pooled, and the median of the pooled data is displayed as the green stippled line indicating baseline odor preference after training, but cleared of associative memory. Relative to this baseline, PREF scores are slightly yet significantly increased for paired training, and are robustly decreased after one-trial unpaired training. **(B)** Analysis of the PREF scores underlying the associative Performance indices (PI) from Figure 8, after three-trial training. This reveals strong paired and unpaired memory relative to baseline. Further details as described in Figure 4. **(C, D)** For a subset of the cases in (A), larvae were video-tracked for offline analyses of their locomotion. This reveals that the behavior of paired-trained and unpaired-

trained animals differed quantitatively in terms of the modulation of head cast (HC) rate (C) as well as HC direction relative to the odor source (D). Relative to baseline, only the modulation of HC rate after unpaired training was significantly different. Corresponding PI and PREF scores can be found in Figure S6. Sketches of larvae (C, D) depict their change in behavior with respect to the odor in the case of positive or negative scores (image courtesy of Naoko Toshima, LIN).

Locomotor 'footprint' of memory after one-trial training

Given the above analyses of the Preference scores, I sought to establish the precise mechanisms that produce these results. Larvae navigate along odor gradients by a series of relatively straight runs, interrupted by lateral head movements (head casts, HCs) that may be followed by turning maneuvers. Appetitive memories after three-trial fructose training have been shown to modulate two aspects of this behavior: the HC rate and the HC direction (Schleyer et al. 2015b, Paisios et al. 2017). To see whether the same is true after one-trial training, I recorded and tracked the animals' behavior in a subset of the experiments shown in Figure 9A. After paired training the HC rate-modulation score was found to be higher than after unpaired training, meaning that after paired training the larvae more strongly increase their HC rate while heading away from the odor source and more strongly decrease their HC rate while heading towards it **(Figure 9C)**. Furthermore, the larvae bias their HC direction more towards the odor source after paired training than after unpaired training, indicated by higher reorientation values **(Figure 9D)**. Interestingly, relative to baseline the only case of significance is for the HC rate-modulation after unpaired training **(Figure 9C, Figure 9D)**, which is in line with the robust learning effects in the unpaired-trained animals according to the more 'macroscopic' analyses in terms of preference scores **(Figure 9A)**.

Fructose memory after one-trial training decays over a few minutes after training

To study the temporal stability of memory after one-trial training with fructose as the reward, larvae were trained and then tested either immediately after training (0 min retention interval), or after retention intervals of respectively 5, 10 or 15 min, which they spent in a water droplet on an otherwise empty Petri dish lid. Appetitive memory was evident immediately after training, whereas results at all later test time-points did not reach significance. Indeed, relative to memory scores immediately after training, scores were decreased when assessed at 5, 10 and 15 min retention intervals **(Figure 10A)**. Thus, fructose memory after one-trial training is transient, lasting for less than 5 min. For three-trial differential conditioning, using multiple short trials with brief breaks between them, fructose memory was reported to be stable for at least 30 min in Neuser et al. (2005). For three-trial, single-odor conditioning, memory scores have been reported to be stable for about 20 min (Kleber et al. 2015). However, under the present conditions, fructose memory after three-trial

training was more transient **(Figure 10B)**. Of note, in this experiment too, the initial memory scores after three training trials were higher than after one training trial **(Figure 10A, B)**, replicating the results from **Figure 8**.

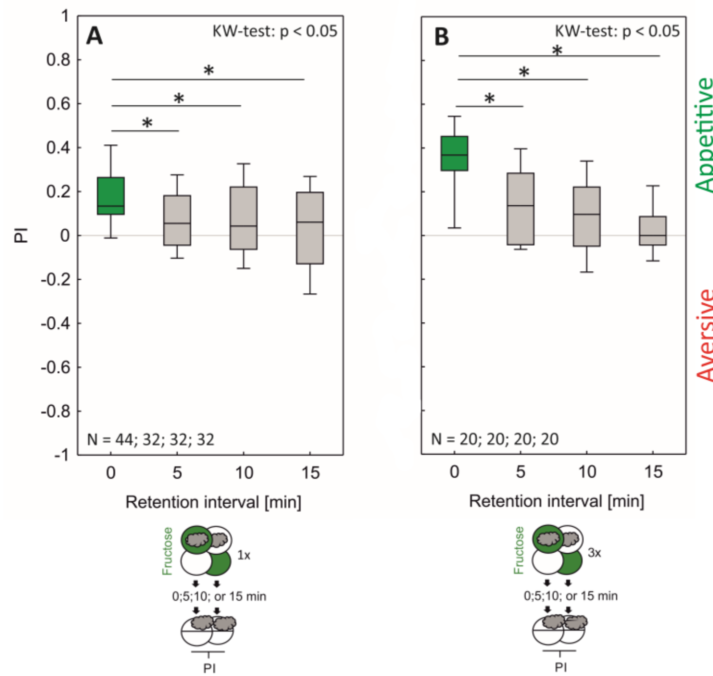

Figure 10: Temporal dynamics of one-trial associative memory using fructose as reward. Larvae underwent training trials of 2.5 min duration, with odor and fructose reward either together (paired) or separate (unpaired). Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. Larvae were tested either immediately after training (retention interval 0 min), or 5, 10 or 15 min after training. The sketches towards the bottom of the figures depict training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate. Regardless of whether training was performed with **(A)** only one trial or **(B)** three trials, memory scores were significant for immediate testing whereas results at the later time-points remained below the statistical threshold. Further details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S7.

One-trial differential conditioning?

Next, I tested whether one-trial memory can also be observed upon differential conditioning. Using fructose as the reinforcer, the larvae either received *n*-amylacetate with reward and 1-octanol without reward (AM+/OCT), or were trained reciprocally (AM/OCT+). Then, I measured the choice between AM and OCT and analyzed the data, with due adjustments, according to equations (1) and (2). This revealed appetitive memory after such one-trial differential conditioning. Again, the behavioral expression of this memory was prevented by testing the larvae in the presence of the fructose reward **(Figure 11A)**. In parallel, the standard one-trial learning experiments in the single-

odor, absolute conditioning paradigm, using either only AM or only OCT as the odor was performed. Confirming our data from **Figure 7** and **Figure 8**, the use of AM as the odor yielded appetitive memory, the behavioral expression of which was abolished when testing was carried out in the presence of the fructose reward **(Figure 11B)**. Surprisingly, however, the same type of experiment did not yield evidence for associative memory when using OCT as the odor **(Figure 11C)**. Thus, I further investigated whether during differential training any memory accrues to OCT, and whether after training with OCT alone a memory for OCT can be revealed by differentially testing the larvae in a choice situation between AM and OCT.

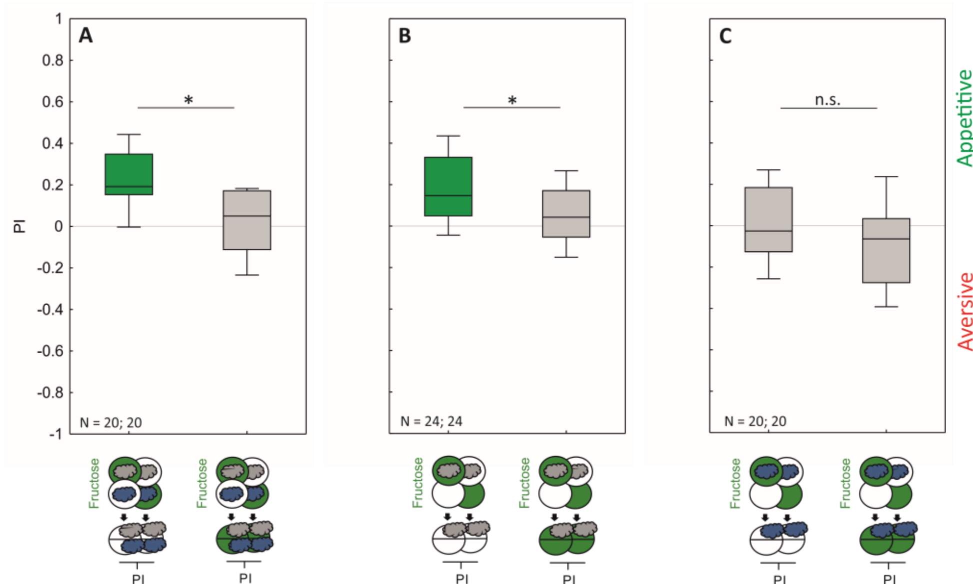

Figure 11: One-trial associative memory for n-amylacetate but not for 1-octanol? Larvae underwent single training trials of 2.5 min duration, with odors and fructose reward at the contingencies indicated. Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. The sketches towards the bottom of the figures depict training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate, and the dark blue cloud the odor 1-octanol. **(A)** Animals were trained differentially, with one of the two odors *n*-amylacetate or 1-octanol paired with fructose and the other odor presented alone. Then, the relative preference between the two odors was determined in a choice test and PIs were calculated. The data show appetitive associative memory when testing was carried out on pure agarose Petri dishes, whereas the behavioral expression of memory was abolished when testing was carried out in the presence of fructose. **(B)** As in (A), but omitting 1-octanol. Appetitive memory for *n*-amylacetate is behaviorally expressed in the absence but not in the presence of fructose (also see Figure 7 and Figure 8). **(C)** As in (A), but omitting *n*-amylacetate. Regardless of the test condition, no associative memory for 1-octanol is detectable. Further details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S8.

Do larvae learn about octanol?

Larvae were differentially trained with fructose as the reward and AM and OCT as odors, as in **Figure 11A**; however, the animals were tested for their preference for OCT alone. This revealed that after differential training the larvae did indeed show an OCT memory **(Figure 12A)**. Moreover, training with OCT alone but differentially testing the larvae for their choice between AM and OCT also resulted in appetitive memory scores **(Figure 12A)**. This means that OCT-memory after one-trial training can be revealed if either training or testing is carried out in a differential manner. But is either differential training or differential testing indeed necessary for OCT memory to become detectable? This is not the case, because when three training trials were performed with only OCT during both training and testing the larvae did reveal OCT memory **(Figure 12B)** (see also Saumweber et al. 2011, Mishra et al. 2010), a memory which, similar to what we observed for AM in **Figure 8**, was not behaviorally expressed in the presence of the fructose reward **(Figure 12C)**.

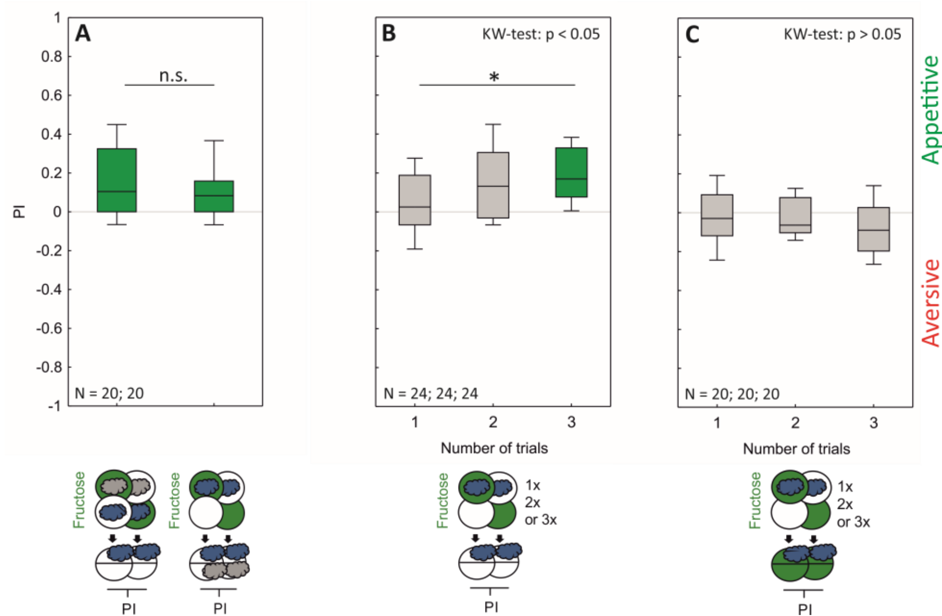

Figure 12: One-trial associative memory for 1-octanol is revealed by differential training or differential testing; associative memory is also revealed by employing multiple training trials. Larvae underwent training trials of 2.5 min duration, with odors and fructose reward at the number and contingencies indicated. Differences in odor preference after such training are quantified by the performance index (PI) and reflect associative memory. The sketches towards the bottom of the figures depict training and testing procedures. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. The grey cloud indicates the odor *n*-amylacetate, and the dark blue cloud the odor 1-octanol. **(A)** Left: Animals were trained differentially, with one of the two odors *n*-amylacetate or 1-octanol paired with fructose and the other odor presented alone. Then, the preference for 1-octanol was determined and PIs were calculated, revealing associative memory for 1-octanol. (A) Right: Animals were trained with 1-octanol either paired or unpaired with the fructose reward. Then, they were tested for their choice between 1-octanol and *n*-amylacetate, revealing that 1-octanol training has established associative memory. **(B)** Animals received either paired or unpaired training with 1-octanol and fructose reward, either once, twice or three times, and

were then tested for their 1-octanol preference. This reveals associative memory for 1-octanol after three but no fewer training trials. **(C)** As in (B) but testing the larvae in the presence of fructose; under these conditions, the behavioral expression of associative memory for 1-octanol was abolished. Further details as described in Figure 4. Preference scores underlying the PIs are documented in Figure S9.

Interim discussion

One-trial memory for some, but not all rewards, and for some, but not all punishments

The present analysis reveals that larvae acquire short-term one-trial associative memory of about equal strength for all sugar types tested **(Figure 4E)**. Thus, it does not seem to be of major importance in this type of assay whether the sugars are both sweet and nutritious (fructose), only sweet (arabinose), or only nutritious (sorbitol) (Fujita and Tanimura 2011, Rohwedder et al. 2012), or indeed whether they promote feeding (Teiichi Tanimura, Nagoya University, Japan, personal communication). In adult flies, one-trial short-term memory has likewise been found to be substantial for both nutritious (fructose, glucose, sucrose) and non-nutritious sugars (arabinose, xylose), but only the nutritious sugars supported appetitive memory persisting for at least 24 hours (Burke and Waddell 2011). In the larva and for the tested case of fructose, there is no evidence of such longer-term appetitive memory **(Figure 10)** (Neuser et al. 2005, Kleber et al. 2015). No significant appetitive memory scores were observed in the larva for aspartic acid, a proteinogenic and according to Sang and King (1961) non-essential amino acid **(Figure 4E)**. Resembling the case of fructose rather than aspartic acid, blue-light activation of the cells covered by the DAN-i1 driver strain reveals robust appetitive memory scores after one training trial **(Figure 6)**.

Regarding taste punishment, one-trial aversive associative memory was shown for high-concentration salt **(Figure 5C)**, matching the report by Widmann et al. (2016), who used a two-odor, differential conditioning paradigm. Also, quinine elicited significant aversive memory scores for the longest trial duration, which was rather weak, such that no significant difference could be detected between groups **(Figure 5B, C)**. We note that, unlike quinine, high-concentration salt may not only affect the taste system of the larvae but also threaten their osmotic balance. Furthermore, exposure of micro-wounds to high-concentration salt may produce itching sensations, potentially in synergy with the activation of multimodal pain sensory neurons, effects that again would not need to be taken into account for quinine. Thus, in the aversive domain, one-trial associative memory might rather be restricted to very strong kinds of punishment that threaten the larvae's bodily integrity. This might not be the case for brief exposures to high-concentration salt **(Figure 5C)**. We note that a trend rather for appetitive memory through such brief exposure **(Figure 5A, C)** would be consistent

with the report by Gerber and Hendel (2006), who showed that low-concentration salt, which might likewise not threaten bodily integrity, has a rewarding effect in larvae (Gerber and Hendel 2006).

Practical implications

The present one-trial version of the paradigm can substitute for the lengthier three-trial version, at least for those reinforcers that indeed support one-trial memory. It is also more resource-friendly, requiring fewer Petri dishes, less agarose and lower quantities of tastant substances, making it environmentally and financially preferable over the three-trial version. For teaching purposes in high school classroom settings or for undergraduate laboratory courses (Michels et al. 2017), one-trial training with a training trial duration of 2.5 min and using single-odor absolute conditioning with fructose as the reward might therefore be the procedure of choice.

Sources of variability

Variability in odor-fructose associative memory scores, and in the preference scores underlying them, is apparently higher after one-trial training than after three-trial training **(Figure 8A, Figure 9A, B)**. Here, the effect of odor exposure might be more pronounced, which decreases odor preference (larvae: Michels et al. 2005, Saumweber et al. 2018, for discussion of earlier work see Gerber and Stocker 2007; adults: Pech et al. 2015, Hattori et al. 2017), and the effect of reward exposure, which increases odor preference compared to innate odor preference (larvae: Michels et al. 2005, Saumweber et al. 2011, Saumweber et al. 2018; adults: not reported). Indeed, one-trial associative memory might be only partially consolidated at the moment of testing, allowing the two afore-mentioned exposure induced processes to influence behavior more strongly. This might make behavior more variable because, as noted above, these processes are of opposite effect on odor preference. In contrast, after repeated training the consolidation of associative memories might already be complete, at least for the early trials, and might dominate behavior.
A second source of variability after one- rather than three-trial training might be related to our current finding that memory after one training trial is apparently dominated by the effects of unpaired rather than paired training **(Figure 9)**. Why would this lead to particularly variable results when only one training trial is used? Within the framework of models of associative learning using prediction-error learning rules (Rescorla and Wagner 1972), one might suggest that the presentation of fructose in the absence of odor establishes context-fructose memory. If subsequently the odor is presented within that same context, a prediction error arises: fructose is predicted by contextual cues, but it is not actually received. This would be the basis for the change in valence of the odor presented during this 'frustrating' experience (reviewed in Schleyer et al. 2018). Obviously, according

to such a scenario the presentation of fructose has to come before the presentation of the odor, as is the case for half of the repetitions within each of our experiments. If the order of presentation is reversed – as is the case in the other half of our samples – the context would still be neutral at the moment of odor presentation. As training progresses with subsequent trials, this variation in the effectiveness of one-trial unpaired training would eventually be diluted out.

Thus, behavior after only one training trial is arguably affected more strongly than after three training trials by the effects of odor exposure and of fructose exposure, and by variations in the effectiveness of learning through unpaired training. If the target associative memory component is weak to begin with, as in the case of aspartic acid and quinine, this may render one-trial associative memory practically undetectable or at least unworkable **(Figure 4D, Figure 5B)**.

The role of differential training and testing for learning about 1-octanol

Of note, one-trial associative memory for 1-octanol requires either differential training or differential testing **(Figure 11C, Figure 12A)**. The requirement for differential training points to a plasticity mechanism during training as a prerequisite for a memory trace for 1-octanol even to be established. This is reminiscent of what Mishra et al. (2010) reported for the larva, and Barth et al. (2014) as well as König et al. (2017) for adult *Drosophila*, in all cases using 3-octanol and 1-octen-3-ol as the odor pair. These authors noted that after absolute, single-odor training the animals fully generalize between these two odors, such that they behave towards the non-trained odor in the same way as towards the trained odor. It is only after differential training that behavior is selective for the trained odor. Barth et al. (2014) further showed that such acuity learning involves both second- and third-order olfactory processing stages.

The requirement for differential testing to reveal one-trial memory for 1-octanol in the present study implies that during testing 1-octanol is processed more effectively for memory retrieval when presented in the context of *n*-amylacetate as a choice alternative than when presented alone. During the test the presence of a second odor gradient, oriented at 180 degrees to the target odor, possibly helps the animals to navigate towards their target.

Stronger, or different, memories after one- versus three-trial training?

Odor-fructose associative memory scores increase across training trials **(Figure 8A)** (see also Neuser et al. 2005). Does this come about by an increase in strength of the association, or by the recruitment of a different, additive kind of process in the trials following the first one? Although the former seems to be the more parsimonious explanation, results from the honey bee suggest that one- and three-trial training establish memories that differ in kind rather than in strength alone. In appetitive

classical conditioning of the proboscis extension reflex (PER), one-trial memory thus begins to decay after about one day, whereas three or more conditioning trials induce memory that is stable for up to several days (reviewed in Giurfa and Sandoz 2012). Critically, unlike one-trial memory, memory after three training trials in this paradigm is largely amnesia-resistant and dependent on translation and transcription (reviewed in Schwärzel and Müller 2006, but see Pamir et al. 2014). It is interesting to note that memories established by one-trial learning might in themselves be a composite, as has been shown by Scheunemann et al. (2013) by varying the number and intensity of individual shock pulses within single training trials in wild-type versus mutant adult *Drosophila*.

We note that an interpretation of acquisition fundamentally different from the above was offered by Pamir et al. (2014). On the basis of the individual-animal performance of honey bees during and after one-trial or multiple-trial PER conditioning, the authors argue that in individual animals learning is actually a step-like process (also see Trabasso 1963 and Ohl et al. 2001 for examples of such one-trial learning during category formation in humans and gerbils, respectively). Once the animals have started to show learned behavior in the second trial, for example, they continue to do so during further acquisition trials. What increases across training trials, the authors argue, is the frequency of individual bees that turn into responders. Thus, the gradual appearance of an 'acquisition curve' is suggested to be an artifact of averaging across animals. Indeed, 24-hour-memory was similarly strong regardless of whether individual bees started to show learned behavior after the first or after subsequent training trials.

'Small-data' learning

In biological systems, one-trial associative learning is not unusual (see Introduction). It should be of evolutionary benefit whenever, despite the limited predictive evidence that only one training trial can offer, the cost of wrongly not-predicting the outcome is higher than the cost of wrongly predicting it. Under such conditions, biological systems are apparently capable of lean, 'small-data' predictive learning that contrasts with the powerful and heavily energy-consuming 'big-data' predictive strategies of artificial intelligence (Halevy et al. 2009, Obermeyer and Emanuel 2016).

Materials & Methods

This study uses established methods for odor-taste associative learning in larval *Drosophila* (Scherer et al. 2003, Gerber and Hendel 2006, El-Keredy et al. 2012, Schleyer et al. 2015a), unless mentioned otherwise using the single-odor, absolute conditioning paradigm established for fructose as tastant by Saumweber et al. (2011). In principle, one group of larvae receives an odor together with a tastant

reinforcer (paired), whereas a second group is presented with the odor and the tastant separately from each other (unpaired). After such training, both groups are tested for their preference for the odor. Differences in odor preferences between paired-trained and unpaired-trained groups thus indicate associative memory. A distinct feature of the present study is that we use only one training trial, unless mentioned otherwise.

Animals

For most experiments 5-days-old, 3rd instar, feeding-stage larvae from the Canton-S wild-type strain (RRID:DGGR_105666) were used. Flies were maintained at 25 °C, 60-70 % relative humidity and a 12/12 h light/dark cycle. Cohorts of approximately 30 larvae were collected from the food vials, rinsed in water, collected in a water droplet and subsequently used in the respective experiment.
Furthermore, transgenically modified larvae were used to optogenetically activate a specific set of neurons. Animals of the effector strain UAS-ChR2-XXL (Bloomington Stock Center no. 58374, RRID:BDSC_58374; Dawydow et al. 2014) were crossed to animals of the split-Gal4 driver strain SS00864-Gal4 (RRID: N/A; kindly provided by HHMI Janelia Research Campus, USA; Saumweber et al. 2018) to obtain double-heterozygous offspring. In the larvae in question, the blue-light-gated cation channel ChR2-XXL can be activated in the neurons of interest. The expression pattern of the SS00864-Gal4 driver strain was confirmed by immunohistochemistry to include the hemispherically unique DAN-i1 neurons, with additional stochastic expression in 1-2 further neurons (Saumweber et al. 2018; see *Immunohistochemistry* section). As the driver control, the DAN-i1 driver strain was crossed to a local copy of w^{1118} (Bloomington Stock Center no. 3605, 5905, 6326, RRID: BDSC_3605). As the effector control, a strain homozygous for both landing sites used for the split-Gal4 (attP40/attP2) (RRID: N/A; kindly provided by HHMI Janelia Research Campus, USA; Pfeiffer et al. 2010), yet without a Gal4 domain inserted, was crossed to UAS-ChR2-XXL. All transgenic flies were raised in darkness with black cardboard wrapped around the food vial.

The continuous protocol for associative learning

For paired training, cohorts of 30 larvae were placed at the center of a Petri dish (9 cm inner diameter; Art-Nr. 82.1472, Sarstedt, Nümbrecht, Germany) equipped with odor containers (see below) and filled with 1 % agarose solution (electrophoresis grade; CAS: 9012-36-6, Roth, Karlsruhe, Germany) supplemented with fructose (FRU; 2 M; CAS: 57-48-7, purity 99 %, Roth, Karlsruhe, Germany) as a taste reward (+). Custom-made Teflon containers of 5 mm diameter contained 10 µl of odor substance. This was 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 (OCT;

CAS: 111-87-5, Merck, Darmstadt, Germany), undiluted. Paraffin has no behavioral significance as an odor (Saumweber et al. 2011). The containers were closed by a lid perforated with 5-10 holes, each of approximately 0.5 mm diameter. At each of the opposing edges of the Petri dish (left or right), one odor container holding AM was placed. Larvae were then free to move about this AM+ Petri dish for 2.5 min, unless mentioned otherwise. Then, they were transferred to a Petri dish which lacked fructose and which featured two empty odor containers (EM), and they were left there for the same amount of time. After such AM+/EM training, they were transferred to the center of a test Petri dish, where an AM odor container was presented on one side and an empty odor container on the opposite side, and were thus tested for their preference for AM. Of note, in this 'continuous' paradigm, odor and reinforcer are paired for the whole trial duration of 2.5 min (or other). Unless mentioned otherwise, the test Petri dish featured only agarose, but no added tastant. After 3 min, 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 side-walls of the Petri dish were counted for the respective side, whereas larvae on the lid were excluded from the analysis. A preference index (PREF) was calculated:

$$PREF = \frac{(\#\mathrm{AM} - \#\mathrm{EM})}{\#\mathrm{Total}} \quad (1)$$

Preference indices may thus range from +1 to −1, with positive values indicating preference and negative values indicating avoidance of AM. Across repetitions of the experiments, in half of the cases the sequence was as indicated (AM+/EM), whereas in the other cases it was reversed (EM/AM+).

The procedure for unpaired training was the same, except that the Petri dishes featured either only AM or only the reward. After such AM/EM+ training (again in half of the cases the sequence was reversed: EM+/AM), the preference test was carried out as above.

From the PREF scores after paired and unpaired training, a performance index (PI) was calculated:

$$PI = \frac{(\mathrm{PREF}\ Paired - \mathrm{PREF}\ Unpaired)}{2} \quad (2)$$

Thus, performance indices may range from +1 to -1. Positive PIs indicate appetitive associative memory, whereas negative values indicate aversive associative memory.

Odor-arabinose associative learning

The procedure was as described above for fructose, except that instead of fructose we used arabinose as a reward (ARA; 2 M; CAS: 10323, purity ≥ 98 %, Sigma-Aldrich, Steinheim, Germany).

Odor-sorbitol associative learning

The procedure was as described above for fructose, except that instead of fructose we used sorbitol as a reward (SOR; 2 M; Art-Nr. 6212.2, purity ≥ 98 %, Roth, Karlsruhe, Germany).

Odor-aspartic acid associative learning

The procedure was as described above for fructose, except that instead of fructose we used aspartic acid as a reward (ASP; 10 mM; CAS: 56-84-8, purity ≥ 99 %, Sigma-Aldrich, Steinheim, Germany).

Odor-quinine associative learning

The procedure was as described above for fructose, with two exceptions. Firstly, instead of fructose we used quinine hemisulfate as punishment (QUI; 5 mM; CAS: 6119-70-6, purity 92 %, Sigma-Aldrich, Steinheim, Germany). Secondly, QUI was present during testing. Learned avoidance of quinine-associated odors can be considered a form of escape behavior that is expressed only if the test situation warrants escape, i.e. if it includes the quinine punishment to motivate escape (Gerber and Hendel 2006, Schleyer et al. 2011, Schleyer et al. 2015a).

Odor-sodium chloride associative learning

The procedure was as described above for quinine, except that instead of quinine we used a high sodium chloride concentration as a punishment (high salt/NaCl; 1.5 M; Art-Nr. 3957.1, purity ≥ 99.5 %, Roth, Karlsruhe, Germany). As with quinine, only when the tests are carried out in the presence of high-concentration salt as the negative reinforcer are the larvae prompted to express aversive memory behaviorally (Gerber and Hendel 2006, Niewalda et al. 2008, Widmann et al. 2016).

Odor-DAN associative learning

All optogenetic experiments were performed inside a custom-made box. Within the box, a light table was equipped with 24 x 12 LEDs with a peak wavelength of 470 nm (Solarox, Dessau-Roßlau, Germany), with a 6 mm-thick diffusion plate of frosted plexiglass on top to ensure uniform light conditions and intensity (120 µW/cm²). The Petri dishes for the learning assay were placed directly on top of the diffusion plate surrounded by a ring of 30 infrared LEDs (850 nm; Solarox, Dessau-Roßlau, Germany) behind a polyethylene diffusion ring that provided illumination. Similar to the associative learning experiment described above, we trained larvae either paired or unpaired with

the reinforcer, which in this case consisted of optogenetic activation of DAN-i1 rather than a tastant. Each trial lasted 2.5 min and the larvae were trained in one training trial only. Then the larvae were transferred to a pure test Petri dish, and their preference for AM as well as the Performance Index was calculated as detailed above. Experimenters were blind to genotype.

Whenever variations in the above paradigms were used, these are mentioned along with the presentation of the results.

Locomotor footprint of memories established by odor-fructose associative learning

Larval behavior was video-tracked and analyzed as described in detail in Paisios et al. (2017). In brief, two aspects of larval chemotaxis were analyzed. Firstly, the modulation of head cast (HC) rate:

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

This measure yields positive scores for attraction, i.e. when larvae systematically perform more head casts while heading away from the odor (i.e. when odor concentration decreases) than while heading towards it (i.e. when odor concentration increases). Conversely, it yields negative scores for aversion. Secondly, the modulation of head cast direction was measured by the reorientation per head cast:

$$Reorientation\ per\ HC = \text{abs(heading angle before HC)} - \text{abs(heading angle after HC)} \tag{4}$$

In this measure, the heading angle describes the orientation of the animal's head relative to the odor, with absolute heading angles of 0° or 180°, for example, indicating that the odor is to the front or to the rear of the larvae, respectively. This measure thus yields positive scores for attraction, i.e. when the head cast directs the larvae towards rather than away from the odor target, whereas it yields negative scores for aversion.

Immunohistochemistry

I undertook larval body wall preparations (N = 4) (see Budnik et al. 2006) on larvae of the same experimental genotype as used for our optogenetic one-trial experiment, for which the DAN-i1 driver strain had been crossed to the UAS-ChR2XXL effector strain. Larvae were individually placed at the center of a well in a custom-made magnetic chamber (kindly provided by Dr. Ulrich Thomas, LIN). Pinning the anterior and posterior ends, larvae were covered with Ca^{2+}-free saline and then dissected

using micro-scissors (No. 15002-08, Fine Science Tools GmbH, Heidelberg, Germany). The internal organs were removed, whereas the central nervous system was left intact. After washing again with Ca^{2+}-free saline, the larvae were fixed in Bouin's solution (HT10132, Sigma-Aldrich, Steinheim, Germany) for 7 min and afterwards larval brains were washed three times consecutively in fresh washing solutions for 10 min each time, using 0.2 % PBT (Triton-X-100, CAS: 9036-19-5, Roth, Karlsruhe, Germany; in 1x PBS) and then another three times every 15 min. The larvae were then transferred into a glass bowl and treated overnight at 4 °C with the primary monoclonal mouse anti-ChR2 antibody (Cat No: 610180, RRID: N/A, ProGen Biotechnik, Heidelberg, Germany) diluted 1:100 in 0.2 % PBT. After three washing steps once every 10 min in 0.2 % PBT, tissue was incubated with a secondary polyclonal Cy3 donkey anti-mouse (Art-Nr. 715-165-151, RRID: AB_2315777, Dianova, Hamburg, Germany) and a polyclonal Alexa Fluor 488 goat anti-horseradish peroxidase (Art-Nr. 123-545-021, RRID: AB_2338965, Jackson Immuno Research, USA), both diluted 1:300 in 0.2 % PBT for one hour. After three final washing steps once every 10 min with 0.2 % PBT, samples were mounted in Vectashield (H-1000-10, Vector Laboratories Inc., Burlingame, USA) on a cover slip. Preparations were examined under a DM6000 CS confocal microscope (Leica, Jena, Germany). All image stacks were analyzed with Fiji Image-J software (RRID:SCR_002285).

Statistics

For the behavioral data, non-parametric statistics were applied throughout. For comparisons with chance levels (i.e. with zero), one-sample sign tests (OSS; corresponding to binom.test) were used (R Core Team (2016)). For between-group comparisons, Kruskal-Wallis tests (KW) and Mann-Whitney U-tests (MWU) were applied where appropriate (Statistica 13, RRID:SCR_014213, StatSoft Inc, Tulsa, USA). We used a Bonferroni-Holm (BH) correction for multiple comparisons to maintain an error rate below 5 % (Holm 1979). I speak of a trend towards significance when a given comparison would be significant without such correction. Data are displayed as box plots with the median as the middle line, the box boundaries as 25 and 75 % quantiles and the whiskers as 10 and 90 % quantiles.

II - Timing-dependent valence reversal in the appetitive domain

Obtaining rewards can drive behavior of humans and animals to a considerable extent. To maximize reward, it is obviously crucial to learn about cues that predict the beginning of a rewarding stimulus, and decades of research have investigated the underlying mechanisms of the positive valence that accrues to cues through this type of associative learning. It is less acknowledged by the scientific community, but not less important for an animal, to learn also about cues that are associated with the termination of a rewarding stimulus. Because the end of something good feels bad, such a cue can thus gain negative valence if it is associated with the termination of a reward. This principle, known as timing-dependent valence reversal, is observed in humans and animals alike (Solomon and Corbit 1974, Hellstern et al. 1998, Gerber et al. 2014, Gerber et al. 2019) but is yet not well understood. The corresponding effect for punishing stimuli has been characterized in more detail: Cues gain negative valence if they predict the beginning of a painful electric shock, and gain positive valence if they are associated with the relief from electric shock. Corresponding results were reported in flies, rodents and humans (reviewed in Gerber et al. 2014, Gerber et al. 2019). In this study, I investigate the features of timing-dependent valence reversal regarding an identified reward-signaling dopaminergic neuron in the larvae of *Drosophila melanogaster*. These animals present an attractive study case because of their numerical simplicity, substantial behavioral complexity and a toolbox of transgenic methods to selectively express any gene in a tissue- or cell-specific manner (Brand and Perrimon 1993, Pfeiffer et al. 2010). In fact, the larval nervous system consists of only 10,000 neurons (Nassif et al. 2003) and the chemical-synapse connectome of their associative memory center was recently reconstructed (Eichler et al. 2017, Saumweber et al. 2018, Eschbach et al. 2020a). The neuronal circuits underlying associative learning in larvae parallel those of adult *Drosophila* and other insects (larvae: Gerber et al. 2009, Thum and Gerber 2019; adults: Modi et al. 2020): The MB, consisting of second-order interneurons called KCs, provides a combinatorial representation of external stimuli, most prominently odors. The axons of the KCs are intersected by mostly dopaminergic modulatory neurons (DANs) as well as MBONs. The area of innervation of individual DANs and MBONs are organized such that they divide the mushroom body into clearly distinct compartments in which the DANs and MBONs of a given compartment form local circuits with the KCs (larvae: Eichler et al. 2017, Saumweber et al. 2018; adults: Aso et al. 2014a, Takeumara et al. 2017). Some DANs, when activated, carry a reward signal, whereas others carry a punishment signal (larvae: Schroll et al. 2006, Rohwedder et al. 2016, Eichler et al. 2017, Saumweber et al. 2018, Eschbach et al. 2020a). In extension of what has been revealed in adults, the working hypothesis is that when such a reward or punishment signal reaches a given compartment, the strength of the

synapses from odor-activated KCs to the MBON(s) of the same compartment is modified; if that same odor is encountered again, the changed odor response of the MBONs then shifts the animal's behavior more towards odor approach or odor avoidance, dependent on the specific association (Cohn et al. 2015, Owald et al. 2015, Takemura et al. 2017). Using odor and electric shock, timing-dependent valence reversal was substantially characterized in adult *Drosophila* (Tanimoto et al. 2004, Yarali et al. 2008, Yarali et al. 2009, Yarali and Gerber 2010, Diegelmann et al. 2013b, Niewalda et al. 2015, Appel et al. 2016). Importantly, also the activation of a single punishing DAN, PPL1-01, can confer both learning about punishment and relief in adults (Aso and Rubin 2016, König et al. 2018), and regarding a broader subset of DANs with rewarding polarity reward versus frustration learning was recently reported (Handler et al. 2019). In larvae, first indications of relief learning using electric shocks did not turn out to be significant (Khurana et al. 2009), and thus so far only the activation of a single DAN of the pPAM cluster, DAN-i1 was found to mediate both reward and frustration memory (Saumweber et al. 2018). In the following I further investigate the capacity of DAN-i1 to bring about oppositely-valenced memories and parametrically describe their similarities and differences.

<u>Results</u>

Optogenetic activation of DAN-i1 mediates timing-dependent valence reversal

Larval offspring of the UAS-ChR2-XXL effector strain crossed to the SS00864 driver strain was used as the experimental genotype. This driver strain strongly and reliably covers the DAN-i1 neuron in both hemispheres, with stochastic expression in 1-2 additional cells **(Figure 13A-C)** (Saumweber et al. 2018). Larvae were trained in a classical conditioning paradigm such that an odor was presented together with optogenetic activation of DAN-i1. Notably, the odor was either presented <u>before</u> DAN-activation (<u>forward</u> conditioning; odor-DAN) or the odor was presented <u>after</u> DAN-activation (<u>backward</u> conditioning; DAN-odor). The relative timing between the onset of DAN activation and odor presentation is called inter-stimulus-interval (ISI), such that by definition negative ISIs indicate forward conditioning, whereas positive ISIs indicate backward conditioning. Odor and reinforcement lasted only 30 s each, not for the whole trial duration ('timed' protocol). I investigated larval behavior for one forward and one backward ISI, respectively, based on previous findings (Saumweber et al. 2018). Each forward or backward paired-trained group of animals was accompanied by a group of unpaired-trained animals, which received odor and DAN activation separate from each other. For a more detailed training procedure see **(Figure S10)**. Three training trials were performed, unless mentioned otherwise, and afterwards larvae were subsequently tested for their odor preference. Replicating the findings from Saumweber et al. (2018), I confirmed appetitive reward memory in the

experimental genotype upon forward conditioning, relative to the respective genetic controls **(Figure 13D)**, as well as aversive frustration memory upon backward conditioning **(Figure 13E)**. Thus, indeed DAN-i1 establishes memories of opposite valence, dependent on evevnt-timing. Next, I asked, whether these two memory types differ in their parametric features.

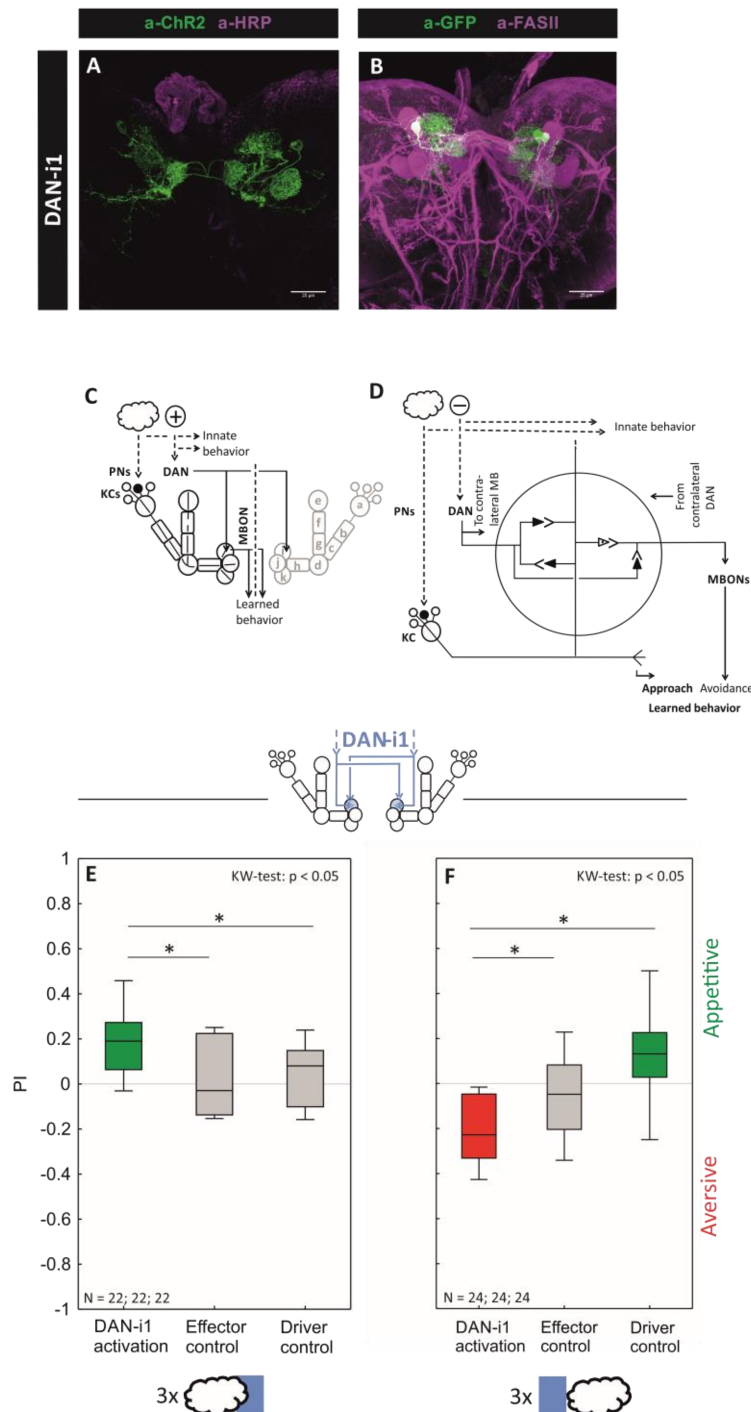

Figure 13: Characterization of the DAN-i1 driver strain. (A) Whole-mount larval brains were prepared from the offspring of the SS00864 driver strain covering DAN-i1 crossed to UAS-ChR2-XXL as the effector strain. Antibody staining with a primary mouse α-ChR2 antibody and a secondary Cy3 goat α-mouse antibody (green) visualizes the expression pattern against a reference background of an Alexa 488 α-HRP antibody staining (magenta). **(B)** In addition, whole mounts were

prepared for the driver strain crossed to pJFRC2-10xUAS-IVS-mCD8::GFP. The respective expression patterns were visualized with a primary rabbit α-GFP antibody and a secondary goat α-rabbit Alexa 488 antibody (green); to discern better the innervation of the respective DANs in the mushroom body a primary mouse α-FAS II antibody and a secondary CY3 goat α-mouse antibody were used. The stainings confirm earlier reports (Saumweber et al. 2018, Weiglein et al. 2019) where reliable and strong expression in the DAN-i1 neuron of both hemispheres with few additional cell bodies was revealed. Data were acquired under a confocal microscope with a 63x glycerol objective. No transgene expression was observed outside the field of view. Scale bars indicate 25 µm. **(C)** Schematic of the compartmental arrangement of the mushroom bodies. Letters a-k indicate compartment identity. As an example for the i-compartment, the mushroom body intrinsic Kenyon cells (KCs) coincidently detect signals from olfactory projection neurons (PNs) as well as intersecting teaching signals from dopaminergic neurons (DANs). This coincidence may lead to change in the connectivity of the subset of KCs in which the coincidence detection took place and the mushroom body output neurons (MBONs). The i-compartment gives rise to one MBON, MBON-i1. Both DAN-i1 and the MBON receive input only ipsilateral to their cell bodies, but provide output towards both hemispheres. **(D)** 'Canonical' compartmental connectivity, for the i-compartment as an example. Filled triangles represent presynapses, forked lines postsynapses. The triangle with a dot indicates experience-dependent depression of the respective presynapse. If the KC-MBON synapse of an avoidance-promoting MBON is depressed, the activity of approach-promoting MBONs from other compartments will prevail, leading to net learned approach. **(E)** Validation of appetitive reward memory as reported in Saumweber et al. (2018) upon forward conditioning at an ISI of -10 s. Larval offspring of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL as the effector strain underwent three training trials pairing the odor n-amylacetate with optogenetic activation of DAN-i1 by blue light, at the indicated inter-stimulus-interval (ISI). Negative ISIs mean that the odor preceded the light activation (forward), whereas positive values mean that light activation preceded the odor (backward). In all cases, reference groups of larvae received DAN-i1 activation unpaired from the odor. The performance index (PI), as a measure for associative memory, reflects the difference in odor preference after paired versus unpaired training. Positive PIs reflect appetitive memory, whereas negative PIs reflect aversive memory. The memory scores of the experimental genotype were compared to genetic controls heterozygous for only the effector, or only the driver, respectively. **(F)** Correspondingly, aversive frustration memory upon backward conditioning at an ISI of 30 s was detected, relative to genetic controls heterozygous for only the effector, or only the driver, respectively. Sample sizes are indicated within the figure. Data are displayed as box plots, with the median indicated by the middle line, the box boundaries indicating 25 and 75 % quantiles, and the whiskers 10 and 90 % quantiles. Red fill indicates aversive frustration memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$); green fill correspondingly indicates appetitive reward memory. Both in (E) and in (F) Kruskal-Wallis tests reveal significance across groups ($p < 0.05$); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p < 0.05$). The training procedure is indicated in sketches to the bottom of (E) and (F): blue bars indicate blue light for optogenetic activation of DAN-i1; white clouds indicate the odor n-amylacetate. The preference values underlying the PIs are documented in Figure S11.

Characterization of memories established by forward and backward conditioning

First, I was interested in whether, similar to the adult case (König et al. 2018) and as predicted by both learning theories (Solomon and Corbit 1974) and common sense, an increase in DAN-activation leads to stronger memory. Thus, groups of larvae were either trained normally, with 30 s of DAN activation, or with a threefold longer (90 s) or three times shorter (10 s) duration of DAN activation.

However, despite detecting significant forward and backward memory for all three durations of DAN-activation, a KW test across groups was not significant for forward conditioning **(Figure 14A)**; and although the duration of DAN activation indeed mattered for backward condition, memory after 90 s DAN activation was not significantly increased compared to the standardly used 30 s of DAN activation (MWU: p = 0.2) **(Figure 14B)**.

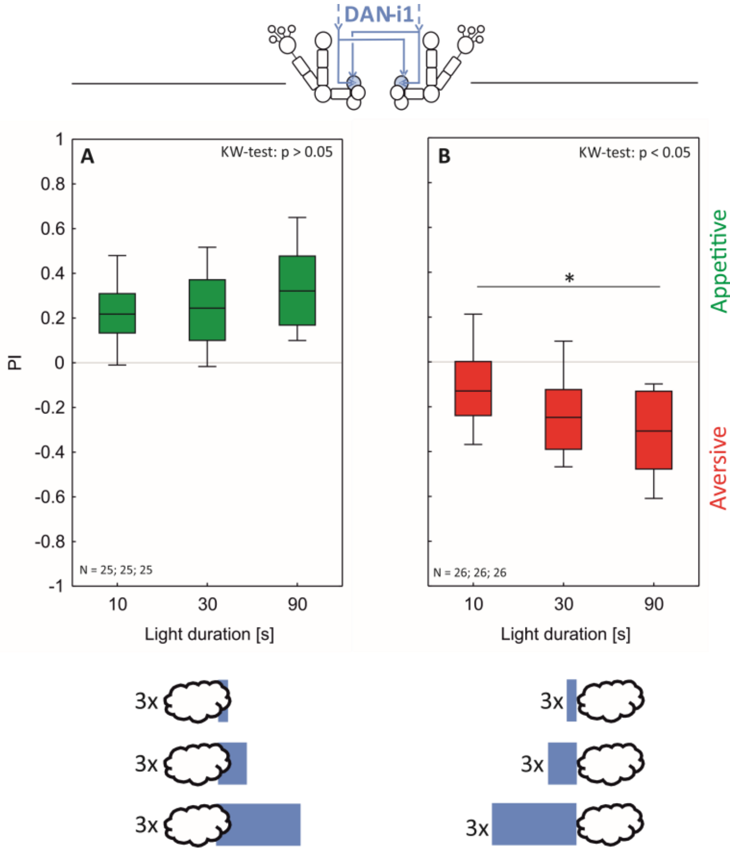

Figure 14. Tripled duration of DAN activation does not increase memory scores. Larval offspring of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-i1 by blue light as in Figure 13. **(A)** The duration of DAN activation during training was either 10, 30, or 90 s; this corresponds to activations of either a third of the duration, of the same duration, or of a duration prolonged threefold relative to those used in Figure 13. For forward ISIs of -10 s, i.e. for cases in which the timing of the onset of DAN-i1 activation relative to odor was maintained but the duration of this activation was varied, animals showed appetitive reward memory for all activation durations tested. **(D)** Similarly, for a backward ISI of 30 s strong aversive frustration memory was detectable across activation durations. Sample sizes are indicated within the figure. Red fill indicates aversive frustration memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests (p < 0.05); green fill correspondingly indicates appetitive reward memory. For (A) a Kruskal-Wallis test was not significance across groups (p > 0.05), whereas for (B) it revealed significance across groups (p < 0.05); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests (p < 0.05). The preference values underlying the PIs are documented in Figure S12. Other details as in the legend of Figure 13.

I next asked whether forward and backward memories brought about by DAN-i1 are also established under aggravated conditions, that is, after training without repetition. Along these lines, the opponent process theory by Solomon and Corbit (1974) predicts that the memory induced by backward conditioning (rather than the memory induced by forward conditioning) is weak after one-trial training and increases only with trial-repetitions. Indeed, the present findings matched this assumption, at least to the extent tested: Forward reward memory after one training trial could be confirmed **(Figure 15A)**, while backward frustration memory was not detectable **(Figure 15B)**. In addition, this result parallels recent findings from Weiglein et al. (2019), which reported that one-trial memory is detectable for most tastant rewards, as well as for activation of DAN-i1, whereas tastant punishments generally require more training trials.

For three-trial training, shortening the inter-trial duration, such that the trial duration was reduced by four min, did not reduce memory scores for forward or backward conditioning **(Figure S14)**. Further experiments are based on this shorter trial duration.

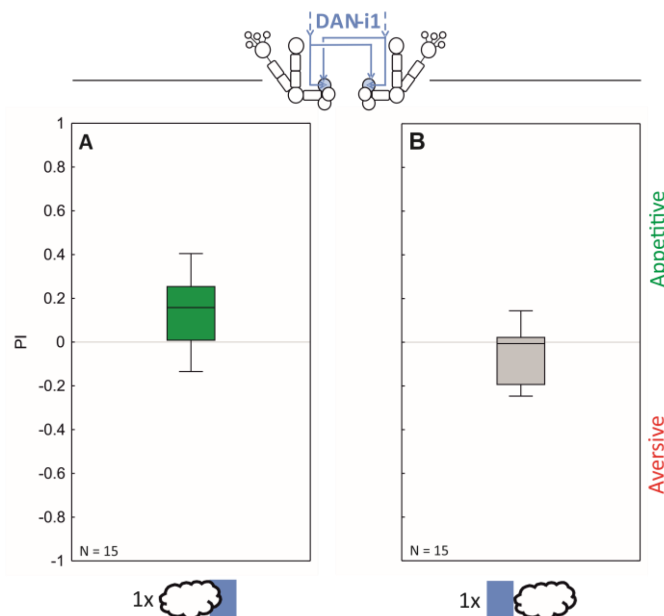

Figure 15: One-trial learning is detectable only after forward odor-DAN conditioning. Larval offspring of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-i1 by blue light as in Figure 13. However, larvae underwent only a single training trial. **(A)** Appetitive reward memory at a forward ISI of -10 s was revealed, **(B)** but no aversive frustration memory at a backward ISI of 30 s. Sample sizes are indicated within the figure. Green fill indicates appetitive reward memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$). The preference values underlying the PIs are documented in Figure S13. Other details as in the legend of Figure 13.

Another major feature of memories is their stability over time. Therefore, it was tested how long memories established through either forward or backward conditioning were still detectable after training. Animals were either tested for their odor preference directly after training (retention interval 0), or either 5, 10, 20 or 40 min after training. To avoid any interference with the memories, larvae spent the waiting time in a water droplet, covered from light. Remarkably, forward reward memory was detectable for up to 40 min **(Figure 16A)**, thus even longer than what was recently reported for fructose memory in larvae (Weiglein et al. 2019). Backward frustration memory, on the contrary, was only observed for up to 10 min after training **(Figure 16B)**.

Taking these results together, the two opposing memory types established by the same neuron, DAN-i1, differ in some respects, most notably in their stability over time. This might suggest distinct underlying molecular mechanisms for forward and backward memories, respectively.

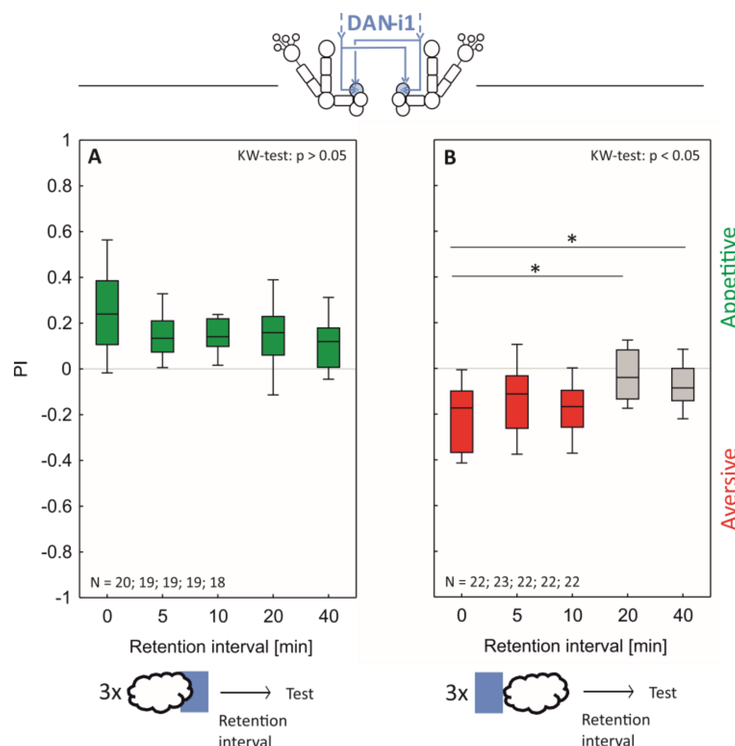

Figure 16. Forward and backward memories differ in their temporal stability. Larval offspring of the driver strain covering DAN-i1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-i1 by blue light as in Figure 13. **(A)** Animals were either tested for their odor preference immediately after training (retention interval 0), or were collected and left to wait in a water droplet for 5, 10, 20, or 40 min until that test was performed. Forward conditioning at an ISI of -10 s leads to appetitive reward memory that is detectable up to 40 min after training. **(B)** For the same retention intervals as in (A), backward conditioning at an ISI of 30 s leads to aversive frustration memory that is detectable only until 10 min after training. Note that training trial duration in these experiments was 8 min instead of previously used 12 min trial durations; see Figure S14. Sample sizes are indicated within the figure. Red fill indicates aversive frustration memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$); green fill correspondingly indicates appetitive reward memory. In (A) a Kruskal-Wallis test

was not significance across groups ($p > 0.05$), whereas for (B) it revealed significance across groups ($p < 0.05$); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p < 0.05$). The preference values underlying the PIs are documented in Figure S15. Other details as in the legend of Figure 13.

How are DAN-i1 mediated memories brought about?

I wondered whether the oppositely-valenced memories brought about by DAN-i1 differ in their locomotor 'footprint', that is, in the specific modulations of behavior they induce. To this end, larvae were video tracked during the test and data from the experiments shown in Figure 13C, D, Figure 14, and Figure S14 were analyzed **(Figure 17)**. Typically, larvae interrupt their relatively straight runs by lateral head movements (head casts, HCs) (Gomez-Marin et al. 2011, Gershow et al. 2012, Gomez-Marin and Louis 2014) that may be followed by turning maneuvers. Appetitive memories, such as after odor-taste reward conditioning, were found to have mainly two effects: For once, paired-trained larvae modulate their HC rate such that they perform more HCs while moving away from the odor and fewer HCs while moving towards it (quantified by a positive HC rate-modulation); and secondly, they direct their HCs more towards the odor (quantified by a more positive reorientation per HC) as compared to animals after unpaired training (Schleyer et al. 2015b, Paisios et al. 2017, Saumweber et al. 2018, Thane et al. 2019, Toshima et al. 2019). Aversive memories are usually characterized by the opposite effects on HC rate and direction (Paisios et al. 2017, Toshima et al. 2019). The run speed was so far not found to be changed through paired-training (Schleyer et al. 2015b, Paisios et al. 2017). It was therefore surprising that the data suggest a weak yet significant difference in run-speed modulation after forward conditioning, but not after backward conditioning **(Figure 17A)**. Paired-trained animals after forward training had a significantly higher HC rate-modulation score than unpaired-trained animals, whereas for backward conditioning it was the other way round **(Figure 17B)**. Similarly, reorientation per HC was significantly higher in paired-trained animals compared to unpaired-trained animals after forward conditioning and vice versa regarding backward conditioning **(Figure 17C)**. Thus, reward memory induced by DAN-i1 activation mostly display a locomotor 'footprint' comparable to taste-reward memories, whereas DAN-i1 frustration memory resembles the locomotor 'footprint' of taste-punishment memories.

Interim discussion

Timing-dependent valence reversal is mediated by some single DANs in Drosophila

The current study presents the first comprehensive parametric investigation of valence reversal in the appetitive domain in larval *Drosophila*. The present results confirm previous findings from Saumweber et al. (2018) which reported that DAN-i1 does not only function as an internal reward

signal but can also induce memories of opposite valence. Thus, if an odor is presented before DAN-activation it gains positive valence leading to reward memory. However, if the odor is presented after the DAN-activation it gains negative valence and larvae express frustration memory **(Figure 18)**. One pleasant event – activation of a rewarding DAN – can induce two types of effect: a cue that predicts the beginning of a pleasant event is approached and is positively remembered, whereas the same cue, after association with the termination of a pleasant event, is avoided and negatively remembered. Up to now, DAN-i1 is the first single DAN in the larva that was found to mediate such oppositely-valenced memories. Whether this ability is shared by DAN-h1, another DAN that can be of rewarding effect (Saumweber et al. 2018), is not known.

Paralleling our findings in the larva, also in adult flies timing-dependent valence reversal can be brought about by DAN-activation. Regarding the appetitive domain, valence reversal was so far only confirmed for a relatively broad set of PAM cluster DANs (Handler et al. 2019). For the aversive domain the PPL1-01 DAN was demonstrated using different optogenetic effectors (Aso and Rubin 2016, König et al. 2018) and less strong for PPL1-06 (König et al. 2018). Interestingly, other single DANs were found to establish only one memory type (Aso and Rubin 2016, König et al. 2018), suggesting heterogeneity in the teaching signal of DANs.

Mechanistic differences between oppositely-valenced memories

One and the same neuron can mediate two opposing memory types, however, these are very unlikely based on the very same underlying molecular pathway. Therefore, I was interested in differences between forward and backward memories, respectively. Indeed, forward reward memory was established after only one training trial, while no backward frustration memory was revealed. Most remarkably, however, was the difference in memory stability after forward and backward conditioning. While forward reward memory was stable for up to 40 min, backward frustration memory was decaying already 10 min after training. This finding should encourage searching for differences in the molecular mechanisms underlying these two memory types.

In adult flies, two hypotheses of how the differences between forward and backward memories could be explained, were recently emerging. On the one hand, König et al. (2018) found that only forward punishment memory mediated by the PPL1-01 DAN was dopamine dependent, as only forward but not backward memory was partially reduced by TH-RNAi within the PPL1-01 neuron. Up to now, however, no potential co-transmitter has been discovered which could account for the backward memory established by the same neuron. Recently Aso et al. (2019) reported that nitric oxide acts as a co-transmitter in DANs to diversify memory dynamics, but it was found to be dispensable for timing-depending valence reversal. Another co-transmitter, which should be present

according to connectomic studies that had identified two different types of vesicles (Takemura et al. 2017) has not yet been confirmed.

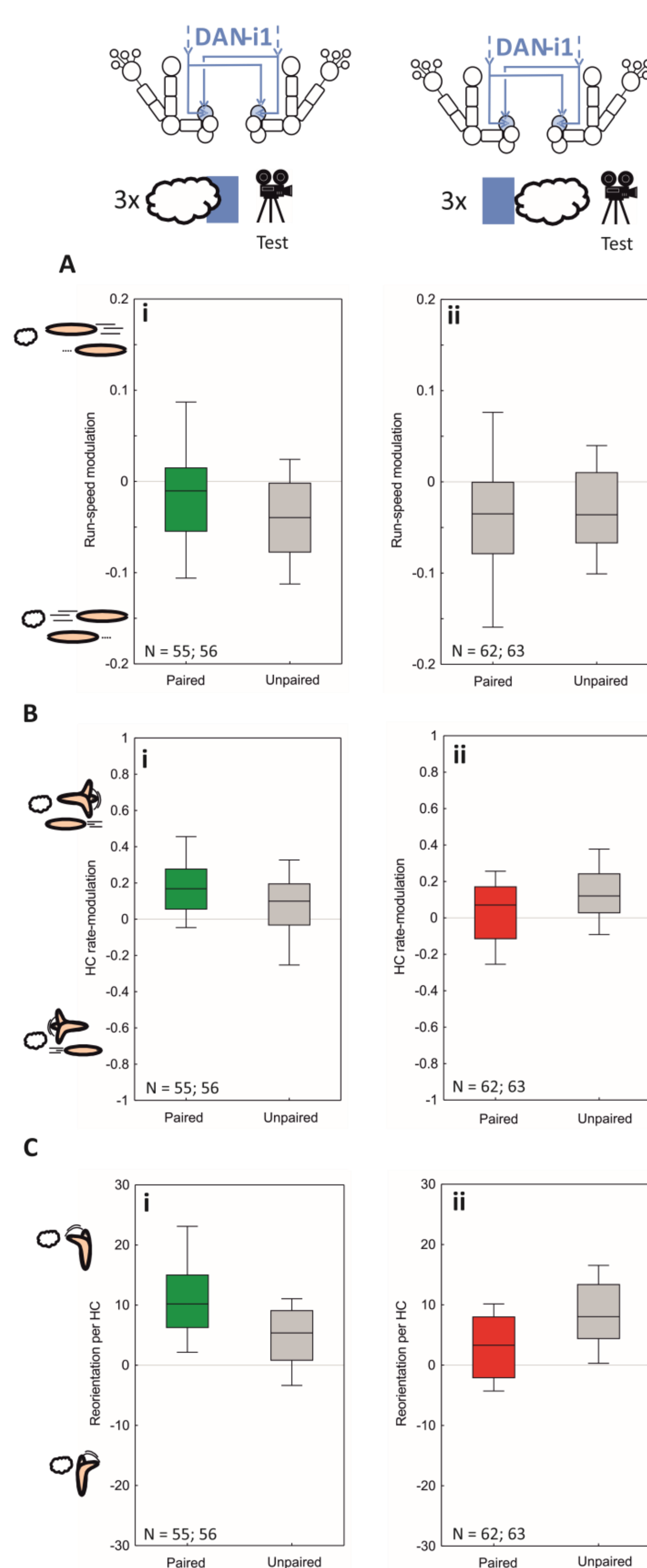

Figure 17. Locomotion footprint of DAN-i1 memories of different valences. Larvae were video-tracked for offline analyses of the modulations of locomotion after paired or unpaired training with odor and DAN activation. **(A)** Surprisingly, a weak yet significant difference in run-speed modulation could be observed between paired and unpaired trained animals for (i) DAN-i1 forward but (ii) not for DAN-i1 backward conditioning. However, paired-trained and unpaired-trained animals differed significantly regarding **(B)** the modulation of head cast (HC) rate in the case of both (i) DAN-i1 forward and (ii) DAN-i1 backward conditioning. **(C)** In addition, paired-trained and unpaired-trained animals for both (i) DAN-i1 forward and (ii) DAN-i1 backward conditioning showed a significant difference in the HC direction relative to the odor. Corresponding PI scores for can be found in Figures 13E, F, Figure 14, and Figure S14. Sketches of larvae depict their change in behavior with respect to the odor in the case of positive or negative scores. Sample sizes are indicated within the figure. Colored fill indicates significant Bonferroni-Holm corrected Mann-Whitney U-tests ($p < 0.05$) for cases reflecting aversive frustration memory (red) and appetitive reward memory (green). The paired training procedure is indicated in sketches toward the top of the figures; blue bars indicate blue light for optogenetic activation of DAN-i1, white clouds indicate the odor n-amylacetate.

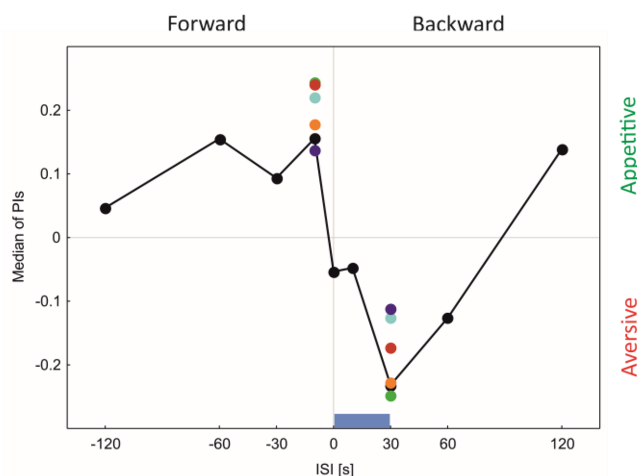

Figure 18. Temporal fingerprints of DAN-i1 teaching signals. For optogenetic activation of DAN-i1 (sketched at the top) the median PI scores obtained in this study are plotted against the timing of this activation in relation to odor presentation (the inter-stimulus-interval, ISI) (black from Saumweber et al. 2018; loc. cit. Fig. 6A; orange Figure 13 E, F; red Figure 16A, B; green Figure 14A, B, turquoise and blue Figure S14A, B, in all cases of the experimental genotype and the standard training procedure).

On the other hand, Handler et al. (2019) put forward a scenario different from the co-transmitter hypothesis. They revealed that two distinct dopamine receptor types, Dop1R1 and Dop1R2, are differentially involved in forward versus backward learning, using relatively broad driver strains for DAN activation. It remains yet to be investigated whether any one of the above described scenarios holds true for the *Drosophila* larva (Eichler et al. 2017).

Implications for addiction?

The present study highlights the importance of timing-dependent valence reversal in the *Drosophila* larva, however, corresponding findings in rodents and man suggest it to be an across-species principle (Gerber et al. 2014, Gerber et al. 2019). Drugs of addiction elicit similar responses as natural rewards (*Drosophila*: Ryvkin et al. 2018, man: Bickel et al. 2018, Solinas et al. 2019) and preocupate the corresponding reward signaling pathways in the brain (reviewed in Bickel et al. 2018, Solinas et al. 2019), including dopaminergic pathways (reviewed in Hyman et al. 2006, Grace et al. 2007). From a basic research perspective, the present results might prompt a consideration of dopaminergic mechanisms also in withdrawal and the avoidance behaviour that patients can go at great length to prevent them. In this sense, results from fly research might inspire a new understanding of the basis of neuropathologies and can be benefitting for studies in vertebrates and man.

Materials & Methods

Throughout all experiments, an established protocol for olfactory associative learning was used with optogenetic activation of a dopaminergic mushroom body neuron (DAN) as a reward (Saumweber et al. 2018). Since the timing between odor presentation (the CS) and DAN activation (the US) was varied, the protocol is referred to as the 'timed' protocol. Thus, the odor was either presented before the DAN-activation (forward conditioning; odor-DAN), or the odor came after the DAN-activation

(backward conditioning; DAN-odor). Along with each group of larvae was presented the odor paired with the DAN-activation (paired), a second group was trained unpaired (odor and DAN-activation separated).

Animals

Experiments were performed using 3rd instar feeding-stage larvae of *Drosophila melanogaster*. Animals were raised on standard food at 25 °C, 60-70 % relative humidity, in a 12 h light/dark cycle. For experiments, approximately 30 larvae were collected from the food vials, washed briefly in water and subsequently used in the respective experiments. In this study, the function of the driver strain SS00864-Gal4 was investigated. This driver strain reliably covers the DAN-i1 neuron, with additional stochastic expression in 1-2 further neurons (Saumweber et al. 2018; **Figure 13A, B**).

To optogenetically activate the specific set of neurons genetically modified larvae were used. Therefore, the animals of the effector strain UAS-ChR2-XXL were crossed to the DAN-i1 driver strain. As respective control, the driver strain was crossed to a local copy of w^{1118}. The effector control was obtained by crossing the UAS-CHR2-XXL strain to a strain carrying both landing sites used for the split-Gal4 (attP40/attP2), but without a Gal4 domain insertion. To prevent unintended activation of ChR2-XXL by daylight animals were kept in darkness. To this end, food vials were covered with black cardboard.

The timed protocol for associative learning

All 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). Directly 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 recording a Camera (Basler acA204090umNIR; Basler, Ahrensburg, 196 Germany) equipped with an infrared-pass filter was placed approximately 25 cm above the Petri dish. At the beginning of the experiment, a cohort of 30 larvae was placed in the middle of a Petri dish (9 cm inner diameter) filled with 1% agarose solution and covered with a perforated lid. Throughout the whole training, larvae stayed on the same Petri dish. In case of forward conditioning at an ISI of -10 s, the odor was presented after 3 min 50 s for 30 s by replacing the regular lid by a lid equipped with four odor-loaded sticky filter papers (n-amylacetate, AM; diluted 1:20 in paraffin oil). Odor presentation ended at 4 min 20 s by replacing the odor-loaded lid with a regular lid. At 4 min, DAN-i1 was optogenetically activated by blue light for 30 s, such that odor and DAN-activation were

overlapping for 20 s (AM+). After 8 min the regular lid was replaced for 30 s by a lid containing four sticky filter papers loaded with paraffin as 'empty control' (P). Paraffin has been shown to not have any behavioral significance as an odor (Saumweber et al. 2011). Three such paired training trials (AM+/P) were carried out, each lasting 12 min. After the third trial, larvae were transferred to a fresh test Petri dish which was closed by a lid equipped with sticky filters loaded with AM or P on opposite sides. A second group of larvae was trained reciprocally in an unpaired manner, such that the odor presentation was temporally seperated from DAN activation by blue light (P+/AM). Note that in half of the cases the tests were carried out with AM on the left, in the other half with AM on the right side of the lid to prevent potential bias of side choice. Furthermore, blue light activation started in half of the cases after 4 min as described, in the other half of the cases light was presented after 8 min **(Figure S10A)**. Correspondingly, for backward conditioning see **Figure S10B**. After the 3 min testing phase, the number of animals (#) on either side and in a 10-mm-wide middle zone was counted. Larvae on the Petri dish lid were excluded, whereas larvae crawling up the side-walls of the were counted for the respective side. A preference index (PREF) was calculated as detailed in *equation (1), Materials & Methods, (I)*, whith due adjustments (P instead of EM). With preference indices ranging from +1 to −1; positive values indicating preference for the odor and negative values indicating avoidance. From the PREF scores after paired and unpaired training, a performance index (PI) was calculated as detailed in *equation (2), Materials & Methods, (I)*, and depicts a measure for appetitive and aversive associative memory, respectively.
Variations of the paradigm are mentioned along with the results. Experimenters were blind to genotype.

Locomotor footprint of timing-dependent DAN-i1 mediated memories

Larval behavior was recorded throughout the test and further analyzed based on Paisios et al. (2017), focusing on three aspects of larval chemotaxis. Very generally, larvae were shown to perform relatively straight forward locomotion (runs), and lateral head movements (head casts; HCs). The latter are often followed by changes in direction. In the present analysis, an HC was detected whenever the angular velocity of a vector through the animal's head exceeded a threshold of 35 °/s and terminated when that angular velocity was falling below that threshold. Only HCs with an HC angle > 20° were taken into account (Schleyer et al. 2015b, Paisios et al. 2017, Thane et al. 2019). Whenever an animal was not doing HCs, this was regarded as a run. We omitted 1.5 s before and after each HC in order to exclude decelerating and accelerating phases before and after an HC, respectively. Run speed was defined as the average speed (mm/s) of the larval midpoint during runs. From this the modulation of run speed was calculated as:

$$Run\ speed\text{-}modulation\ = \frac{Run\ speed\ towards - Run\ speed\ away}{Run\ speed\ towards + Run\ speed\ away} \tag{5}$$

A negative Run speed-modulation indicates odor aversion, as it would mean that animals modify their run speed such that they speed up whenever they head away from the odor. Positive Run speed-modulation indicates odor attraction, as the animals would slow down whenever they head towards the odor. As the second aspect of chemotactic locomotion the modulation of HC rate (HCs per second, HC/s) was calculated as in *equation (3)*, *Materials & Methods, (I)*. Positive scores thus mean that larvae perform more head casts while moving away from the odor than while moving towards it, which would indicate odor attraction. By contrast, negative scores would indicate odor aversion. The third aspect investigated was the modulation of HC direction, as measured by the reorientation per HC, following *equation (4), Materials & Methods, (I)*. At absolute heading angles of 0° or 180° the odor would be to the front or rear of the larva, respectively. Positive scores mean that the head cast directs the larva towards the odor, indicating attraction, whereas negative scores indicate aversion. All the measures were compared between paired- and unpaired-trained animals to determine the impact of associative memory.

Immunohistochemistry

To confirm the expression pattern of the SS00864-Gal4 driver strain, whole mounts of larval brains of the same genotype as used for the optogenetic experiments were analyzed. Therefore, larval brains were dissected in Ca^{2+}-free saline in a microtiter plate on ice. In the next step, Bouin`s solution was added to gain a 1:3 Bouin's-Ca^{2+}-free saline mix. In this mix, brains were fixed for 7 min at room temperature (RT). Afterwards, the larval brains were washed three times consecutively in fresh washing solutions for 10 min each time, using 0.2 % PBT followed by incubaton with the primary monoclonal mouse anti-ChR2 antibody diluted 1:100 in 0.2 % PBT overnight at 4°C. To provide humidity, well plates were equipped with a wet paper stripe and covered in tinfoil. On the consecutive day, larval brains were washed three times every 10 min in 0.2% PBT. Afterwards brains were incubated for one hour at RT on a shaker with a secondary Cy3 goat anti-mouse (Art-Nr. 115-165-071, RRID: AB_2338687, Jackson Immuno Research, Pennsylvania, USA) and a secondary polyclonal Alexa Fluor 488 goat anti-horseradish peroxidase, both diluted 1:300 in 0.2 % PBT. Following three final washing steps with 0.2 % PBT once every 10 min, samples were mounted in Vectashield (H-1000-10, Vector Laboratories Inc., Burlingame, USA) on a cover slip.

In addition, we prepared larval brain whole mounts from crosses of the respective driver strain and the pJFRC2-10xUAS-IVS-mCD8::GFP effector strain (RRID: N/A; kindly provided by HHMI Janelia Research Campus, USA; Pfeiffer et al. 2010). This enables a more detailed visualization of the

mushroom body as reference for the DAN innervation. Larvae were dissected in Ca^{2+}-free saline and brains were collected in 15 µl Ca^{2+}-free saline in a microtiter plate on ice. Afterwards the brains were transferred into 4 % PFA (J19943, Alfa Aesar, Ward Hill, USA; in PBS) and fixed for 30 min on a shaker. Subsequently, brains were washed three times in 0.2 % PBT once every 10 min. They were overnight incubated with the primary antibody mixture, which consisted of 4 % normal goat serum (NGS; Art-Nr. 005-000-121, Jackson Immuno Research, Pennsylvania, USA), a primary polyclonal rabbit anti-GFP antibody (A-11122, RRID: AB_221569, Invitrogen, Carlsbad, USA), diluted 1:1000 and a primary monoclonal mouse anti-FAS II antibody (1D4 anti-Fasciclin II – DSHB, RRID: B_528235, DSHB, Iowa, USA), diluted 1:50 in 0.2 % PBT, at 4 °C on a shaker. On the following day, brains were washed 6 times, once every 10 min with 0.2% PBT and afterwards incubated for one hour at RT on a shaker with the secondary antibody mixture, which consisted of a secondary polyclonal Alexa 488 goat anti-rabbit antibody (A32731, RRID: AB_2633280, Thermo Fisher Scientific, Waltham, USA), diluted 1:200, and secondary polyclonal goat anti-mouse CY3 (Art-Nr. 115-165-071, RRID: AB_2338687, Dianova, Hamburg, Germany), diluted 1:200 in 0.2% PBT. After 6 further washing steps, once every 10 min, brains were mounted in Vectashield on a cover slip. Preparations were examined using a DM6000 CS confocal microscope (Leica, Jena, Germany) and analyzed with Fiji Image-J software. Note that plates were on a shaker while washing and incubating.

Statistics

Statistical analyses as detailed in *Statistics*, *Material & Methods, (I).*

III - Timing-dependent valence reversal in the aversive domain

Avoiding punishment can be a powerful goal of behavior. Accordingly, animals and humans alike are able to learn predictors of the occurrence of punishment, a process that has been studied in detail across species. It is less widely acknowledged, however, that learning can also take place from the termination of punishment. Indeed, delivering versus terminating punishment can induce affect of opposite valence. It feels bad to receive punishment but it feels good to be relieved from it (Solomon and Corbit 1974) **(Figure 19A)**, resulting in aversive and appetitive learning, respectively, of the associated cues. Such learning is observed in animals as well as humans and is referred to as timing-dependent valence reversal (reviewed in Gerber et al. 2014, Navratilova et al. 2015, Gerber et al. 2019). The same dichotomy applies for reward processing, with opposite sign (Hellstern et al. 1998). Timing-dependent valence reversal features prominently in many computational models of reinforcement learning (overview in Malaka 1999) and is arguably essential for adapting to the causal event-structure of the world. Here, I investigate timing-dependent valence reversal in the larvae of the fruit fly *Drosophila melanogaster* as mediated by two identified dopamine neurons recently found to confer teaching signals for associative learning in the aversive domain (Eschbach et al. 2020a).

Drosophila is a suitable model system for such an endeavor because it combines convenient experimental tractability by means of genetic manipulation, robust behavioral paradigms for associative learning, a high degree of similarity to humans at the molecular level, and a numerically simple brain. The learning of associations between odor and electric shock punishment has been studied in particular detail in adult flies (Heisenberg 2003, McGuire et al. 2005, Cognigni et al. 2018, Aso and Rubin 2020, Boto et al. 2020). In brief, this association process takes place in the KCs of the MB, a third-order brain structure in the insects providing a combinatorial, specific, and sparse representation of the environment, including odors. Along their elongated axonal fibers, the KCs also receive intersecting input from mostly dopaminergic neurons (DANs) that can be broadly classified as mediating modulatory teaching signals concerning either punishment or reward. The coincidence of the activation of DANs and the specific set of KCs representing the odor can lead to presynaptic plasticity at the KCs-to-MBON synapse. The MBONs can be broadly categorized as either approach- or avoidance promoting. DANs and MBONs overlap in a regionally confined way along the KC fibers, establishing a compartmental organization. Typically, punishment-DANs are matched up with approach-promoting MBONs, and reward-DANs with avoidance-promoting MBONs. Upon odor-shock coincidence, synaptic strength between the odor-activated KCs and approach-promoting MBONs is reduced, such that for a punished odor the balance between approach and avoidance is shifted in

favor of avoidance. A similar organization, in separate mushroom body compartments and their respective DANs and MBONs, underlies reward learning, and likely the learning about punishment and reward in larval Drosophila as well (Gerber and Stocker 2007, Thum and Gerber 2019) **(Figure 19)**.

Using the association of odor with electric shock punishment in adult *Drosophila*, timing-dependent valence reversal was reported by Tanimoto et al. (2004) and subsequently analyzed in some detail (Yarali et al. 2008, Yarali et al. 2009, Yarali and Gerber 2010, Diegelmann et al. 2013b, Niewalda et al. 2015, Appel et al. 2016; also see Vogt et al. 2015). Strikingly, timing-dependent valence reversal was also found for the optogenetic activation of the DAN known as PPL1-01 (Aso and Rubin 2016, König et al. 2018; for a broader set of DANs: Handler et al. 2019). Other DANs also confer aversive teaching signals but with different 'temporal fingerprints' and no – or at least no robust – timing-dependent valence reversal (Aso and Rubin 2016, König et al. 2018). Thus, I ask whether such qualitative differences among punishment DANs in the temporal fingerprint of their teaching signals are found in larval *Drosophila* as well, suggesting heterogeneity in DAN function as a general principle.

Larval *Drosophila* are an emerging study case for learning and memory, sharing the above-mentioned experimental advantages of adult flies – yet at about 10-fold lower cell numbers (reviewed in Gerber and Stocker 2007, Thum and Gerber 2018). Thanks to this numerical simplicity, a complete light microscopy atlas of its neurons and their chemical-synapse connectome is within reach (Li et al. 2014, Gerhard et al. 2017). In particular, all KCs and their pre- and postsynaptic partners have been reconstructed (Eichler et al. 2017, Saumweber et al. 2018; also see Selcho et al. 2009, Pauls et al. 2010b, Rohwedder et al. 2016). Likewise, all pre- and postsynaptic partners of the DANs innervating the mushroom body have been uncovered (Eschbach et al. 2020a). For a subset of these DANs, transgenic drivers for studying their individual behavioral function are available. It has turned out that optogenetic activation of either of two of them can confer a rewarding effect (DAN-i1, DAN-h1; Saumweber et al. 2018), whereas at least two other DANs can be punishing (DAN-f1, DAN-d1 and possibly also DAN-g1; Eschbach et al. 2020a) (regarding broader sets of neurons see Schroll et al. 2006, Rohwedder et al. 2016, Almeida-Carvalho et al. 2017, Eichler et al. 2017). In the appetitive domain, Saumweber et al. (2018) showed that the DAN-i1 teaching signal can confer timing-dependent valence reversal, whereas DAN-h1 has not yet been tested in this regard. Here, I focus on the aversive domain and ask whether the teaching signals from the respective DANs can establish timing-dependent valence reversal.

Results

DANs and drivers

At least two DANs have previously been reported to confer punishing effects: presenting an odor together with optogenetic activation of either DAN-f1 or DAN-d1, and possibly of DAN-g1, was found to establish odor avoidance in a subsequent test (Eschbach et al. 2020a). These neurons receive input from ascending pathways mediating aversive somatosensory cues and innervate the intermediate vertical lobe, the lateral appendix, and the lower vertical lobe compartments of the mushroom body, respectively. Within these compartments they host reciprocal synapses with the KCs, and connect to the compartments' cognate MBONs. Outside the mushroom body they receive ascending input from i.a. touch and pain sensory pathways, as well as feedback originating from the MBONs **(Figure 19B-D, Figure 20)**.

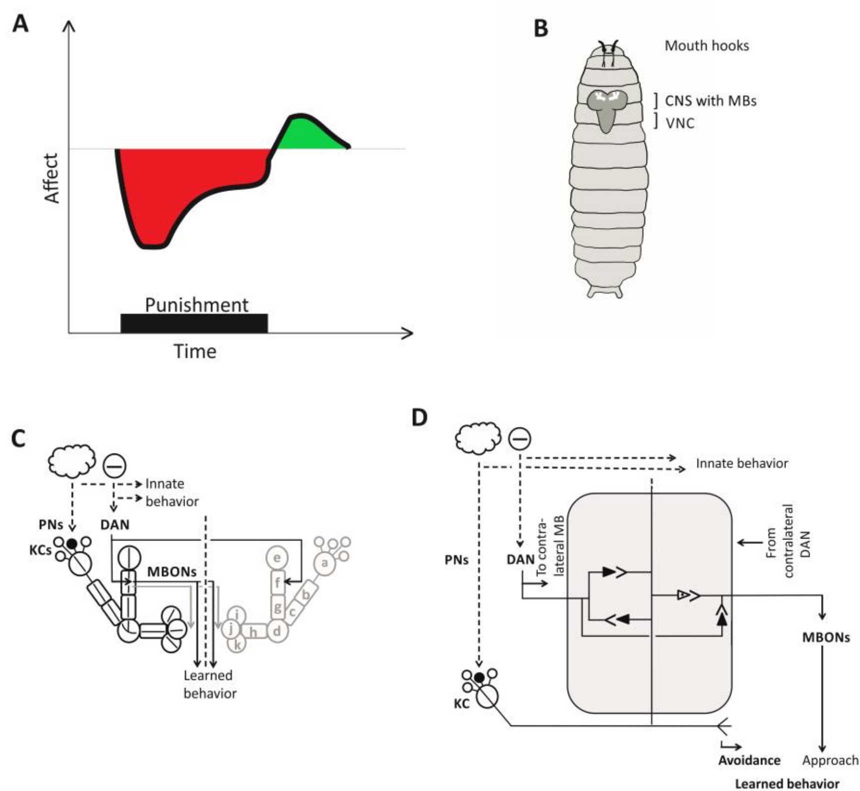

Figure 19. Topology and connectivity of the larval learning and memory center. (A) Schematic of the time course of affect upon receiving a negative stimulus such as punishment. Initially negative affect (red) dominates, followed upon termination by less intense positive affect (green) (after Solomon and Corbit 1974). **(B)** Sketch of a *Drosophila* 3rd instar larva showing its body, the mouth hooks, brain hemispheres and ventral nerve cord (VNC), and mushroom bodies (MBs, white). **(C)** Schematic of the compartmental arrangement of the mushroom bodies. Letters a-k indicate compartment identity. As shown for the f-compartment as an example, at the mushroom body intrinsic Kenyon cells (KCs) a coincidence of signals from olfactory projection neurons (PNs) and intersecting teaching signals from dopaminergic neurons (DANs) can

be detected. Such a coincidence can lead to a change in the connection from the subset of KCs in which the coincidence was detected onto the mushroom body output neurons (MBONs). The f-compartment gives rise to two MBONs, MBON-f1 and MBON-f2. Both DAN-f1 and these MBONs receive input only ipsilateral to their cell bodies, yet provide output towards both hemispheres. **(D)** 'Canonical' compartmental connectivity, for the f-compartment as an example. Filled triangles represent presynapses, forked lines postsynapses. The triangle with a dot indicates experience-dependent depression of the respective presynapse. If the KC-MBON synapse of an approach-promoting MBON is depressed, the activity of avoidance-promoting MBONs from other compartments will prevail, leading to net learned avoidance.

Before studying these neurons functionally, the expression pattern of the driver strains covering them was confirmed, reporting strong and reliable expression in DAN-f1, DAN-d1 and DAN-g1 from the respective drivers **(Figure 20A-D, Figure S16)**. Whereas for DAN-f1 and DAN-d1 these driver strains are also specific in expression, the driver covering DAN-g1 shows additional expression in the ventral nerve cord **(Figure S16)** (see also Eschbach et al. 2020a), prompting the restriction of the following functional analyses to DAN-f1 and DAN-d1.

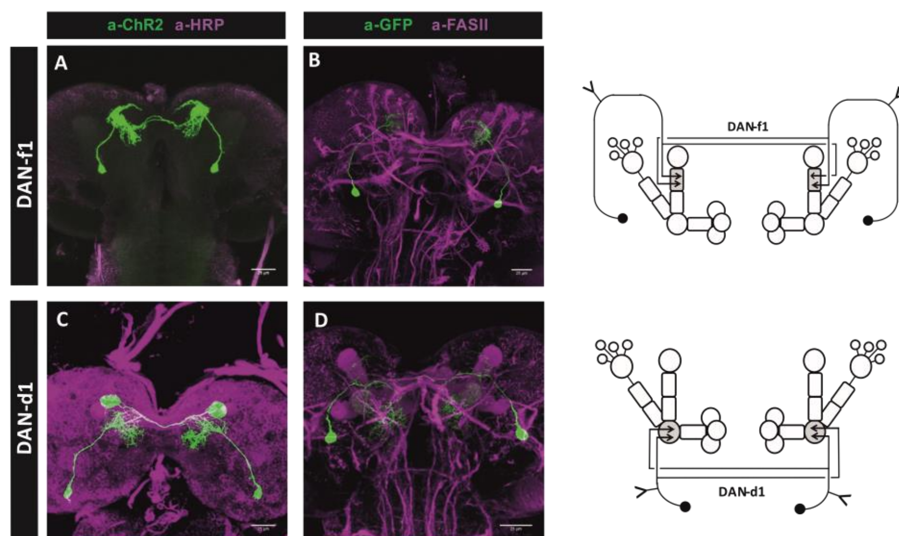

Figure 20. Characterization of driver strains covering DAN-f1 and DAN-d1. (A) Whole-mount larval brains were prepared from the offspring of the driver strain covering DAN-f1 (SS02180) crossed to UAS-ChR2-XXL as the effector strain. Antibody staining with a primary mouse α-ChR2 antibody and a secondary Cy3 goat α-mouse antibody (green) visualizes the expression pattern against a reference background of an Alexa 488 α-HRP antibody staining (magenta). **(B)** Additional whole mounts were prepared for the driver strain crossed to pJFRC2-10xUAS-IVS-mCD8::GFP, and expression patterns visualized with a primary rabbit α-GFP antibody and a secondary goat α-rabbit Alexa 488 antibody (green); to discern better the innervation of the respective DANs in the mushroom body a primary mouse α-FAS II antibody and a secondary CY3 goat α-mouse antibody were used. Data were acquired under a confocal microscope with a 63x glycerol objective. No transgene expression was observed outside the field of view. Scale bars indicate 25 µm. **(C-D)** Same as in (A-B), for the driver strain covering DAN-d1 (MB328b). To the right of the panels a schematic of DAN-f1 and DAN-d1 is shown, respectively.

Temporal fingerprint and parametric features of the DAN-f1 teaching signal

To characterize the teaching signal from DAN-f1 its temporal 'fingerprint' was determined. That is, ChR2-XXL was expressed in DAN-f1 and optogenetically activated it with blue light at specific times relative to odor application. Specifically, the time from the onset of the 30-s light pulse to the beginning of the 30-s odor application is defined as the inter-stimulus-interval (ISI). As per convention, negative ISIs indicate that the odor is presented first and is followed by DAN-f1 activation in training (forward conditioning, odor-DAN), whereas positive ISIs indicate by contrast that DAN-f1 activation comes first and is followed by the odor (backward conditioning, DAN-odor). In both cases, reference groups are presented with the odor unpaired from DAN-f1 activation; the performance index (PI), as a measure of associative memory, reflects the difference in odor preference after training at the respective ISI versus the odor preference in the reference group. Positive PIs therefore reflect appetitive memory, whereas negative PIs reflect aversive memory.

The present results show that the relative timing of odor application and DAN-f1 activation has a strong impact on memory scores, as indicated by a significant difference across groups **(Figure 21A)**. To see whether the aversive punishment memory after forward conditioning with an ISI of -10 s can be confirmed, I repeated the experiment including the appropriate genetic controls. Aversive memory was observed in the experimental genotype that expressed ChR2-XXL in DAN-f1, but not in the genetic controls heterozygous for only the ChR2-XXL effector, or only the DAN-f1 driver construct, respectively **(Figure 21B)**; indeed, memory scores in the experimental genotype differed from either of the genetic controls.

Relative to genetic controls, the trend for appetitive relief memory shown in Figure 21A was likewise verified for backward conditioning at an ISI of 60 s **(Figure 21C)**. In fact, appetitive relief memory was further confirmed in a replication of the experiment, as well as both for slightly shorter and for slightly longer backward ISIs **(Figure S18)**. Of note, it is expected in theory for aversive punishment memory to be stronger than appetitive relief memory (Solomon and Corbit 1974) and that for 'real' electric shock punishments this is indeed the case (Gerber et al. 2019).

Furthermore, we found that aversive punishment memory decayed over time, remaining detectable until 10 min after training, whereas appetitive relief memory, starting out somewhat less strong already, was undetectable from 5 min on **(Figure 22A, B)**. This difference in the temporal stability of these memories qualitatively matches what has been reported for adult Drosophila (using odors and electric shock: Diegelmann et al. 2013b). Notably, both punishment and relief memory require several training trials to be established: neither memory type was detectable after only one training trial **(Figure S20)**, consistent with previous findings indicating that associative learning about punishments generally warrants multiple-trial training in the larva (Weiglein et al. 2019). Interestingly, neither punishment memory nor relief memory increased by tripling the duration of

DAN-f1 activation during training **(Figure 22C, D)**. This suggests that it is the timing of the onset and the offset, respectively, of DAN-f1 activation that is the major determinant for the temporal fingerprint of the teaching signal. Next, I asked whether the teaching signal from activation of the DAN-d1 neuron, the second of the neurons under study, shares these features.

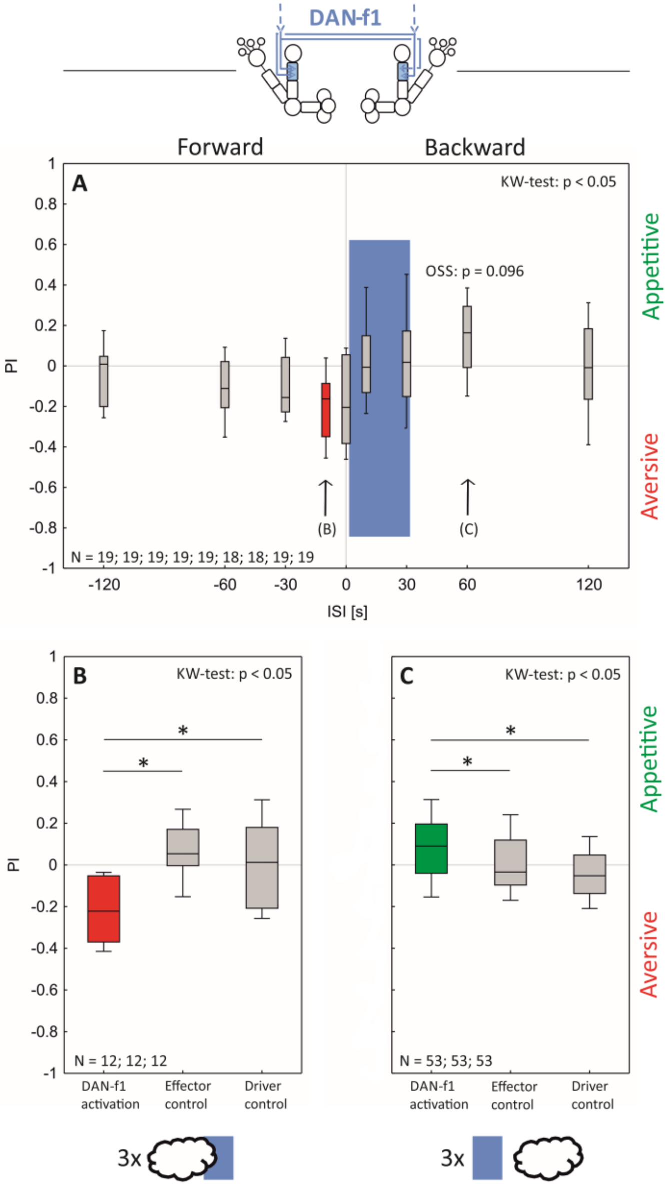

Figure 21. Temporal fingerprint of the DAN-f1 teaching signal. Larval offspring of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL as the effector strain underwent three training trials pairing the odor n-amylacetate with optogenetic activation of DAN-f1 by blue light, at the indicated inter-stimulus-interval (ISI). Negative ISIs mean that the odor preceded the light activation (forward), whereas positive values mean that light activation preceded the odor (backward). In all cases, reference groups of larvae received DAN-f1 activation unpaired from the odor. The performance

index (PI), as a measure for associative memory, reflects the difference in odor preference after paired versus unpaired training. Positive PIs reflect appetitive memory, whereas negative PIs reflect aversive memory. **(A)** The relative timing of the odor and the DAN-f1 activation had a significant impact on memory performance ($p < 0.05$ in a Kruskal-Wallis test). Forward conditioning resulted in aversive punishment memory (ISI = -10 s), whereas in this dataset only a tendency for appetitive relief memory upon backward conditioning (ISI = 60 s) was observed. **(B)** Validation of aversive punishment memory upon forward conditioning at an ISI of -10 s, in comparison to genetic controls heterozygous for only the effector, or only the driver, respectively. **(C)** Appetitive relief memory upon backward conditioning at an ISI of 60 s, relative to genetic controls heterozygous for only the effector, or only the driver, respectively. Sample sizes are indicated within the figure. Data are displayed as box plots, with the median indicated by the middle line, the box boundaries indicating 25 and 75 % quantiles, and the whiskers 10 and 90 % quantiles. Red fill indicates aversive punishment memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$); green fill correspondingly indicates appetitive relief memory. Both in (B) and in (C) Kruskal-Wallis tests reveal significance across groups ($p < 0.05$); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p < 0.05$). The training procedure is indicated in sketches to the bottom of (B) and (C): blue bars indicate blue light for optogenetic activation of DAN-f1; white clouds indicate the odor n-amylacetate. The preference values underlying the PIs are documented in Figure S17.

Temporal fingerprint and parametric features of the DAN-d1 teaching signal

For DAN-d1 too, the timing of its activation relative to odor application had an impact on memory scores, as indicated by a significant difference across groups **(Figure 23A)**. In this case, however, the results suggest a single peak of aversive punishment memory at an ISI of about -10 s. Indeed, punishment memory for an ISI of -10 s was confirmed in a repetition of the experiment including genetic controls **(Figure 23B)**. Although the initial results were not suggestive of any appetitive relief memory **(Figure 23A)**, I wondered whether relative to genetic controls, rather than relative to chance level (PI = 0) as in Figure 23A, relief memory might come to light. However, for an ISI of 30 s, which appeared to be the relatively most promising candidate based on the ISI curve **(Figure 23A)**, this was not the case **(Figure 23C)**.

The aversive punishment memory established through the DAN-d1 teaching signal was no longer detectable by 5 min after training **(Figure 24A)**. A comparison across retention intervals did not reach significance, probably due to a floor effect. Similar to DAN-f1, one training trial was not sufficient to establish a memory with the DAN-d1 teaching signal **(Figure S23)**. For the DAN-d1 teaching signal too, tripling the duration of activation did not increase the aversive punishment memory, suggesting that in the case of DAN-d1 it is also the onset of activation that is critical for an effective teaching signal **(Figure 24B)**. Notably, an increase in the duration of activation did not reveal appetitive relief memory through DAN-d1, either **(Figure 24C)**.

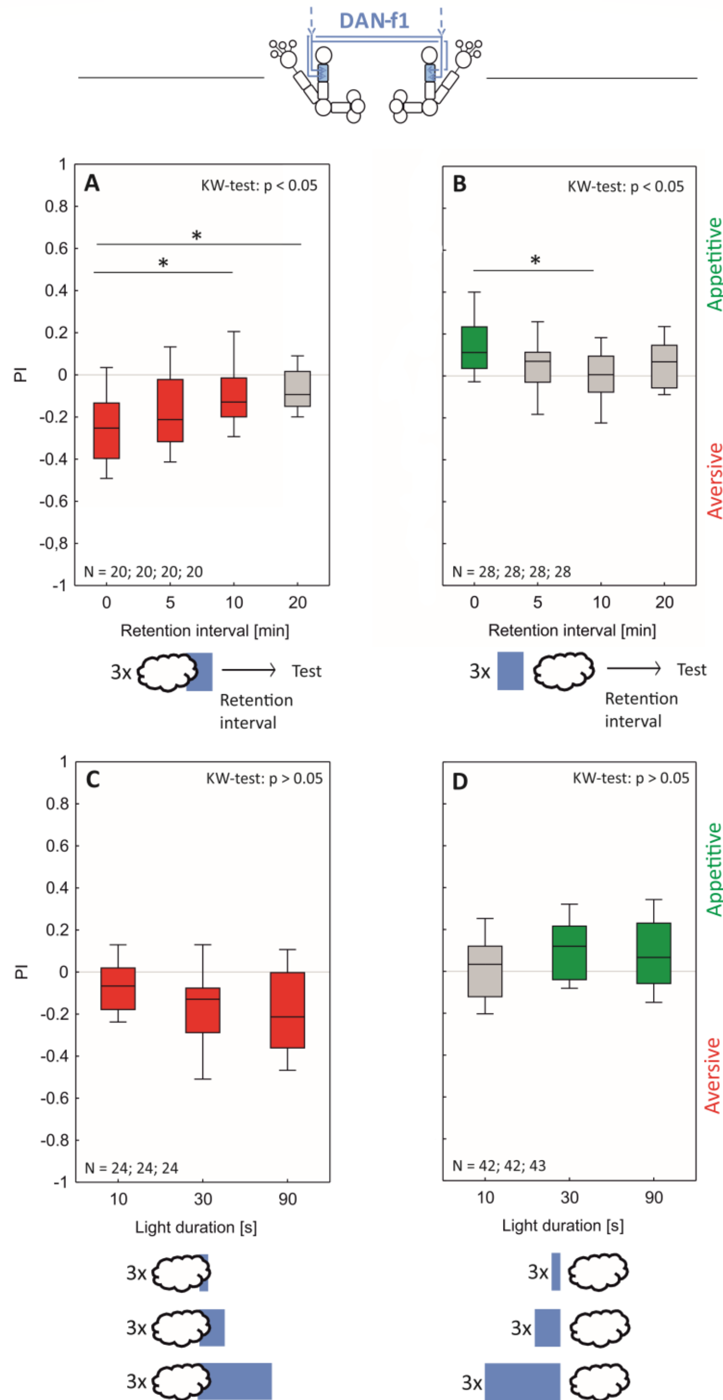

Figure 22. **Parametric features of the DAN-f1 teaching signal.** Larval offspring of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-f1 by blue light as in Figure 21. **(A)** Animals were either tested for their odor preference immediately after training (retention interval 0), or were collected and left to wait in a water droplet for 5, 10, or 20 min until that test was performed. Forward conditioning at an ISI of -10 s leads to aversive punishment memory that is detectable until at least 10 min after training. **(B)** For the same retention intervals as in (A), backward conditioning at an ISI of 60 s leads to appetitive relief memory that is detectable only immediately after training. **(C)** The duration of DAN-activation during training was either 10, 30, or 90 s; this corresponds to activations of either a third of the duration, of the same duration, or of a duration prolonged threefold relative to those used in Figure 21. For forward ISIs of -10 s, i.e. for cases in which the timing

of the onset of DAN-f1 activation relative to odor was maintained but the duration of this activation was varied, animals showed aversive punishment memory for all activation durations tested. **(D)** For a backward ISI of 60 s, i.e. for cases with a constant timing between the offset of DAN-f1 activation and odor, comparably strong appetitive relief memory was detectable across activation durations. Sample sizes are indicated within the figure. Red fill indicates aversive punishment memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$); green fill correspondingly indicates appetitive relief memory. Both in (A) and in (B) Kruskal-Wallis tests reveal significance across groups ($p < 0.05$), whereas this was not the case for (C) and (D); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p < 0.05$). The preference values underlying the PIs are documented in Figure S19. Other details as in the legend of Figure 21.

Specifically how do DAN-f1 and DAN-f1 memories affect behavior?

Given that forward conditioning with both DAN-f1 activation and DAN-d1 activation can establish punishment memories, I wondered whether these memories differ in how they specifically affect microbehavior. As recounted above, on a Petri dish surface, *Drosophila* larvae typically move in a zig-zagging way, alternating between periods of relatively straight runs and lateral movements that we call head casts (HCs) (Gomez-Marin et al. 2011, Gershow et al. 2012, Gomez-Marin and Louis 2014). After odor-taste punishment training, aversive memories have been shown not to affect run speed, but can be characterized by a decrease in the number of HCs when moving away from the odor versus when moving towards the odor (i.e. a decrease in HC rate-modulation), and a decreased propensity of HCs to align the larvae towards the odor (a decrease in the reorientation per HC) (Paisios et al. 2017). From offline analyses of video recordings of the combined experiments shown in Figures 21-24 and Figure S18, I observed the same to be the case for aversive punishment memories established by forward conditioning with either DAN-f1 activation **(Figure 25Ai, Bi, Ci)** or DAN-d1 activation **(Figure 25Aii, Bii, Cii)**. Of note is that appetitive memories are usually characterized by the opposite modulations of HC rate and direction (Schleyer et al. 2015b, Paisios et al. 2017, Thane et al. 2019). Regarding the (relatively weak) relief memory established through backward conditioning with DAN-f1, an increased propensity to align towards the odor, but no modulation of HC rate was detected **(Figure 25Aiii, Biii, Ciii)**.

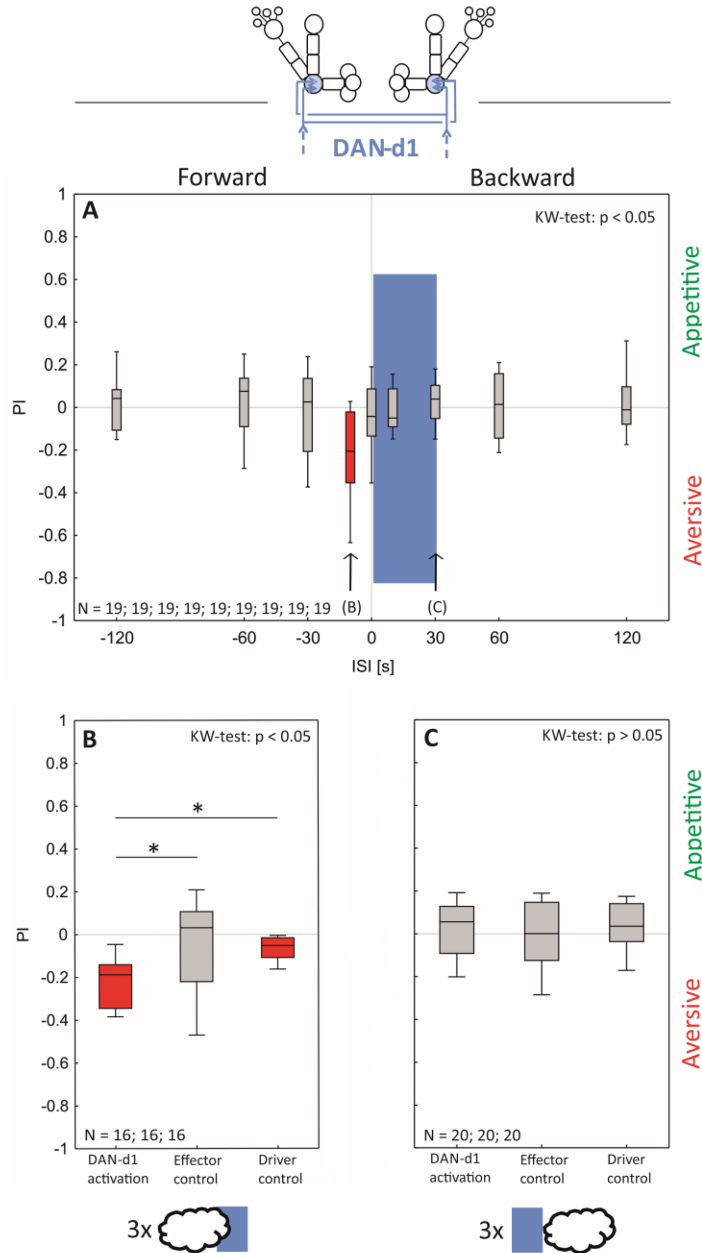

Figure 23. Temporal fingerprint of the DAN-d1 teaching signal. Larval offspring of the driver strain covering DAN-d1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-d1 by blue light as in Figure 21. **(A)** The relative timing of the odor and the DAN-d1 activation had a significant impact on memory performance ($p < 0.05$ in a Kruskal-Wallis test). Forward conditioning resulted in aversive punishment memory (ISI = -10 s). **(B)** Validation of aversive punishment memory upon forward conditioning at an ISI of -10 s, in comparison to genetic controls heterozygous for only the effector, or only the driver, respectively. (C) Also relative to genetic controls, no appetitive relief memory was observed upon backward conditioning at an ISI of 30 s, confirming the lack of any trend for such relief memory relative to chance level (PI = 0) in (A). Sample sizes are indicated within the figure. Red fill indicates aversive punishment memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-

sample sign tests ($p < 0.05$). In (B) a Kruskal-Wallis test reveals significance across groups ($p < 0.05$) whereas such a comparison was not significant in (C) ($p > 0.05$); * refers to Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p < 0.05$). The training procedure is indicated in sketches to the bottom of (B) and (C): blue bars indicate blue light for optogenetic activation of DAN-d1; white clouds indicate the odor n-amylacetate. The preference values underlying the PIs are documented in Figure S21. Other details as in Figure 21.

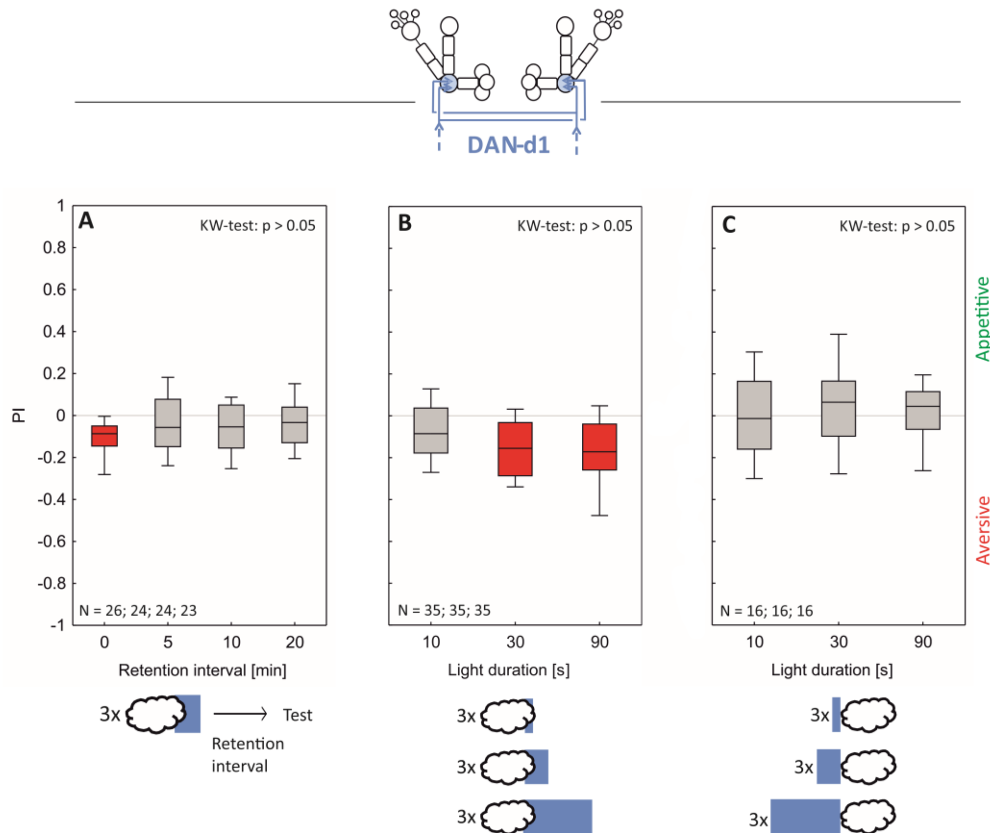

Figure 24. Parametric features of the DAN-d1 teaching signal. Larval offspring of the driver strain covering DAN-d1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-d1 by blue light as in Figure 21. **(A)** As in Figure 22A animals were either tested directly after the training (retention interval 0), or they were collected after the training, left to wait in a water droplet for either 5, 10, or 20 min, and only then tested for their preference. Forward conditioning at an ISI of -10 s led to punishment memory when animals were tested immediately after training, whereas no such aversive memory was observable for any other retention interval. **(B)** As in Figure 22C, the duration of activation during training was either 10, 30, or 90 s, and thus either a third of the duration, the same duration, or a duration prolonged threefold relative to those used in Figures 21 and 23. For forward ISIs of -10 s, i.e. for cases in which the timing of the onset of DAN-d1 activation relative to odor was maintained but the duration of activation was varied, there was no significant effect across activation durations. Testing each case against chance levels (PI = 0) suggests that animals showed aversive punishment memory for activation durations of 30 and 90 s but not for shorter durations. **(C)** For the backward ISI of 30 s, i.e. cases with a constant timing between the offset of DAN-d1 activation and odor, no appetitive relief memory was detectable, irrespective of activation duration. Sample sizes are indicated within the figure. Red fill indicates aversive punishment memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$). Neither in (A), (B), or (C) did Kruskal-Wallis tests reveal

significance across groups ($p < 0.05$). The preference values underlying the PIs are documented in Figure S22. Other details as in the legend of Figure 21.

Interim discussion

Heterogeneity in the temporal fingerprints of teaching signals in Drosophila *and its implications*

The present study reveals qualitative differences in the temporal fingerprint of teaching signals from two larval DANs in the aversive domain **(Figure 26A, B)**. Optogenetic activation of DAN-f1 can mediate both punishment memory upon forward conditioning with an odor and relief memory upon backward conditioning, and can thus establish timing-dependent valence reversal. In contrast, for DAN-d1 only punishment memory upon forward conditioning is observed, with a relatively narrow window of effective intervals compared to DAN-f1. Similar heterogeneity of teaching signals in the aversive domain has been reported in adult *Drosophila*, with timing-dependent valence reversal observed for PPL1-01 but not – or not robustly – for the other tested cases (Aso and Rubin 2016, König et al. 2018). Thus, in the aversive domain teaching signals from different DANs allow for more or less broadly defined coincidences with environmental cues to be established, with some DANs actually reflecting the relative temporal structure within the aversive event.

In the case of the larva a neuron that 'mirrors' the teaching signal of DAN-f1 in the appetitive domain has been found (Saumweber et al. 2018) **(Figure S24)**. Forward conditioning of an odor with DAN-i1 activation establishes learned odor approach (reward memory), whereas backward conditioning establishes odor avoidance ('frustration' memory). Whether this temporal fingerprint is shared by DAN-h1, the other DAN that can be of rewarding effect (Saumweber et al. 2018), is not known. In adults and regarding the appetitive domain, Aso and Rubin (2016) found relatively broad windows of coincidence for two sets of DANs from the PAM cluster (defined by the drivers MB213B and MB315C + MB109B), yet no timing-dependent valence reversal in either case. More recently, Handler et al. (2019) used a behavioral paradigm that allows training with more precise timing and revealed timing-dependent valence reversal in the appetitive domain for a relatively broad set of DANs from the PAM cluster (defined by the R58E02 driver).

In any event, in the case of the larva the present study together with Saumweber et al. (2018) suggests that the elegantly simple architecture of the single, identified DAN-f1 and DAN-i1 neurons mediates oppositely valenced teaching signals for the occurrence and the termination of aversive and appetitive events, respectively **(Figure S24)**. This is consistent with the scenario put forward by Handler et al. (2019) for adults, more broadly referring to DANs of the PPL cluster versus those of the PAM cluster (defined by the drivers R58E02 and 52H03, respectively), and to classical theoretical proposals of reinforcement learning (Malaka 1999). Indeed, such an organization of an association system should be versatile enough to decipher the causal structure within events (Dickinson 2001), in

particular regarding events of motivational significance. In contrast, DANs establishing coincidence – such as DAN-d1 with its notably narrower effective time window – may rather allow two coincident inputs to be bound together into one mnemonic object. In what he called 'an experiment into synthetic psychology', such a separation into event- and object-learning has been proposed by Braitenberg (1984).

Molecular mechanisms of timing-dependent valence reversal

The molecular mechanisms underlying timing-dependent valence reversal are beginning to be uncovered in adults. In an explant brain preparation and with respect to the appetitive domain, Handler et al. (2019) found that forward pairing of Kenyon cell activity and activation of the above-mentioned, relatively broad set of PAM neurons leads to a depression of the KC-to-MBON synapse in the γ4 compartment, whereas backward pairings lead to potentiation. These effects are abolished in Dop1R1 and Dop1R2 receptor mutants, respectively. Strikingly, the optima for coincidence detection in these two molecular pathways are slightly offset, such that cAMP signals mediated via the Dop1R1/Gαs/AC pathway peak for coincidence, whereas the Ca^{2+} signals mediated via the Dop1R2/Gαq/IP3 pathway peak for short backward intervals. At the behavioral level, using a high-temporal-resolution assay, neither mutant can follow repeated reversals of forward and backward conditioning; notably, the net effect of such repeated reversals in Dop1R1 mutants corresponds to backward conditioning (frustration memory), whereas in Dop1R2 mutants it corresponds to forward conditioning (reward memory). These findings suggest that the concerted action of the Dop1R1 and Dop1R2 pathways underlies timing-dependent valence reversal.

Regarding the association of odor and electric shock, both forward and backward conditioning are impaired upon a lack of the protein synapsin (Niewalda et al. 2015). Synapsin is an evolutionarily conserved presynaptic protein with a high number of phosphorylation sites, and consensus motifs for multiple kinases (reviewed in Diegelmann et al. 2013b; see also Niewalda et al. 2015, Kleber et al. 2015, Blanco-Redondo et al. 2019). Synapsin regulates the balance between reserve and readily-releasable synaptic vesicle pools, and hence synaptic efficacy, across species (Hilfiker et al. 1999, Benfenati 2011, Diegelmann et al. 2013b). This raises the possibility that molecular cascades originating from the Dop1R1 and Dop1R2 pathways are integrated on synapsin as a common effector.

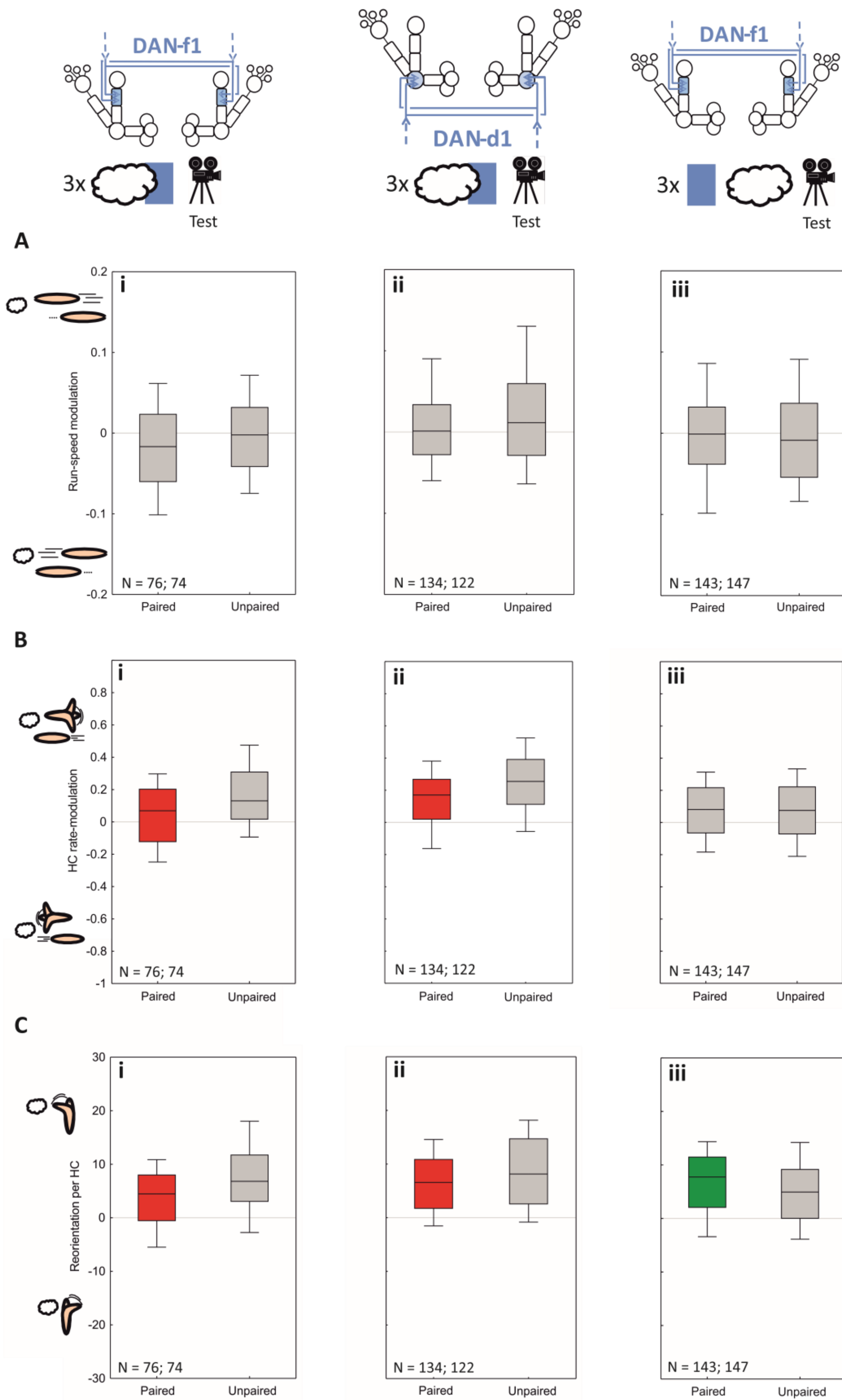

Figure 25. Specifically how do DAN-f1 and DAN-d1 memories affect behavior? Larvae were video-tracked for offline analyses of the modulations of locomotion after paired or unpaired training with odor and DAN activation. **(A)** No significant difference in run speed-modulation was observed between paired and unpaired trained animals for (i) DAN-f1 forward and (ii) DAN-d1 forward conditioning, nor for (iii) DAN-f1 backward conditioning. However, paired-trained and

unpaired-trained animals differed significantly regarding **(B)** the modulation of head cast (HC) rate in the case of both (i) DAN-f1 forward and (ii) DAN-d1 forward conditioning, such that after paired training larvae make fewer HCs while heading away from the odor source, and more HCs while heading towards it. (iii) For DAN-f1 backward conditioning no significant difference in the modulation of HC rate was observed. **(C)** In addition, paired-trained and unpaired-trained animals for both (i) DAN-f1 forward and (ii) DAN-d1 forward conditioning showed a significant difference in the HC direction relative to the odor, such that after paired training larvae direct their HCs more away from the odor source than after unpaired training. (iii) For backward conditioning with DAN-f1, the opposite was observed. Corresponding PI scores for (i) can be found in Figures 21 and 22; for (ii) in Figures 23 and 24; and for (iii) in Figures 21, 22 and S18. Sketches of larvae depict their change in behavior with respect to the odor in the case of positive or negative scores. Sample sizes are indicated within the figure. Colored fill indicates significant Bonferroni-Holm-corrected Mann-Whitney U-tests ($p < 0.05$) for cases reflecting aversive punishment memory (red) and appetitive relief memory (green). The paired training procedure is indicated in sketches to the top of the figures: blue bars indicate blue light for optogenetic activation of the respective DAN; white clouds indicate the odor *n*-amylacetate.

Interestingly, in parallel to the 'canonical' punishment memory component established via dopamine signaling from the PPL1-01 neuron during forward conditioning, nitric oxide signaling from this neuron supports an appetitively valenced memory component (Aso et al. 2019). Such nitric oxide signaling seems to be dispensable for relief memory after backward conditioning (Aso et al. 2019; loc. cit. Figure 5-S3). This would be consistent with the above scenario of timing-dependent valence reversal via the concerted action of the Dop1R1 and Dop1R2 pathways. However, König et al. (2018) found that an RNAi knock-down of the tyrosine hydroxylase (TH) enzyme in PPL1-01 impairs punishment memory through forward conditioning with this neuron, but not relief memory established by backward conditioning. This raises the possibility of a non-dopaminergic mechanism for relief memory formation, or at least a mechanism not affected by TH-RNAi in the PPL1-01 neuron. Of note, in order to account for the heterogeneity of teaching signals from DANs, the scenario of Dop1R1/R2 function conferring timing-dependent valence reversal would suggest a correspondingly heterogeneous expression of these two receptors across compartments, which to the best of my knowledge has not been observed.

In summary and with the above-mentioned caveats in mind, the best working hypothesis still seems to be that timing-dependent valence reversal by the activation of DANs in adult *Drosophila* comes about through the differential recruitment of Dop1R1 and Dop1R2 signaling. Whether this holds true for the larva, too, whether it applies for 'real world' reinforcers such as sugar or electric shock, whether such a scenario can explain the heterogeneity in the temporal fingerprint of teaching signals from dopaminergic neurons, and whether this reflects an across-species principle, remains to be determined.

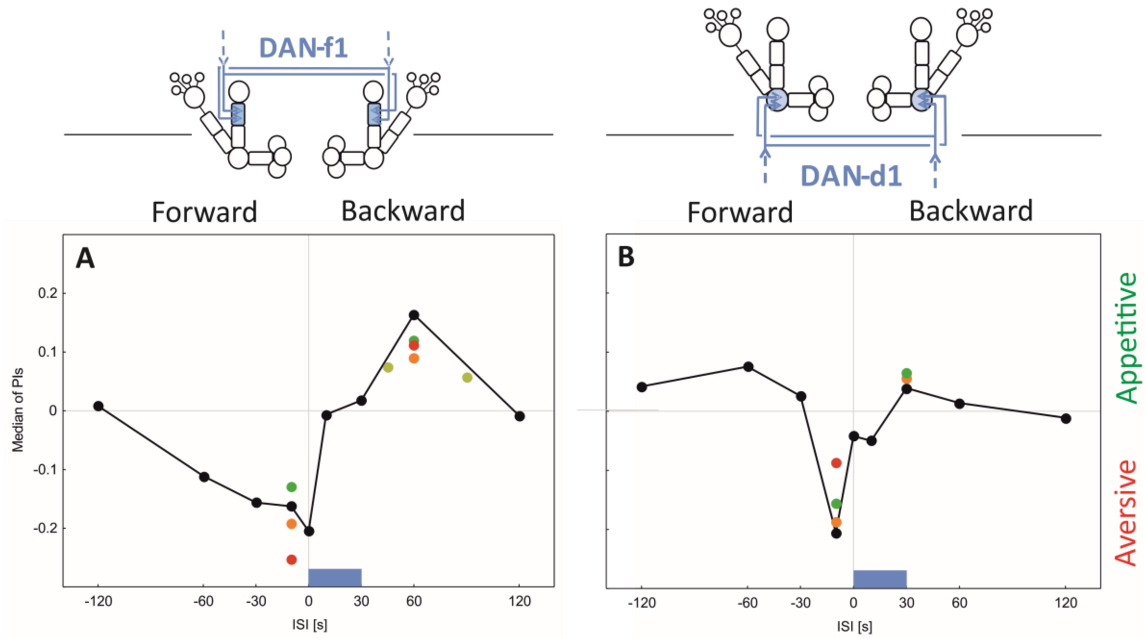

Figure 26. Temporal fingerprints of DAN-f1 and DAN-d1 teaching signals. (A) For optogenetic activation of DAN-f1 (sketched at the top) the median PI scores obtained in this study are plotted against the timing of this activation in relation to odor presentation (the inter-stimulus-interval, ISI) (black Figure 21A; orange Figure 21B, C; red Figure 22A, B; green Figure 22C, D; beige Figure S18A, in all cases of the experimental genotype and the standard training procedure). **(B)** As in (A), for DAN-d1 (black Figure 23A; orange Figure 23B, C; red Figure 24A; green Figure 24B, C).

Materials & Methods

This study uses an established protocol for olfactory associative learning with teaching signals from the optogenetic activation of individual DANs instead of a real reward or punishment (Saumweber et al. 2018). In brief, one group of larvae receives an odor together with the optogenetic activation of a DAN (paired) whereas a second group receives the odor separate from DAN activation (unpaired). Since odor presentation and DAN activation are relatively short, and because in the paired condition the relative timing of odor and DAN activation is the key experimental variable throughout this study, the present protocol is called 'timed' protocol. Specifically, in the paired cases the odor is either presented before DAN activation (forward conditioning: odor-DAN), or after DAN activation (backward conditioning: DAN-odor) at the intervals specified below. In all cases, a final test determines the level of odor preference in paired-trained versus unpaired-trained larvae.

Of note, in Pavlovian terminology, the odor is the conditioned stimulus (CS), DAN activation the unconditioned stimulus (US), and the difference in odor preference between paired-trained versus unpaired-trained larvae our measure of the conditioned response (CR).

Animals

We used 5-day-old, 3rd instar, feeding-stage larvae throughout the experiments. Animals were raised on standard food and maintained at 25 °C, 60-70 % relative humidity and a 12/12 h light/dark cycle. Cohorts of approximately 30 larvae were collected from the food vials, rinsed in water, collected in a water droplet and subsequently used in the respective experiment. In order to investigate the effect of DAN activation, we crossed animals of the effector strain UAS-ChR2-XXL to one of the following Gal4 driver strains, namely SS02180-Gal4, reliably covering the DAN-f1 neuron, or MB328b-Gal4, reliably covering the DAN-d1 neuron, as well as SS01716-Gal4, reliably covering the DAN-g1 neuron (RRIDs: N/A; all driver strains kindly provided by HHMI Janelia Research Campus, USA). In the offspring of these crosses, the respective DAN can be activated by blue light. All three DANs have previously been shown to mediate punishment (Eschbach et al. 2020a). The expression pattern of all driver strains used in this study was confirmed by immunohistochemistry, using either the same UAS-ChR2-XXL effector strain, or pJFRC2-10xUAS-IVS-mCD8::GFP (see section *Immunohistochemistry*). As the driver control, the respective driver strain was crossed to our local copy of w^{1118}. We obtained the effector control by crossing the UAS-ChR2-XXL strain to flies carrying both landing sites used for the split-GAL4 (attP40/attP2) but lacking an inserted GAL4 domain. To prevent ChR2-XXL from being activated by daylight, the flies were raised in food vials wrapped in black cardboard.

The timed protocol for associative learning – short trial duration

Optogenetic experiments were performed inside a custom-made box, equipped as described in *Material & Methods, (II).* Following the short version of the timed protocol **(Figure S14)**, one training trial lasted 8 min, during which time the larvae stayed on the same Petri dish and the Petri dish lid alone was exchanged either with a lid equipped with four odor-loaded sticky filter papers (*n*-amylacetate; diluted 1:20 in paraffin oil) or a 'mock control', with four sticky filter papers loaded with paraffin. Paraffin has been shown not to have behavioral significance as an odor (Saumweber et al. 2011). Three training trials were performed, unless mentioned otherwise. Larvae that crawled onto the lid during training were excluded from the experiments.

Following established protocols (Saumweber et al. 2011, Michels et al. 2017, Saumweber et al. 2018), the larvae were either trained to associate the odor with the optogenetic activation of the respective DAN (paired), or they received odor and DAN activation separately (unpaired). Both odor presentation and DAN activation lasted 30 s, unless mentioned otherwise. Critically, in the paired case the larvae received odor presentation and DAN activation at different relative timings (the inter-stimulus-interval, ISI, defined as the time interval from the onset of odor presentation to the onset of DAN activation). As an example of paired training for an ISI of -10 s, the animals were placed on a

Petri dish and after 1 min 50 s they were presented with the odor for 30 s **(Figure S25A, top)**. DAN activation started at minute 2 by turning on the blue light, i.e. 10 s after the onset of the odor (ISI -10 s), and lasted for 30 s, too. After the end of the DAN activation an additional 3.5 min were allowed to pass, before at minute 6 a 30-s presentation of paraffin as the odor-solvent followed to equate handling with the unpaired group. Then the larvae were left untreated until minute 8, when the clock was reset and the next training trial was started. Of note is that the sequence of events during the training trials, i.e. presentation of paraffin or of odor with DAN activation, was reversed in half of the cases. For each group paired-trained with a given ISI, an unpaired group was run. In this case odor was presented after 2 min and paraffin as the solvent after 5:50 min with DAN activation starting after 6 min **(Figure S25A, bottom)**. Again, the sequence of these events was reversed in half of the cases.

After such training, the larvae were transferred to a test Petri dish, also filled with 1 % agarose. The testing lid was equipped with two filter papers on opposite sides; one was loaded with the odor, the other with paraffin. The test was carried out in the presence of the blue light; this was done because punishment-related learned behavior is a form of learned escape which is facilitated under aversive conditions (Gerber and Hendel 2006, Schleyer et al. 2011). After 3 min, the number of larvae (#) on the odor side, on the paraffin side, and in a 10-mm-wide middle zone was counted. Larvae on the lid were excluded from the analysis, whereas larvae crawling up the side-walls of the Petri dish were counted for the respective side. A preference index (PREF) was calculated as follows, separately for the paired-trained and the unpaired-trained animals as detailed in *equation (1), Materials & Methods, (I)*, whith due adjustments (P instead of EM). Preference indices may range from +1 to −1, with positive values indicating preference and negative values indicating avoidance of the odor. From the PREF scores after paired and unpaired training, a performance index (PI) was calculated as in *equation (2), Materials & Methods, Chapter (I).* Performance indices may also range from +1 to -1. Positive PIs indicate appetitive associative memory, whereas negative values indicate aversive associative memory.

In cases of genetic controls being trained and tested along with the experimental genotype, vials were coded and the experimenters were thus blind to genotype.

Microbehavioral effects of associative memories

The behavior of larvae during the test situation was video-tracked and analyzed as described in detail in Paisios et al. (2017). In general, larvae alternately perform relatively straight forward locomotion, called runs, and lateral head movements, called head casts (HC), which are often followed by changes in direction. This leads to the typical zig-zagging pattern of the locomotion of larvae on a Petri dish surface (Gomez-Marin et al. 2011, Gershow et al. 2012, Gomez-Marin and Louis 2014).

Here, 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 that threshold again. If the angular velocity of a vector through the animal's tail at the same time exceeded a threshold of 45 °/s (a somewhat 'funny' walk, happening very rarely), this event was not counted as an HC. In accordance with previous studies, only HCs with an HC angle > 20° were taken into account (Schleyer et al. 2015b, Paisios et al. 2017, Thane et al. 2019). The time when an animal was not head-casting was regarded as a run, omitting 1.5 seconds before and after an HC to exclude the decelerating and accelerating phases that usually happen before and after an HC, respectively.

Three aspects of these behaviors were analyzed. The first refers to the run speed, i.e. to the average speed (mm/s) of the larval midpoint during runs. The modulation of run speed was calculated following *equation (5), Material & Methods, (II)*. Thus, if animals modified their run speed such that they speeded up whenever they headed away from the odor and slowed down whenever they headed towards an odor, we would obtain a negative Run speed-modulation, indicating odor aversion. To judge the impact of associative memory on run speed, these measures were compared between paired-trained and unpaired-trained animals.

The second aspect of chemotactic locomotion refers to the rate of HCs (HCs per second, HC/s). The modulation of HC rate was calculated as in *equation (3), Materials & Methods, (I)*. Positive scores thus mean that larvae perform more head casts while moving away from the odor than while moving towards it, which would indicate odor attraction. By contrast, negative scores would indicate odor aversion. Again, to judge the impact of associative memory on HCs, these measures were compared between paired-trained and unpaired-trained animals.

The third aspect investigated was the modulation of HC direction, which is measured by the reorientation per HC as detailed in *equation (4), Materials & Methods, (I)*. The heading angle indicates how the head of the larva is oriented relative to the odor. Thus, at absolute heading angles of 0° or 180° the odor would be to the front or rear of the larva, respectively. Positive scores occur when the head cast directs the larva towards the odor, indicating attraction. Again, negative scores indicate aversion, and comparisons between paired- and unpaired-trained animals were used to determine the impact of associative memory.

Immunohistochemistry

Whole mounts of larval brains of the respective experimental genotype were prepared following the protocol detailed in *Immunohistochemistry, Materials and Methods, (II).*

Statistics

Statistical analyses as detailed in *Statistics*, *Material & Methods, (I).*

General discussion

Associative learning enables animals to increase predictability, while reducing uncertainty. However, associative learning warrants relatively complex cognitive operations. What are the neural mechanisms within the brain underlying a given, properly predictive behavioral output? Thanks to decades of work of numerous neurobiologists across disciplines we have a basic understanding of brain structure, neuronal signaling as well as the homology or analogy across model organisms from different phyla (e.g. arthropods versus chordates). However, we are just beginning to grasp the interplay of different subsets of neurons, their respective transmitter, receptors and downstream signaling cascades. The *Drosophila* larva possesses a brain that is numerically and genetically tractable, such that its reward system can be investigated at single cell level. The present study delivers novel insights into reinforcement learning, such as lean one-trial learning. It further describes the role of individual dopaminergic mushroom body input neurons of distinct polarity for reinforcement learning. Specifically, the capability of DANs to bring about oppositely-valenced memories dependent on the event-timing is under investigation. Indeed, this phenomenon, known as timing-dependent valence reversal might depict an across species principle.

In **(I)** the basic memory faculties of little *Drosophila* 'maggots' have been characterized, namely their capability of one-trial learning, that is learning without repetition. To this end, larvae were trained to form an association between various kinds of commonly used taste rewards or punishments and an odor within one training trial. Interestingly, most rewards established appetitive memory, while aversive memory brought about by punishing tastants was only detectable for long training trials and higher sample sizes. This result underpins the importance of food rewards, especially sugars, for the larva, the feeding stage of the *Drosophila* fly. Punishments might only induce associative one-trial learning if the exposure is long enough, or if the larva's bodily integrity is threatened. Further parametric investigations on one-trial fructose learning revealed that more-trial training causes significantly higher memory scores. However, both memories established by one-trial and more-trial training decayed almost immediately, suggesting that rather protein synthesis independent short-term memory is supported by this kind of training regimen. The insights from this study are helpful for the design of larval associative learning experiments, e.g. to save experimental time and resources; moreover, they contribute to the general understanding of reward versus punishment learning. Based on these findings corresponding experiments considering memory strength and stability were implemented in experiments for the follow-up investigations of DAN function.

II portrays memories established by optogenetic activation of the rewarding DAN-i1 paired with an odor. Animals were trained in a timed protocol, that is, either odor was presented <u>before</u> DAN

activation (forward conditioning, odor-DAN), or after the DAN activation (backward conditioning, DAN-odor). Different inter-stimulus-intervals (ISIs), which are defined as the time between the onset of DAN activation and the onset of odor presentation, had previously been investigated in some detail (Saumweber et al. 2018). Of note, DAN-i1 establishes two opposing types of memory, dependent on the event-timing, reward versus frustration memory, respectively. This study confirmed the two memory types and characterized their properties. In line with the results regarding one-trial learning in (I), one-trial DAN memory was only established after appetitive forward conditioning. While tripling the duration of DAN activation did neither increase forward or backward memory scores, interestingly, memory after forward conditioning was stable for up to 40 min. Thus, forward odor-DAN memory was not only drastically longer stable than odor-fructose memory, as described in (I), but also compared to backward DAN-odor memory, which was detectable only up to 10 min after training. These differences in forward versus backward memories prompt the question whether the two opposing memory types are established via the same molecular pathway and indeed depend on dopamine signaling only.

Given these results, I was interested to see in **(III),** whether valence reversal is a common principle of DANs across valence domains and if so, whether also the properties of forward versus backward memories would be comparable. Two out of three DANs that had previously been described to signal punishment (Eschbach et al. 2020a) were under investigation. Notably, a timed function for DAN-f1 was described. Specifically, DAN-f1 mediated punishment memory upon forward conditioning and relief memory upon backward conditioning. Thus, similar to DAN-i1, two opposing types of memory were formed with regard to the event-timing. Similar as in (II) for DAN-i1, tripling the duration of DAN-f1 activation did not increase memory scores. Contrastingly, learning theories predict that relief memory scores ought to be increased after a longer painful period (Solomon and Corbit 1974). As the design of the timed protocol did not allow for more than tripling the duration of DAN activation, it was not possible to investigate even longer durations without major changes of the protocol. Thus, it remains unclear whether indeed relief memory scores are independent of the duration of preceding painful stimulus, or whether this was just not detectable within the range of durations of our experiments. Paralleling the findings in (II), forward DAN-f1 memory was stable for at least up to 10 min, while backward DAN-f1 memory was only detectable directly after training. According to the results, features of forward versus backward memories indeed seem to be comparable across valence domains. Forward memories refer to all memory types established through odor-DAN conditioning (appetitive domain: reward; aversive domain: punishment), whereas backward memories refer to all memory types established through DAN-odor conditioning (appetitive domain: frustration; aversive domain: relief; see **Figure 3**). This hints to a different underlying molecular mechanism for forward versus backward memories. Interestingly, valence reversal was not revealed

for the second candidate, DAN-d1, under investigation. Upon forward conditioning, a sharp time window for the occurance of punishment memory was observed. Hence, some but not all DANs share the principle of valence reversal. This heterogeneity is indeed interesting in itself and promts the question of underlying molecular differences between DANs 'with' and DANs 'without' valence reversal.

In general, I am interested in very basic principles of reinforcement learning, which I followed up upon in different ways as detailed in the present work. One-trial learning as well as timing-dependent valence reversal for both the appetitive and aversive domain depict across-species principles. Thus, the results from this study do not only contribute to the general understanding of reward and punishment processing in the insect mushroom body but novel insights into shared principles are also of translational value. This is especially interesting with respect to neuropathologies that have a perturbation of the dopamine system in common. Understanding the neuronal mechanism of the across-species principle of timing-dependent valence reversal could provide valuable information for novel pharmacological or therapeutic treatments.

In the following, the circuit principles, especially concerning DAN function, which are known to contribute to memory formation in *Drosophila* are being discussed. Further on, the findings of the present study regarding different levels of heterogeneity in DAN signaling are considered in the context of the learning and memory circuitry. Possible underlying neural mechanisms for timing-dependent valence reversel are proposed and its relevance as an across-species principle is highlighted in comparison to recent data in vertebrates. Clinical implications of valence reversal are indicated.

A mechanism for memory formation in *Drosophila*

For decades researcher have been grappling with the question how memories are established and where they are represented within the brain. Although it was known for quite some time that olfactory associative learning takes place in the MB of insects (de Belle and Heisenberg 1984, Heisenberg et al. 1985, Dubnau et al. 2001, Heisenberg 2003), only recent studies revealed that the memory engrams are stored as sparse DAN-mediated modifications in the connectivity of the KC-to-MBON network (Tomchik and Davis 2009, Gervasi et al. 2010, Aso et al. 2014b, Boto et al. 2014). DANs themselves receive sensory input from the periphery most likely via acetylcholine binding to nicotinic acetyl-choline receptors (nAChRs) and glutamate binding to *N*-methyl-D-aspartate (NMDA) receptors (Xia et al. 2005, Qin et al. 2012, Ueno et al. 2017). Of note, details about these pathways are not fully elucidated. Coincidence detection of dopamine release from DANs, which further activates the Dop1R1/Gαs signaling cascade, and an odor evoked rise in Ca^{2+} levels in the axons of

spiking KCs leads to increased rutabaga encoded adenylyl cyclase activity (Kim et al. 2007, Busto et al. 2010, Boto et al. 2014, Tomchik and Davis 2009, reviewed in Boto et al. 2020). In the following, cAMP subsequently, among other things, activates protein kinase A (PKA) as a prerequisite for memory aquisition (Skoulakis et al. 1993, Gervasi et al. 2010). The formation of long-term memory is 'cAMP-responsive element binding transcription factor' (CREB) dependent (reviewed in Dubnau and Tully 1998, Widmer et al. 2018). It is likely that Dop1R1 does not only alter cAMP levels but in addition recruits the mitogen-activated protein kinase (MAPK) cascade which in turn potentially modifies synaptic output of the MB by acting on molecules such as integrin and fasII (reviewed in Guven-Ozkan and Davis 2014). These mechanisms result in presynaptic changes of the KC-to-MBON connectivity, such that activity of MBONs is favoured which, depending on the compartment in question, are either approach- or aversion-promoting. Specifically, in the case of appetitive associations, activity of aversion-mediating MBONs is depressed, whereas in the case of aversive associations, activity of MBONs mediating appetitive responses is depressed (Owald and Waddell 2015, Hige et al. 2015b, Berry et al. 2018). Although learning is primarily based on depression of the KC-to-MBON synapse, as described above, scenarios involving synaptic potentiation have also been reported. This is for example the case for backward conditioning, that is, when reinforcement preceeds the odor cue (Handler et al. 2019), or for either prolonged or intense DAN activation in absence of an odor (Cohn et al. 2015, Hattori et al. 2017, Berry et al. 2018, reviewed in Modi et al. 2020).

Of note, ongoing DAN activity without simultaneous odor-evoked KC activity and thus low Ca^{2+} levels facilitates memory decay ('forgetting') via Dop1R2 and the scaffold protein Scribble which interacts with the 'small G- protein Ras-related C3 botulinum toxin substrate 1' (Rac 1) (Kim et al. 2007, Shuai et al. 2010, Berry et al. 2012, reviewed in Guven-Ozkan and Davis 2014), possibly by acting on the KC-to-MBON synapse to induce recovery from synaptic depression (Berry et al. 2018). Although it appears to be clear, that mechanisms for resisting memory decay must also exist in order to preserve meaningful memory engrams, these remain yet to be elucidated (Berry et al. 2018). Thus, both the formation and disruption of a given memory trace can be brought about by the activity of one and the same DAN. How this two-receptor model for memory management actually operates such that MBONs can read a clear acquisition- or forgetting-signal has long been a mystery. Revisiting the hypothesis of differences in downstream signaling cascades, it was found that Dop1R1 strongly couples to Gαs and increases cAMP levels, whereas Dop1R2 preferentially couples to Gαq to mobilize Ca^{2+} from internal stores (Himmelreich et al. 2017, reviewed in Verlinden 2018). However, the Gαq signaling cascade was recently also shown to be required for the learning-associated depression of the KC-to-MBON synapse in γ-lobe KCs via type A muscarinic acetyl-choline receptors (mAChR-A) (Bielopolski et al. 2019). Interestingly, the α'-lobe compartment in adult flies was found to be

resistant to memory decay as a result of DAN activation without odor presentation (Aso and Rubin 2016). This finding is in line with previous studies, suggesting a role in long-term appetitive memories regarding nutrient value (Yamagata et al. 2015, Huetteroth et al. 2015). Very generally, distinct cell types can be attributed specific roles in learning and memory in adult flies: output from γ- and α'/β'-lobe neurons is required during learning and shortly afterwards (Zars et al. 2000, Krashes et al. 2007, Qin et al. 2012), whereas output from α/β-lobe neurons is necessary for memory recall (Dubnau et al. 2001, McGuire et al. 2001). Whether a similar rational applies for the distinct MB compartments in larvae has not yet been resolved. Taken together, memory updates could be implemented by two different strategies, depending on MB compartment. On the one hand, while a new memory is established, the old memory decays. On the other hand, a new memory is formed although the old memory remains. This leads to the question how long-term memory is generally established. In principle, short-term memories could simply be converted into long-term memories due to chemical changes at the synapse. Secondly, long-term memories could be formed in parallel in different compartments (for discussion see Aso and Rubin 2016). A model posed by Felsenberg et al. (2017) suggests that extinguishing a reward memory demands the formation of a novel aversive memory within a separate compartment. A similar process requiring the formation of an oppositely valenced complementary memory was reported for the extinction of aversive memories (Felsenberg et al. 2018).

Most remarkably, the MB circuitry seems to be much more complicated than previously thought. Several micro-circuits have been detected in recent connectomic studies in both larval and adult *Drosophila* (larva: Eichler et al. 2017; adult flies: Takemura et al. 2017) which had previously been completely overlooked. These 'unknown unknowns' drastically changed the view of the interconnectivity within the MB. As one of these newly discovered connections, DANs maintain direct synaptic contact to MBONs which were indeed shown to express dopamine receptors (Crocker et al. 2016, Eichler et al. 2017, Takemura et al. 2017) and could thus directly change their excitability (reviewed in Modi et al. 2020). The opposing effects of the excitatory DAN-to-MBON connection and the learning-induced depression of the KC-to-MBON synapse might serve to ensure that learned behavior does not only take into account reward memory but rather the gain in reward if learned behavior is indeed expressed (Schleyer et al. 2011, Schleyer et al. 2015a). Possibly DANs also receive GABAergic feedback originating mostly through one or multiple synaptic steps from MBONs to regulate pre-synaptic DAN activity (larva: Eschbach et al. 2020a; adult flies: Pavlowsky et al. 2018). This shows striking parallels to the feedback loop discovered in rodents which serves to prevent over-activation of DAN signaling (Edwards et al. 2017, Groessl et al. 2018, reviewed in Karam et al. 2019). In the *Drosophila* larva, further feedback from MBONs to DANs is provided by recently described pre-modulatory neurons. These neurons either provide input from MBONs (called FBNs; one-step

feedback neurons), input from other FBNs (called FB2Ns; two-step feedback neurons), or yet different input (called FFNs; feedforward neurons), some of them being inhibitory, others excitatory (Eschbach et al. 2020a). Of note, activation of inhibitory feedback neurons induces memories which are of opposite valence to the activation of the DAN they inhibit (Eschbach et al. 2020a). A possible function of this additional feedback layer might be to encode contextual information or the internal state of the animal and enhance the connectivity between DANs and MBONs such that the output from functionally distinct regions of the MB could influence the activity of a given DAN during memory formation (Eschbach et al. 2020a). This scheme greatly increases the flexibility of the learning system and enables more complex learning tasks such as second-order conditioning (Tabone and de Belle 2011). Additionally, the newly discoverd KC-to-DAN connections could serve to further increase DAN activity by providing positive feedback from a coincidently activated KC to the respective DAN (Cervantes-Sandoval et al. 2017, reviewed in Boto et al. 2020). Recent data in the larva suggest that the KCs, most likely indirectly, communicate feedback to DANs, also independent of acetylcholine signaling, via neuropeptide F to potentially stabilize odor-reward memories (Lyutova et al. 2019). Although no hard evidence for a DAN-to-DAN connectivity is available (no chemical synapses were detected in connectomic studies which, however, does not rule out the existence of electrical synapses) feedback among compartents was confirmed, notably of the same lobe, such that memory formation in one compartmet could potentially affect DANs of an adjacent compartment (Eichler et al. 2017). Apart from the above described within-compartment connectivity, some DANs (e.g. PPL1 in adult flies) themselves exert control on associative learning by inhibiting the APL neuron through Dop2R and its downstream molecules (Zhou et al. 2019). Of note, it was reported that a reduction of DAN activity can have reinforcing effects. More specifically, thermogenetic activation of PAM-γ3, one of the few punishing PAM cluster DANs in adults, paired with an odor, induced robust aversive memory, whereas inhibiting this subset of neurons established appetitive memory. This surprising effect of change in memory valence was shown to come about by allatostatin A (AstA), which signals a sugar reward through inhibition of PAM-γ3 neurons (Yamagata et al. 2017). The bidirectional role of PAM-γ3 underpins the functional heterogeneity of dopamine neurons in the MB (Yamagata et al. 2017). Taking everything together, associative learning in the MB requires orchestrated interplay of the distinct neuron groups. Further research is yet warranted to ascertain the function of all newly discovered connections.

Apart from the within MB circuitry, relatively little is known about the network downstream of MBONs. The plasticity-driven fine tuning of MBONs in the MB network stands in stark contrast to the stereotypic output from lateral horn (LH) neurons and could even override innate responses from the LH (Hige et al. 2015a). However, as research had focused almost exclusively on the role of the MB in learning and memory, knowledge about the LH is rare. This is also because of two main caveats;

firstly, genetic reagents to specifically lable LH neurons were lacking (Dolan et al. 2018, Dolan et al. 2019), and secondly, the LH neuropil does not have prominent anatomical landmarks to categorize neurons (Frechter et al. 2019). The functional division between MB and LH might be less pronounced, given the results from recent studies which identified connections between MBONs and LH cells. Indeed, some LH cell types were found to be relevant for innate olfactory responses but in addition also necessary for memory retrievel (Dolan et al. 2018, Dolan et al. 2019, Lerner et al. 2020). How the extensive interactions between LH and MB contribute to neural modulation needs to be further investigated. In addition to the MBON-to-LH neuron connection described in adult flies (Dolan et al. 2018, Dolan et al. 2019, Lerner et al. 2020), EM reconstructions in the larva revealed additional LH neuron-to-MBON connectivity, as well as connections from both MBONs and LH neurons onto downstream convergence neurons which suggestively integrate innate and learned valence (Eschbach 2020b).

After introducing the circuitry as a whole with its 'knowns' and 'unknowns', I discuss in the following sections how individual DANs differ from each other and can thus contribute differentially to the formation of associative memories.

Heterogeneity part I: DANs have a specific 'polarity'

To understand the functionality of the MB circuitry as a whole it is crucial to study the features of individual DANs and their impact on memory formation. Appetitive or aversive teaching signals are relayed by different DANs, depending on the collection of ascending input they receive. Thus, the most obvious criterion to functionally distinguish DANs is by the polarity of the valence they can confer to associated cues. Of note, DANs with different polarity are also regionally separated, in that sense that they innervate distinct sections of the MB. In the larva, the medial lobe receives input from rewarding DANs (Rohwedder et al. 2016, Saumweber et al. 2018), while the vertical lobe and the lateral appendix are innervated by punishing DANs (Schroll et al. 2006, Eschbach et al. 2020a). Similarly, in adult flies PAM cluster DANs were shown to be mostly rewarding (Burke et al. 2012, Liu et al. 2012, Yamagata et al. 2015), and PPL DANs mostly punishing (Schwaerzel et al. 2003, Claridge-Chang et al. 2009, Aso et al. 2010, Aso et al. 2012, Galili et al. 2014). However, surprisingly little is known about the kind of appetitive and aversive information that is conveyed by a given DAN. Put to the extreme, individual DANs might mean a specific kind of reward or punishment, or DANs of similar polarity could redundantly signal any appetitive or aversive stimulus. To the extent that data is available, it is suggestive of a partially specific, partially combinatorial encoding of reward and punishment type, respectively. Rohwedder et al. (2016) found that individual medial lobe DANs in the larva signal nutrient value versus sweetness, respectively. DAN-h1 of the medial lobe was later on discovered to mediate sugar- but not aspartic acid- or salt-learning (Saumweber et al. 2018).

Accordingly, in adult flies, DANs can be classified as sweetness or nutrient value conveying, depending on whether they express the OAMB octopamine receptor or not (Burke et al. 2012, Huetteroth et al. 2015, Yamagata et al. 2015). While octopamine signals reinforcement memory of sweet taste through subsets of PAM cluster DANs, octopamine is dispensable for reinforcement memory of nutritious sugars (Burke et al. 2002). Interestingly, sugars with only a sweet taste component elicit only short-term memory, whereas sugars offering a nutrient value lead to the formation of long-term memory (Burke and Waddell 2011). Of note, it was shown that the activity of aversive DANs is increased after ingestion of sugars with nutrient value, possibly to signal satiety (Krashes et al. 2009, Musso et al. 2015). The association of water as a reward is also accomplished via DANs in adult flies (Lin et al. 2014, Senapati et al. 2019). Whether DANs are also involved in amino acid learning remains yet unknown, but some DANs were shown to harbor the amino acid sensor GCN2 (Bjordal et al. 2014).

Thus, while there seems to be discrimination in DAN signaling with regard to appetitive stimuli, this pertains to a lesser extend for aversive stimuli. In the larva, three individual DANs were described to signal punishment: DAN-f1 and DAN-g1 of the intermediate and lower vertical lobe and DAN-d1 of the lateral appendix (Eschbach et al. 2020a; see (III): for DAN-f1 **(Figure 21)** and DAN-d1 **(Figure 23)**). To further test punishment encoding across aversive DANs, specific somatosensory-related neurons were optogenetically activated as a type of punishment. These include mechanosensory neurons, nociceptive neurons and Basin neurons that integrate both mechanosensory and nociceptive inputs. Changes in calcium transients in the respective DANs in response to the activation of the different somatosensory-related neurons were recorded. Interestingy, DAN-f1 responded to the activation of nociceptive neurons and mechanosensory neurons, DAN-d1 responded to the activation of nociceptive neurons and Basin neurons. By contrast, DAN-g1 responded nonselectively to all tested punishments (Eschbach et al. 2020a). In adult flies, aversive DANs of the PPL cluster were described to be required for both electroshock and heat learning (Schwaerzel et al. 2003, Claridge-Chang et al. 2009, Aso et al. 2010, Aso et al. 2012, Galili et al. 2014).

Generally, the segregation of ‘good’ and ‘bad’ food components, and reinforcer in general, at the level of DANs is essential to enable independent coding of the respective memories (reviewed in Das et al. 2016). Some DANs seem to be at least partially selective, more so for rewards than for punishments, although further investigations in this matter need to be undertaken. Most strikingly, as discussed by Schultz (2010), dopamine neurons signal a ‘common currency reward signal’ and thus relay pure appetitive valence and no sensory information. This feature of DANs enables the comparision of the effect size between distinct rewards or punishments, respectively (Schultz 2010). Contrasting the case of *Drosophila*, in mammals the mostly homogeneous response of DANs to

appetitive stimuli is suggestive of a rather broad tuning, while distinct dopamine neurons respond to distinct aversive stimuli (Matsumoto and Hikosaka 2009, Fiorillo et al. 2013).

Apart from a given polarity, however, DAN signaling is even more complex and greatly depends on the event-timing between a given reinforcer and an odor cue. The additional layer of heterogeneity in DAN signaling is elaborated in the following.

Heterogeneity part II: Some, but not all DANs mediate timing-dependent valence reversal

Just like nearly everything in life has two sides, a given CS such as an odor cue can be remembered as something positive or negative, depending on its timing relative to the US (Solomon and Corbit 1974, Solomon 1980, Wagner 1981). If an odor precedes an appetitive or an aversive stimulus, reward or punishment memory is established. However, it is less acknowledged, that the backward pairing of appetitive or aversive stimuli and odor can also bring about associative memory, namely frustration or relief memory, respectively. Of note, timing-dependent valence reversal can be easily overlooked if the measure is not bivalent, if the timing of the stimuli is chosen in an unfortunate way, or if the number of training trials is low.

Remarkably, timing-dependent valence reversal has finally been confirmed across valence domains in both larval and adult *Drosophila*. Early data on odor-shock learning in the larva were suggestive of an effect of event-timing, however, only strong punishment memory scores but yet no significant relief memory scores could be confirmed (Khurana et al. 2009). Further studies used optogenetic activation of DANs as reinforcement rather than 'real world' rewards or punishments. Preliminary results from optogenetically activating a broad TH-Gal4 driver strain, which expresses the *Drosophila* TH gene in dopaminergic neurons and thus covers most of the DANs (Friggi-Grelin et al. 2003) except for the majority of the pPAM DANs (Yarali and Gerber 2010) led to aversive punishment memory upon short forward pairing of odor and DAN activation (data not shown). Appetitive relief memory upon backward pairing of DAN-activation and odor could not be verified in direct comparison to genetic controls (data not shown). Based on the results from a screen which revealed that two individual DANs (-i1 and -h1 of the pPAM cluster) were sufficient as a reward (Saumweber et al. 2018), the ability of individual DANs to bring about oppositely valenced memory types was further investigated. Indeed, DAN-i1 activation reliably established both appetitive reward memory upon forward conditioning, and aversive frustration memory upon backward conditioning (Saumweber et al. 2018; see (II): **Figure 13, Figure 18**). As a next step, we asked, whether valence reversal in the larva can also be validated for individual DANs with aversive polarity. One candidate neuron under investigation, DAN-f1 which innervates the intermediate vertical lobe, was in fact found to mediate aversive punishment memory upon forward conditioning as well as appetitive relief memory upon backward conditioning ((III): **Figure 21, Figure 26**). Intriguingly, the timed function of the two

individual DANs was similarly shaped yet flipped, according to the polarity of the DAN. As discussed above, another DAN, DAN-d1, of the aversive domain did not support valence reversal ((III): **Figure 23, Figure 26**). This is interesting enough and confirms that DANs do not only differ in their polarity but also in the temporal 'fingerprint' of their teaching signal. So far only one DAN per valence domain (DAN-i1 and DAN-f1) was confirmed to mediate timing-dependent valence reversal in the larva, and one DAN was shown to have a punishing effect with a single optimum (DAN-d1) **(Figure 27)**.

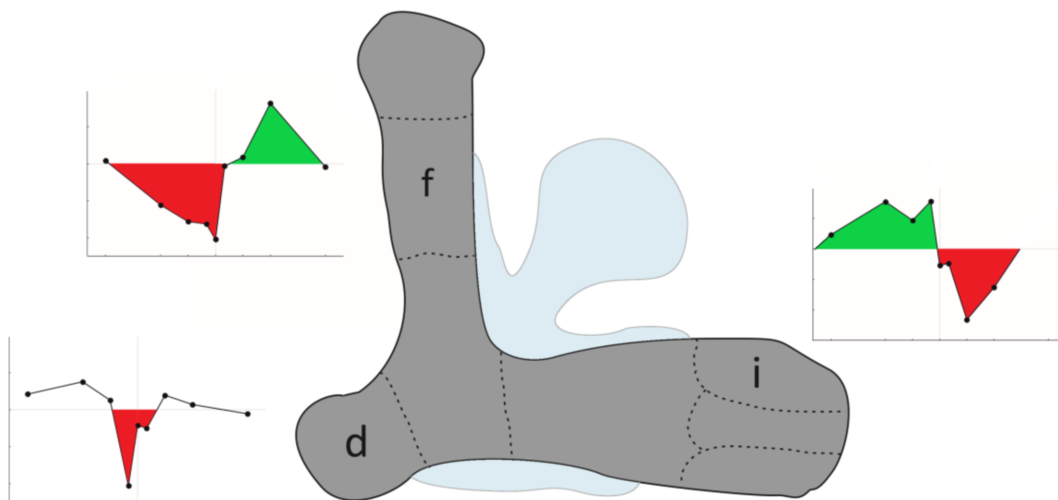

Figure 27: Some, but not all DANs mediate timing-dependent valence reversal in larval *Drosophila*. Three out ouf eight DANs in L3 larvae were described in some detail. Dependent on the event timing, DAN-f1 with punishing 'polarity', innervating the intermediate vertical lobe of the mushroom body established forward punishment and backward relief memory, respectively (see graph to the top left; (III): Figure 21). Another punishing DAN, DAN-d1, innervating the lateral appendix elicited forward punishment memory only (see graph to lower left; (III): Figure 23). One rewarding DAN, DAN-i1, innervating the upper toe oft he medial lobe, was found to also bring about two opposing memory types, dependent on the event-timing. These are forward reward and backward frustration memory, respectively (see graph on the right; Saumweber et al. 2018). Thus, some, but not all DANs are capable of timing-dependent valence reversal, such that DANs do not only have a specific polarity, but also display further heterogeneity in their teaching signal.

Are these de facto the only DANs with this capability? A handful of DANs remain yet to be tested in this respect. The driver strain covering the third DAN with aversive polarity, DAN-g1, innervating the lower vertical lobe, includes strong additional expression in the ventral nerve cord. DAN-h1, innervating the shaft of the MB, was not further investigated since the respective split-GAL4 driver strain also stochastically coveres DAN-i1 in at least one hemisphere and also because appetitive scores upon optogenetic activation of that driver strain could only be partially reproduced (data not shown). Activation of DAN-k1 of the pPAM cluster was so far not found to be rewarding (Saumweber et al. 2018) and DAN-c1 did not elicit appetitive or aversive memory (Eschbach et al. 2020a). For DAN-j1 of the pPAM cluster, apparently no split-Gal4 driver strain could be generated up to now.

Thus, investigations of timing-dependent valence reversal brought about by other DANs would currently not come without caveats, clearly pointing to a need for novel single DAN driver strains.

Is a similar heterogeneity in DAN signaling observed in adult *Drosophila*? Preceding first results on valence reversal in larvae, it was found that flies form aversive punishment memory, if an odor precedes an electric shock, and appetitive relief memory, if the event-timing is reversed (Tanimoto et al. 2004, Yarali et al. 2008, Yarali et al. 2009, Yarali and Gerber 2010). Similarly, Aso and Rubin (2016) observed timing-dependent valence reversal when odor and optogenetic DAN activation with CsChrimson as the optogenetic effector were presented at different timings relative to each other. Interestingly, DANs innervating different MB compartments employed heterogeneity in their signaling. While activation of PPL1-01 revealed aversive punishment memory upon forward conditioning as well as appetitive relief memory upon backward conditioning, activation of other PPL DAN driver strains led exclusively to forward punishment memory. Regarding rewarding PAM cluster DANs, reward memory upon forward conditioning was confirmed for all tested driver strains, but in none of the cases backward frustration memory was detected (Aso and Rubin 2016). Shortly after, König et al. (2018) confirmed the capability of PPL1-01 to mediate timing-dependent valence reversal, using ChR2-XXL as the optogenetic effector. While PPL1-01 activation resulted in sufficiently strong forward and backward memory scores, another DAN, PPL1-06, established the opposing memories to a lesser extent. Further aversive DANs exclusively revealed punishment memory (König et al. 2018) (for an overview see **Table 1**).

Table 1. DANs in adult flies tested for timing-dependent valence reversal.

REFERENCE	DRIVER STRAIN	SHORT NAME	CELL TYPE	FORWARD	BACKWARD
(1); (2)	MB320C	PPL1-01	PPL1-γ1ped	x	x
(1); (2)	MB099C; MB296B	PPL1-03	PPL1-γ2α'1	x	N/A
(1); (2)	MB630B; MB099C	PPL1-06	PPL1-α3	x	x
(1)	MB441B	PAM-12	PAM-γ3	x	N/A
(2)	MB043B	PAM-11	PAM-α1	x	N/A
(2)	MB213B	PAM-04 and PAM-10	PAM-β2 and PAM-β1	x	N/A
(2)	MB315C and MB109B	PAM-01 and PAM-02	PAM-γ5 and PAM-β'2a	x	N/A

(1) König et al. 2018, (2) Aso and Rubin 2016; x = memory detected; N/A = memory not detected

Just recently, Handler et al. (2019) showed in yet a different paradigm and for a broader set of rewarding DANs both reward and frustration memory.

Thus, across studies it is indeed the case that in larvae as well as in adult flies that different DANs operate with distinct learning rules: Some, but not all DANs dispose of the property to mediate timing-dependent valance reversal. Given that fact, I was wondering, what the actual molecular requirement for timing-dependent valence reversal might be and whether and what differences

might be observed between forward versus backward memories. In addition, the question remains to be answered, why some but not all DANs show valence reversal and how this reflects in the underlying molecular features of the DANs that explicitely do or do not support valence reversal. Towards this end, the next paragraph is taking into account insights from the present study as well as novel results regarding both larval and adult *Drosophila*.

Heterogeneity part III: Differences in DAN-mediated forward and backward memories

Strikingly, some DANs have the ability to establish two opposing types of memories. Across valence domains, similarities between memory types established by forward pairing of odor-DAN activation and backward pairing of DAN-odor activation have been observed, respectively ((II): **Figure 13**; (III): **Figure 21**). Apparently, these similarities seem to be consistent across developmental stages, suggesting a shared mechanism for forward memory types as well as for backward memory types. Specifically, memories established by forward conditioning are consistently stronger than memories established by backward conditioning ((III): **Figure 21, Figure 22**; Tanimoto et al. 2004, Yarali et al. 2008, Yarali et al. 2009, Yarali and Gerber 2010, Diegelmann et al. 2013b, Niewalda et al. 2015, Appel et al. 2016, Aso and Rubin 2016, König et al. 2018) as was predicted by traditional learning theories (Solomon and Corbit 1974, Solomon 1980). It is only for DAN-i1 that forward and backward memory scores are comparably high.

Which factors are determining forward versus backward memory strength, respectively? Previous studies in adult flies showed that, although punishment memory scores did not change much, relief memory scores are increased when i) more electric shock pulses distributed over a prolonged time period are used, ii) a quantitatively stronger effector (ChR2-XXL instead of ChR2-XXM) is deployed or when iii) more trials are applied. The latter (six versus one trial training) led to an impressive six-fold increase in relief memory scores (König et al. 2018). These findings fancy the rationale that prolonged punishment should lead to stronger relief memories. Accordingly, in humans showing risk-seeking behavior (e.g. bungee jumping) the aversive component gets less, while the relief component gets stronger with repetitions of the dangerous activity (see Solomon and Corbit 1974). In the larval case, however, no significantly increased forward or backward memory was observed, even when the duration of DAN-activation was tripled. Potentially, effect sizes could already be at their maxima, or limitations of our protocol might prevent memory scores from being raised higher. Of note, in the larval optogenetic paradigms constant blue light is used for DAN-activation. Results from studies on ChR2 (not the later version ChR2-XXL which was used in the present study) warn against spike frequency adaptation, as a fast, initial decay in firing rate, followed by overall cessation of firing of the cell later on, was reported (Pulver et al. 2009). To ensure a stable firing rate over several minutes, pulsed blue light stimulation (as it is mostly used for adult fly conditioning) is recommended (Pulver

et al. 2009). Alternatively, another optogenetic effector could be applied to see whether this affects the timed function observed in larval DANs, or whether backward memory scores can be increased. Often, CsChrimson is used as optogenetic effector, mainly to reduce light-induced behavioral artefacts as the wavelength for CsChrimson activation, 720 nm, was previously reported to be outside the fly's photoreceptor light absorption spectra (Minke & Kirschfeld 1979, Salcedo et al. 1999). Other studies, however, suggest that flies might still, although less well, perceive light of this wavelength (Klapoetke et al. 2014). Interestingly, in the context of prolonged DAN-activation in larvae, we noticed that the onset of DAN-activation matters to establish forward memories, while rather the offset of DAN-activation is of relative importance for backward memories to be formed.

Another major difference between forward and backward memories lies in their stability; with forward memories being stable for a relatively long time-span, while backward memories are quickly decaying ((II): **Figure 16**; (III): **Figure 22**). Although relief memory is only shortly after training detectable in adult flies as well, no direct comparison of the decay rates can be made, due to the pronounced difference in memory strength after forward and backward conditioning (Yarali et al. 2008, Diegelmann 2013b). In case of DAN-i1, however, where forward and backward memory scores are comparably high ((II): **Figure 16**), the conclusion that forward reward memory is longer detectable than backward frustration memory is indeed valid.

These basic parametric similarities in larval and adult *Drosophila* forward and backward memories, respectively, may hint at similar underlying molecular properties. In adult flies, some first attempts have been made to disentangle the molecular background of punishment versus relief memory. It was found that cold-shock amnesia fully abolishes relief memory, while only a partial reduction was observed for punishment memory, suggesting that the latter composes of an amnesia-sensitive and an amnesia-resistant component (Diegelmann et al. 2013b). Further investigations focused on one evolutionary conserved candidate gene, *synapsin*, coding for the synapsin protein, which had previously been shown to determine memory strength by regulating the recruitment of reserve-pool vesicles during learning (Klagges et al. 1996, Hilfiker er al. 1999, Nuwal et al. 2011, Diegelmann et al. 2013a). In mutant larvae lacking synapsin, memory scores were partially reduced (Michels et al. 2005, Michels et al. 2011). Fittingly, while forward memory scores were reduced in mutant adult flies, backward memory was fully abolished (Niewalda et al. 2015). This is reminiscent of what has been found regarding cold-shock amnesia in the study from Diegelmann et al. (2013b) and is indicative of punishment memory being composed of two distinct short-term memory components: One amnesia-resistant, synapsin-independent component and an amnesia-sensitive, synapsin-dependent component. Contrastingly, relief memory seems to harbor only the latter memory component (Diegelmann et al. 2013b, Niewalda et al. 2015). Despite this difference, the results suggest at least partially shared molecular properties of punishment and relief memory. This is

consequential for clinical applications, and potentially constrains pharmacological approaches to selectively target only one excessively pronounced type of memory, since it might as well interfere with the opposing memory type und thus be of detrimental impact (for discussion see the *Clinical implications* section).

As discussed above, there are considerable differences between forward and backward memory types. What, however, are the underlying molecular properties of the oppositely valenced memory types? The hypothesis that a co-transmitter in DANs might be responsible for either forward or backward memory has been around for some time. In larval *Drosophila* the majority of DANs was reported to harbor only small dense-core vesicles, while only DAN-c1 and -g1 contained both small dense-core and small clear vesicles. DAN-g1 was even found to have large dense-core vesicles in addition to the other two vesicle types (Eichler et al. 2017; loc. cit. Extended Data Figure 7). Contrastingly, both PAM cluster and PPL DANs in adults were shown to possess both dense-core and clear vesicles (Takemura et al. 2017; loc. cit. Table 1). While small dense-core vesicles (40-60/60-120 nm) harbor biogenic amines including dopamine, small clear-core vesicles (40-60 nm) store acetylcholine and amino acid transmitters, and large dense-core vesicles (90-250 nm) transport neuropeptides (Purves et al. 2001). Most likely, due to the packaging in different vesicles types, the co-transmitters need not be released simultaneously and can potentially serve distinct processes. To study, whether indeed both forward and backward processes are dopamine dependent, König et al. (2018) used TH-RNAi in the PPL1-01 neuron, which mediates timing-dependent valence reversal (Aso and Rubin 2016, König et al. 2018), while simultaneous optogenetically activating it. Of note, this approach reduced punishment memory scores by half, while relief memory scores remained unaffected (König et al. 2018). Respective experiments are currently being executed regarding the larval DAN-i1 and DAN-f1.

Continuing the quest for a potential co-transmitter in DANs, Aso and colleagues reported the expression of nitric oxide synthase (NOS), an enzyme required for the synthesis of nitric oxide (NO) in the terminals of some subsets of adult *Drosophila* DANs (Aso et al. 2019). While dopamine activates the cAMP signaling cascade, NO binds to soluble guanylate cyclase (sGC), triggering the cyclic guanosine monophosphate (cGMP) pathway. Interestingly, when distinct clusters of DANs in a dopamine deficient background (Riemensperger et al. 2011) were activated, a robust associative memory of opposite valence was observed, compared to the memory established upon activation of the same DAN cluster in wildtype flies (Aso et al. 2019). This valence-inversed phenotype both in punishing as well as rewarding DANs was indeed dopamine-dependent as it could be rescued by L-Dopa and carbidopa feeding. However, activation of some other DAN clusters in the dopamine deficient background led sometimes to memory of the usual valence, or no memory was detectable at all. This suggests that DAN clusters differ in their respective co-transmitter, either with a different,

or a similar sign of action as dopamine, or one which cannot establish associative memory in the absence of dopamine. NO, however, was found to be dispensible for timing-dependent valence reversal (Aso et al. 2019). So far, no other candidate co-transmitter could be identified which might potentially be involved in either forward or backward learning.

Another possible mechanism for timing-dependent valence reversal could be the differential involvement of Dop1R1 and Dop1R2 as reported by Handler et al. (2019). This is specifically not because of differences in DAN activity or differential levels of dopamine release after forward versus backward pairing. Indeed, while Dop1R1/Gαs signaling senses the coincidence of odor and DAN activity during associative learning, Dop1R2/Gαq signaling depends on the temporal sequence of these events (Handler et al. 2019). Unexpectedly, Dop1R2 was recently found to also stabilize appetitive memory via the Raf/MAPK pathway in KCs (Sun et al. 2020). Further studies imply that Dop1R2 detects changes in dopamine levels after learning events (Berry et al. 2012, Musso et al. 2015, Ichinose et al. 2017, Plaçais et al. 2017). Distinct downstram signaling cascades are likely to be responsible for the differential involvement of Dop1R2 in given neural processes. While Dop1R2 acts on Gαq to induce forgetting (Berry et al. 2012) and mediates backward memories (Handler et al. 2019), it is signaling via the Raf/MAPK pathway, possibly activating CREB (Sun et al. 2020).

Summing up, two main hypotheses are around how forward versus backward memories might differ with regard to their underlying molecular pathways. Firstly, as forwarded by Handler and colleagues, a differential involvement of dopamine receptor types (Handler et al. 2019). Since not all DANs confer timing-dependent valence reversal, this might hint at an uneven expression of Dop1R1 and Dop1R2 across compartments which was so far not observed (Kondo et al. 2020). Secondly, forward versus backward memories could depend on the release of different transmitters from the DANs. Forward memories seem to be mostly dopamine dependent, while backward memories might depend on the involvement of a co-transmitter, as suggested by the findings from König et al. (2018). Whether any of the two hypotheses holds true for the larval case remains yet to be elucidated.

In the following chapter will be discussed what might account for timing-dependent valence reversal on the synaptic level and how it fits in with existing learning theories.

<u>Learning theories can indeed explain timing-dependent valence-reversal</u>

How can a cue acquire either of two opposing valences? Going back to classical learning theories, the opponent-process theory (Solomon and Corbit 1974, Solomon 1980) offers an explanation for the existence of timing-dependent valence reversal and even predicts the relative strength of the opposing memory types. Their theory claims that the primary affective process (a-process), which begins and terminates with a given stimulation, elicits an additional opponent process (b-process), which is characterized by its rather weak effect size and its slow decay (Solomon and Corbit 1974).

However, their theory does not give an explanation for the neural implementation of timing-depenent valence reversal. A critical factor for a CR to occur is the probability for a CS to be more often paired with a given US than to be present without the latter. In addition, the timing between CS and US is supposed to be critical. The temporal coding hypothesis claims that animals do not only learn about the actual order of CS and US, but more importantly, about their temporal relationship (Savastano and Miller 1998, Arcediano and Miller 2002, Arcediano et al. 2003). Consequently, according to the temporal coding hypothesis backward learning can occur. The animals learn the temporal relationship between DAN activation and odor presentation, such that the odor becomes an offset signal for something good (frustration memory is formed), or for something bad (relief memory is formed). However, following the assumptions of the temporal coding hypothesis, the temporal order of CS and US should not matter such that forward and backward conditioning should produce similarly strong memory scores. That is not, however, what is experimentally observed: Forward memory scores are generally more pronounced than backward memory scores (Yarali et al. 2008, Aso and Rubin 2016, König et al. 2018, (III): **Figure 22**). This result can best be understood with the opponent-process theory described above (Solomon and Corbit 1974, Solomon 1980). Overall, timing-dependent valence reversal can be explained by a combination of traditional learning theories, although the underlying neuronal mechanisms were unknown at the time of their announcement and mostly still remain unsolved up to date.

<u>How is timing-dependent valence reversal realized in the brain?</u>

What is the cellular basis of timing-dependent valence reversal? To tackle that question, one has to go back to the actual question that has impelled scientists for more than a century, namely what is memory? The search for an answer to this question finally resulted in the concept of an engram: Upon memory acquisition connections between neurons are strengthened due to simultaneous excitation (Semon 1921). Some years later, Donald Hebb proposed that apart from an enhancement of synaptic strength also morphological changes of the involved synaptic contacts can be observed (Hebb 1949, Bliss and Lømo 1973). Indeed, further research confirmed that activity-induced long-term potentiation (LTP) and long-term depression (LTD) are dependent on the changes in number and shape of dendritic spines during learning events (Matsuzaki et al. 2004), although this does not have to be the case for all the synapses of a given neuron (Bliss and Collingridge 1993, reviewed in Poo et al. 2016). The memory mechanisms of LTP and LTD seem to be opposing at first glance but actually rather play together though the concept is still divisive. According to one concept of how a strong skew in synaptic weight comes about, some respective synapses are strengthened on the expense of others. This was termed the 'robbing Peter to pay Paul' principle, in the sense that the 'rich get richer' while the 'poor get poorer' (reviewed in Poo et al. 2016). Different from that, a local

regulation could take place, such that if some spines are strengthened, a mechanism kicks in which weakens neighboring spines to compensate for the weight change (reviewed in Poo et al. 2016). While the time window allowing LTP or LTD to occur is relatively broad (approx. 20-100 ms) and requiring only coincidence activity, the later discovered spike timing-dependent plasticity (STDP) changed the view of Hebb's learning rule. In contrast to LTP and LTD, STDP adjusts the strength of neural connections based on correlations at millisecond-scale (approx. 20 ms) in the firing of action potentials/spikes between pre- and postsynaptic neurons. In this sense, if an input spike to a neuron happens to be directly before the output spike of the neuron, the respective input is strengthened, while if the input spike occurs just after the output spike, the respective input is weakened. Notably, the sequence of pre- and postsynaptic spiking is important, rather than just the coincidence activity as in the case of LTP or LTD (reviewed in Poo et al. 2016). Thus, STDP is thought to at least partially offer an explanation for activity-dependent development in the brain (Levy and Steward 1983, Debanne et al. 1994, Markram et al. 1997, Bi and Poo 1998, Markram et al. 2011).

Is STDP facilitating learned modifications? Given that the brain must have a way to retain information about a cue predicting reward or punishment, until the actual occurrence of reward or punishment, the question arises what the neural representation for this could be. STDP has been proposed as possible mechanism to establish such associations. Indeed, STDP has been validated at the synapses between KCs and β-lobe output neurons in the locust, supporting the synchronous flow of olfactory information for means of odor discrimination (Cassenaer and Laurent 2007, discussed in Masse et al. 2009). By contrast, Ito et al. (2008) reasoned that STDP alone cannot explain associative olfactory learning in the moth *Manduca sexta.* When they varied the temporal overlap between odor-evoked spikes and a reward, learning was actually decreased the more CS and US were overlapping. Their results suggest that temporal contiguity between spike representation of CS and US is not necessary to enable learning. While it remains not yet fully understood how associative learning at a relatively slow timescale could be connected to STDP, requiring highly precisely timed spiking, neuromodulators might be in line for offering a possible mechanism to bring the different timescales together (Drew and Abbott 2006, Pawlak et al. 2010, Brzosko et al. 2019). Timing rules for synaptic plasticity are presumably attuned to the respective behavioral and functional requirements of a given circuit, even if this contradicts the STDP rules of close temporal correlations (Suvrathan 2019).

In *Drosophila*, the adenylate cyclase-based model was the prevailing model to explain the effect of event-timing on learning, and of associative learning in general, over the past years (Heisenberg 2003, Yarali et al. 2012; for corresponding work in Aplysia see Yovel and Abrams 1992, Abrams et al. 1998). According to this hypothesis, the bidirectional regulation of the adenylate cyclase is the underlying key mechanism. The difference in the behavior is supposed to come by the differential levels of cAMP. For punishment learning cAMP levels are increased, whereas for relief learning cAMP

levels are decreased, similar to the rodent STDP-based model by Drew and Abbot (2006). Thus, both forward and backward learning are brought about through output of the very same KC (Yarali et al. 2012). Until then, the Heisenberg fly model did not consider the activity of MBONs necessary for the induction of plasticity. However, MBONS could actually contribute to associtive learning via the DAN-to-MBON connection. Whether STDP at the KC-to-MBON synapse contributes to timing-dependent valence reversal in *Drosophila,* is not yet clear (Suvrathan 2019). Could the STDP principle apply for the results of the present study? Firstly, the odor is presented on sticky filter papers at the lid of the Petri dish and is probably not immediately sensed by the larva crawling on the substrate but rather needs time to equally distribute. Secondly, although the onset of DAN activation should be precise, the closing of the light-gated cation channel ChR2-XXL is delayed for several seconds (Dawydow et al. 2014). Thus, a temporal correlation at millisecond scale is unlikely. However, STDP in the MBON could contribute to timing-dependent valence reversal. While the odor signal is relayed via the KC-to-MBON synapse, reward or punishment signal could be relayed via the recently discovered DAN-to-MBON connection (Eichler et al. 2017). This hypothesis, though, warrants further investigations, e.g. blocking different dopamine receptors at the level of the MBONs.

Timing-dependent valence reversal was described in larval and adult *Drosophila*, and first data suggest mechanisms of how forward and backward memories can be established by one and the same dopaminergic neuron. In addition, current models offer an explanation how timing-dependent valence reversal can be implemented at the synaptic level. Understanding this phenomenon in the fly is especially interesting because novel insights could spark research questions in vertebrates. The next chapter sheds light on what is known about timing-dependent valence reversal in vertebrates, including humans, and the so far known neural mechanisms.

Timing-dependent valence reversal: an across species principle?

Timing-dependent valence reversal has emerged as an interesting study case in *Drosophila*, and was described across species. In the past years several studies reported this principle and tried to characterize its properties. Opposing memories were brought about by odor-shock/shock-odor training in adult flies (Tanimoto et al. 2004, Yarali et al. 2008, Yarali et al. 2009), or by odor-DAN/DAN-odor training in adult flies (Aso and Rubin 2016, König et al. 2018, Handler et al. 2019) and in larvae (Saumweber et al. 2018, (II): **Figure 13**; (III): **Figure 21**). Paralleling these findings, memories of opposite valence were established when flies were conditioned with chromatic visual cues and electric shocks (Vogt et al. 2015). This implies that event-timing affects memory valence across different modalities (Vogt et al. 2015). Early studies on the honey bee suggest that timing-dependent valence reversal can be detected in other insects, too. Hellstern et al. (1998) presented sucrose and an odor paired at various timings and measured the proboscis extension reflex (PER). For forward

pairing reward memory was observed, while for backward pairing they reported what they called 'backward inhibitory learning'. This can be viewed as frustration memory since the bees learn that the odor means the offset of a given reward (Hellstern et al. 1998).

Paralleling the results in invertebrates, both rodents and humans express two opposing types of memories, conditioned fear and relief, depending on the order of events (Andreatta et al. 2010, Andreatta et al. 2012, Andreatta et al. 2016). Forward conditioning with a cue (CS) followed by the shock (US) results in an increase in startle amplitude while if the shock is followed by the cue a decrease in startle amplitude is observed (bivalent measure) (Andreatta et al. 2010, Andreatta et al. 2012, Mohammadi et al. 2014, Andreatta et al. 2016, Luck and Lipp 2017). Interestingly, the two memory types can be dissected on the neural level: Fear learning in rodents requires the amygdala, while relief learning recruits reward circuits, involving the NAC and the striatum (Andreatta et al. 2012, Mohammadi and Fendt 2015). Similarly, in humans fear is mediated via activity in the amygdala and relief via striatal activity including the NAC (Andreatta et al. 2012). Contrasting what has been reported in *Drosophila*, relief memory seems to be rather elicited by reward circuitries and not mediated by punishment mediating neurons (Andreatta et al. 2012, reviewed in Navratilova et al. 2015). For a comprehensive juxtaposition of the vertebrate and *Drosophila* dopamine system see Scalpen and Kaun (2016).

Interestingly, patients rate an event as negative, when being asked, although they react with relief (Andreatta et al. 2010). Mechanistically, a neutral cue gains positive valence after an aversive implicit event, although explicitly the overall aversiveness of the event prevails. Thus, supporting the dual-process theory (Strack and Deutsch 2004), implicit and explicit valence can be dissociated depending on the order of events (Andreatta et al. 2010, Luck and Lipp 2017). Importantly, relief and safety learning, that is learning that the CS explicitly unpaired with the aversive US predicts the absence of the latter (see Mohammadi et al. 2014), underly different neural mechanisms: while the NAC is dispensable for safety learning (Mohammadi et al. 2014), it is required for relief conditioning. Specifically, co-activation of accumbal NMDA receptors and D1 receptors is required for the acquisition of relief memory (Bergado Acosta et al. 2017). Further investigations suggest that besides the D1 recepor also D2/3 receptors are of relevance for relief memory acquisition, while only the D1 receptor is necessary for the expression of conditioned relief (Bergado Acosta et al. 2017, Mayer et al. 2018). Interestingly, the direct pathway via D1 receptors promotes reward-seeking behaviors, while the other pathway via D2 receptors facilitates punishment-avoidance behaviors (Hikida et al. 2010, Kravitz et al. 2012, Yawata et al. 2012). When is which pathway favoured? Indeed, the key determinants are differences in dopamine levels, differences in the firing pattern of dopamine neurons which leads to an activation of D1 or D2 receptors, respectively, the differential binding affinity of the two receptor types, and the differential expression of the receptors (Richfield et al.

1989, Hikosaka 2007, Hikida et al. 2010, Kravitz et al. 2012, Yawata et al. 2012). Contrastingly, in *Drosophila* it is not known what are the key determinants for the Dop1R1 and the Dop1R2 pathway, respectively. At least in adult flies, differences in dopamine levels did not have any impact on the downstream pathway (Handler et al. 2019).

Relatively little is known about how relief learning is brought about. Presumably, the NAC receives dopaminergic input from mesolimbic VTA neurons (Fallon and Moore 1978) which were reported to exhibit phasic activity after the termination of an aversive event (Brischoux et al. 2009, Mayer et al. 2018). Consolidation into long-term relief memory occurs similar as for other types of reward conditioning (Hernandez et al. 2002, Blaiss and Janak 2007) at the NAC and is dependent on de novo protein synthesis (Bruning et al. 2016). Previously, dopamine was thought to mediate only appetitive valence (Mirenowicz and Schultz 1996) while serotonin (5-HT) delivers aversive signals (Daw et al. 2002, reviewed in Boureau and Dayan 2011). Dopamine signals though, appear to be more diverse than previously assumed, although most likely, individual DANs do not transfer single motivational signals but rather a range of signals brought about by various neural processes (Bromberg-Martin et al. 2010, reviewed in Schultz 2017, apes: Fiorillo et al. 2013).

Despite some mechanistic differences, the overall principle of timing-dependent valence reversal is strikingly similar across species. This allows for a comparision of basic underlying circuit principles between insects and vertebrates, including humans. In the following, the importance of timing-dependent valence reversal for patients suffering from a disturbance in the dopamine system is highlighted.

Clinical implications

Just as dopamine is of obvious importance not only in the fly but also in the vertebrate reward system, any change in dopamine levels can be of drastic impact. Disturbances in dopamine signaling are associated with a number of neuropathologies and psychiatric disorders in humans. Critically, basically all pharmacological approaches target the dopamine levels in a systemic way. That is, they respectively reduce or increase dopamine levels within any brain area and including the whole body. Thus, pharmacological treatments might help to get imbalanced dopamine levels in a given brain area back to basic levels, but unwittingly cause severe side effects by changing the dopamine levels elsewere in brain and body. Thus, it is crucial to understand exactly how dopamine modulates behavior, e.g. via mediating associative learning, and to elucidate the underlying molecular mechanisms. Novel insights from *Drosophila* or rodent research might stir the development of more targeted pharmacological or therapeutic treatments.

Patients with a number of neuropathologies suffer from either one problem: too strong, or too weak forward or backward learning, respectively. Thus, the majority of current treatments acts onto the

imbalanced part of the dopaminergic system, however, not taking into account that unwittingly the counterside of the system might also be affected by the treatment, if similar neural or molecular mechanisms apply for forward and backward learning.

In risk seeking behavior, for example, forward punishment learning is too weak, wheareas backward relief learning is far too strong. More specifically, the offset of extreme sports such as bungee-jumping, free climbing or a roller-coaster ride brings about strong relief feelings, thus potentially explaining the attraction to dangerous activities (Wang and Tsien 2011). With repetitions, the aversive part generally habituates, whereas the relief part increases (see Solomon and Corbit 1974). A related scenario may apply for the Stockholm syndrome and self-cutting injury **(Table 2)**.

Contrastingly, in patients suffering from post-traumatic stress disorder (PTSD) e.g. traumatized war veterans, forward punishment learning is too strong and backward relief learning too weak. Patients with PTSD exhibit over-generalization of fear as well as pronounced deficits in both safety and relief learning. Although they are capable of discriminating between danger and safety, they fail to inhibit fear responses in a safe situation (reviewed in Grillon 2002, Zweifel et al. 2011, Jovanovic et al. 2012). Related scenarios may apply for anxiety or panic disorders and phobias **(Table 2)**.

Accordingly, imbalances in forward versus backward learning in the appetitive valence domain can be causal for neuropathologic diseases. That is, for example, in patients with major depression forward reward learning is too weak, and accordingly, backward frustration learning is too strong. Thus, regarding major depression, changes in reward processing were observed (Forbes et al. 2007, Forbes et al. 2009) which find expression in reduced experience of positive affect (Clark and Watson 1991). This may comprise the motivation to obtain rewards, the behavioral expression of reward-seeking, or the hedonic aspects of reward-experiencing (Clark and Watson 1991). For this reason, patients with major depression are less likely to make decisions with a potentially rewarding outcome since rewards in general are less salient to them (Forbes et al. 2007). On the level of the neural network, reduced responses in reward-related areas such as the striatum (Forbes et al. 2006) or the caudate (Forbes et al. 2009) were observed in patients with early-onset depression (Forbes et al. 2006), while depression in adult patients is accompanied by reduced activity in striatal areas (Surguladze et al. 2005, Epstein et al. 2006) but also enhanced activation in medial prefrontal cortex areas which are thought to be related to sadness and social cognition (Keedwell et al. 2005).

What if, in contrast, forward reward learning is too strong and backward frustration learning is too weak? One prominent example, and beyond that also one of the major public health problems, is addiction. Both natural rewards and drugs of addiction elicit similar responses, including the desire to obtain them, the active pursuit of them and the accompanied rapid learning of predictive cues (reviewed in Bickel et al. 2018); a phenomenon described across species including *Drosophila* (reviewed in Ryvkin et al. 2018). The positive reinforcement itself then leads to an increase in the

frequency of motivated behaviors aiming at obtaining the reward. Addiction is best characterized by three distinct stages: intoxication, withdrawal and preoccupation. All drugs of addiction (opiates, psychostimulants such as cocaine and amphetamine, nicotine, alcohol and marijuana), independent of their individual mechanism of action, have one common feature: they precipitate an increase in synaptic dopamine levels in the NAC which receives its dopaminergic input mainly from the VTA (reviewed in Bickel et al. 2018, Solinas et al. 2019). Additionally, the amygdala and the prefrontal cortex (PFC) play a role in establishing reward-related memories, while the dorsal striatum, which in turn receives dopaminergic input from the substantia nigra (SN), is essential for memory consolidation after repeated administration of drugs of addiction (reviewed in Hyman et al. 2006). Drug-related distortions in the PFC are thought to foreclose flexible behavioral responses, confining the addict to a state of drug-seeking behavior (Grace et al. 2007). Apart from that, drug-induced extensive elevations of dopamine levels might eventually induce a positive prediction error, signaling the reward to be better than expected (Kamin 1969, Schultz 2010). Normally, rewards lose value if they are delayed, or will not suffice to activate dopamine neurons anymore, if repetitively the same reward is present (Schultz 2010). This might explain why we always want more and want it now. Strikingly, drugs of addiction seem to constantly signal a positive prediction error and thus manipulate behavior towards permanent and increased drug consumption (reviewed in Hyman et al. 2006, Bickel et al. 2018). Thus, withdrawal from drugs of addiction is extraordinary difficult. Even more so, if not only a bodily but in addition a mental addiction is manifested (for theories of addiction see Bickel et al. 2018). Dopamine neurons in patients on cold turkey, which is defined as the abrupt and complete cessation of taking the respective drug of addiction, are thought to signal a strong negative prediction error, such that patients suffer from heavily negative feelings and potentially negatively associate external cues with the missing of the drug of addiction they are craving for. A hypoactivity of the dopaminergic system accompanied by a hyperactivation of the stress system is observed on the neural level (reviewed in Solinas et al. 2019). Treatment episodes for patients are usually lengthy and relapse risk is high. This is due to long-term synaptic changes including changes in synaptic weight, CREB induced dendritic spine formation, Delta-FosB production and accumulation, as well as remodelling of synaptic connections across diverse brain structures (reviewed in Hyman et al. 2006, reviewed in Schultz 2017, Solinas et al. 2019). A possible therapy approach could be to make patients continuously pushing aside their drug of addiction, e.g. a cigarette, to make it less desirable and thus weaken the excessive forward reward learning part. According to the ideomotor principle there is mutual causation between psychological occurances and overt behavior (Melcher et al. 2013). Thus, like for the facial feedback hypothesis (‘I smile because I am happy’ versus ‘I am happy because I smile’; McIntosh 1996), moving something

repeatedly towards oneself can make the object desirable, while moving the object away makes it aweful.

Thus, across valence domains, if forward learning is decreased or increased, this changes backward learning to the opposite direction, to be increased or decreased, respectively (and vice versa) The previous examples indicate that any change in this balance can have drastic implications **(Table 2)**. Studying timing-dependent valence reversal is of importance to gain detailed understanding of the properties and the underlying molecular mechanisms of the oppositely-valenced memories.

Table 2. Neuropathological diseases sorted by their imbalance

Punishment: too weak; Relief: too strong	Relief: too weak; Punishment: too strong
Risk-seeking behavior	Post-traumatic stress disorder
Stockholm syndrome	Phobia
Self-cutting injury	Panic disorder
Frustration: too weak; Reward: too strong	Reward: too weak; Frustration: too strong
Addiction	Major depression

Summary and outlook

The surprisingly large variety of complex behaviors paired with genetic tractability brought *Drosophila* to the forefront of behavioral genetics and neurosciences (reviewed in Sokolowski 2001). The impact of dopaminergic modulatory function is universally observed across the animal kingdom. In vertebrates, however, several thousands of dopamine neurons are involved in the reward system and thus the complexity constitutes a considerably difficult barrier to study dopamine function (reviewed in Waddell 2010). The fly dopaminergic system has been comprehensively dissected, prompting that dopamine is the critical signaling molecule necessary for appetitive and aversive associative learning (Schwaerzel et al. 2003, Schroll et al. 2006, Kim et al. 2007, Claridge-Chang et al. 2009, Aso et al. 2010, Aso et al. 2012, Burke et al. 2012. König et al. 2018, Aso and Rubin 2016, Aso et al. 2019, Handler et al. 2019). Although it is not possible to compare vertebrate and invertebrate brain structures directly, novel insights in simple circuit motives in *Drosophila* can be of translational value for vertebrate and even medical research.

The combination of connectomic data and studies of the role of individual neurons, neurotransmitter release, changes at the receptor level or downstream signaling cascades, helps to generate a comprehensive circuit model of the mushroom body, the center for learning and memory in flies. The present study contributes to this model by adding behavioral and optogenetic analyses of reinforcement learning. More specifically, by contributing a parametric investigation of lean one-trial learning and by describing the role of single dopamine neurons in timing-dependent valence reversal. The further characterization of oppositely-valenced memories suggests different molecular

mechanisms for forward versus backward memory types, respectively. These mechanisms remain yet to be elucidated, not only in DANs that do support valence reversal, but also in the DANs that specifically do not support valence reversal. Thus, future research should address the necessity of dopamine as well as the involvement of potential cotransmitters, the function of different dopamine receptor types and downstream pathways.

Although the mushroom body has been comprehensively studied over the past decades, many major questions still demand further investigations. Taking a closer look at the so far discovered connections within the mushroom body, it seems like ‘everyone’ is talking to ‘everyone’. What sounds like recipe for chaos could actually serve to add flexibility and computational power to the neural network. It is essential to gain further understanding of the connectivity ‘road map’. This prompts the question why only very few connections do not or only hardly exist, e.g. KC-to-PN, or MBON-to-KC. Indeed, that is just what most likely accounts for the observed polarity. Alarmingly, hemispheric interactions have so far mostly been neglegted and we still lack the understanding of the ‘blueprint’ of the MB, that is, why is the higher-order brain structure exactly shaped the way it is? One should reckon with the fact that the geometry of the MB matters, e.g. the way the odor information travels from the calyx through the different compartments. Future research should also revisite the question where plastic changes occur. The commonly accepted hypothesis suggests the KC-to-MBON synapse as the place of where the memory engram resides, however, differential plasticity at the level of single dendritic spines remains to be investigated.

Analyzing and understanding principles of neural circuits depicts a research endeavour with long-term translational value, not only for across species translational approaches, but also for modeling of artificial neural networks, or robotics. Connectivity, such as feedback and feedforward motives, convergence versus differgence, voltage changes, changes in synaptic weigth to name but a few, can be inspiring for engineering. Disentangling the role of individual neurons in neural networks might expedite neuromorphic engineering, for example machine learning and bioinspired learning rules in artificial neural networks. Their underlying algorithms approach problems by learning rules from data. Thus, data on individual neurons within a network will help to make machine learning more predictive and in turn stimulate new research ideas (Obermeyer and Emanuel 2016, Dasgupta et al. 2017, Pfeiffer and Pfeil 2018).

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Data documentation

The behavioral raw data are documented on the enclosed data storage device provided along with the final version of the Thesis.

Abbreviations in alphabetical order

ABBREVIATION	FULL NAME
5-HT	serotonin
AD	transcription-activation domain
AL	antennal lobe
AM	n-amylacetate
amn	amnesic
AN	antennal nerve
APL neuron	anterior paired lateral neuron
ARA	arabinose
ASP	aspartic acid
AstA	allatostatin A
BH	Bonferroni-Holm
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
ChR	channelrhodopsin
CR	conditioned response
CREB	cAMP-responsive element binding (transcription factor)
CRISPR/Cas9	clustered regularly interspaced palindromic repeats/CRISPR associated
CS	conditioned stimulus
DAN	dopaminergic mushroom body input neuron
DAT	dopamine active transporter
DBD	DNA-binding domain
dnc	dunce
DO	dorsal organ
DOG	dorsal organ ganglion
Dop1R1	dopamine 1-like receptor 1
Dop1R2	dopamine 1-like receptor 2
Dop2R	dopamine 2-like receptor
DopEcR	dopamine ecdysteroid receptor
DPO	dorsal pharyngeal organ
DPS	dorsal sense organ
EM	empty (odor container)
FB2N	two-step feedback neuron
FBN	one-step feedback neuron
FFN	feedforward neuron
FRU	fructose
GABA	γ-aminobutyric acid
GR	gustatory receptor
GRN	gustatory receptor neuron
HC	head cast
IR	ionotropic receptor
ISI	inter-stimulus-interval
KC	kenyon cell
KW	Kruskal-Wallis
L1	first larval stage
L3	third larval stage
LH	lateral horn
LN	lateral neuron
LTD	long-term depression
LTP	long-term potentiation
mAChR-A	type A muscarinic acetyl-choline receptors

continued

ABBREVIATION	FULL NAME
MAPK	mitogen-activated protein kinase
MB	mushroom body
MBIN	mushroom body input neuron
MBON	mushroom body output neuron
MWU	Man-Whitney U-test
NAC	nucleus accumbens
nAChRs	nicotinic acetyl-choline receptors
NaCl	sodium chloride
NGS	normal goat serum
NMDA	*N*-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
NpHR	halorhodopsin
OAN	octopaminergic mushroom body input neuron
OCT	1-octanol
OR	olfactory receptor
OR83b	orco
ORN	olfactory receptor neuron
OSS	one-sample sign test
PAM	protocerebral anterior medial
PER	proboscis extension reflex
PFA	paraformaldehyde
PFC	prefrontal cortex
PI	performance index
PKA	protein kinase A
PN	projection neuron
pPAM	primary-lineage PAM
PPK	pickpocket
PPL	paired posterior lateral
PPS	posterior sense organ
PREF	preference index
PTSD	post-traumatic stress disorder
QUI	quinine
Rac 1	small G- protein Ras-related C3 botulinum toxin substrate 1
rsh	radish
RT	room temperature
rut	rutabaga
sGC	soluble guanylate cyclase
SN	substantia nigra
sNPF	short-neuropeptide F
SOG	subesophageal ganglion
SOR	sorbitol
STDP	spike timing-dependent plasticity
TH	tyrosine hydroxylase
TO	terminal organ
TRP channel	transient receptor potential channel
UAS	upstream activating sequence
US	unconditioned stimulus
VMAT	vesicular monoamine transporter
VO	ventral organ
VPS	ventral sense organ
VTA	ventral tegmental area

Key resources table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-ChR2 mouse, monoclonal	ProGen Biotechnik, Heidelberg, Germany	Cat. No.: 610180
Cy3 donkey anti-mouse, polyclonal	Dianova, Hamburg, Germany	Art-Nr. 715-165-151 ; RRID: AB_2315777
Cy3 goat anti-mouse, polyclonal	Jackson Immuno Research, Pennsylvania, USA	Art-Nr. 115-165-071 ; RRID: AB_2338687
Alexa Fluor 488 goat anti-horseradish peroxidase, polyclonal	Jackson Immuno Research, Pennsylvania, USA	Art-Nr. 123-545-021 ; RRID: AB_2338965
anti-GFP rabbit, polyclonal	Invitrogen, Carlsbad, USA	A-11122 ; RRID: AB_221569
anti-FAS II mouse, monoclonal	DSHB, Iowa, USA	1D4 anti-Fasciclin II - DSHB ; RRID: B_528235
Alexa 488 goat anti-rabbit, polyclonal	Thermo Fisher Scientific, Waltham, USA	A32731 ; RRID: AB_2633280
Chemicals		
agarose, electrophoresis grade	Roth, Karlsruhe, Germany	CAS: 9012-36-6
D-fructose	Roth, Karlsruhe, Germany	CAS: 57-48-7
n-amylacetate	Merck, Darmstadt, Germany	CAS: 628-63-7
paraffin	AppliChem, Darmstadt, Germany	CAS: 8042-47-5
1-octanol	Merck, Darmstadt, Germany	CAS: 111-87-5
D-arabinose	Sigma-Aldrich, Steinheim, Germany	CAS: 10323
D-sorbitol	Roth, Karlsruhe, Germany	Art-Nr. 6212.2
aspartic acid	Sigma-Aldrich, Steinheim, Germany	CAS: 56-84-8
quinine	Sigma-Aldrich, Steinheim, Germany	CAS: 6119-70-6
sodium chloride	Roth, Karlsruhe, Germany	Art-Nr. 3957.1
Bouin's solution	Sigma-Aldrich, Steinheim, Germany	HT10132
Vectashield	Vector Laboratories Inc., Burlingame, USA	H-1000-10
Triton-X-100	Roth, Karlsruhe, Germany	CAS: 9036-19-5
PFA 4 % (in PBS)	Alfa Aesar, Ward Hill, USA	J19943
NGS	Jackson Immuno Research, Pennsylvania, USA	Art-Nr. 005-000-121
Fly strains		
Csgpu		RRID:DGGR_105666
UAS-ChR2-XXL	Dawydow et al. 2014	Bloomington Stock Center no. 58374; RRID:BDSC_58374
SS02180-Gal4 (covering DAN-f1)	Eschbach et al. 2020	kindly provided by HHMI Janelia Farm; RRID: N/A
SS01716-Gal4 (covering DAN-g1)	Eschbach et al. 2020	kindly provided by HHMI Janelia Farm; RRID: N/A
MB328b-Gal4 (covering DAN-d1)	Eschbach et al. 2020	kindly provided by HHMI Janelia Farm; RRID: N/A
SS00864-Gal4 (covering DAN-i1)	Saumweber et al. 2018	kindly provided by HHMI Janelia Farm; RRID: N/A
w^{1118}		Bloomington Stock Center no. 3605, 5905, 6326; RRID: N/A
attP40/attP2	Pfeiffer et al. 2010	kindly provided by HHMI Janelia Farm; RRID: N/A
pJFRC2-10xUAS-IVS-mCD8::GFP	Pfeiffer et al. 2010	kindly provided by HHMI Janelia Farm; RRID: N/A
Software		
Fiji Image-J		RRID:SCR_002285
Statistika 13	StatSoft Inc., Tulsa, USA	RRID:SCR_014213
Corel Draw X6	Corel Corporation, Ottawa, Canada	RRID:SCR_013674
Other		
Petri dish 9 cm inner diameter	Sarstedt, Nümbrecht, Germany	Art.Nr.: 82.1472
micro scissors	Fine Science Tools GmbH, Heidelberg, Germany	No. 15002-08
pinceers 55 (for larval whole mount preperation)	Fine Science Tools GmbH, Heidelberg, Germany	11255-20

Supplemental data

I - Single-trial learning with appetitive and aversive tastant reinforcement in larval *Drosophila*

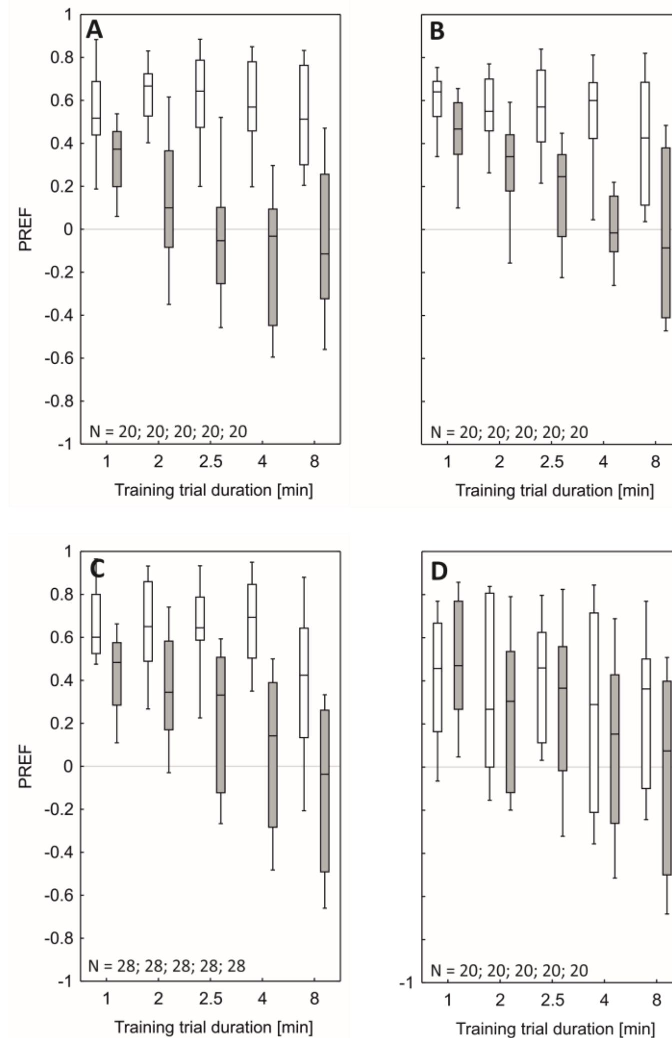

Figure S1: Preference scores underlying the performance indices from Figure 4. Documentation of preference scores (PREFs) after paired (open boxes) and unpaired (boxes with grey fill) training for the indicated training trial durations and the respective appetitive reinforcer **(A)** fructose, **(B)** arabinose, **(C)** sorbitol and **(D)** aspartic acid. Box plots show the median as the middle line, the 25/75 % quantiles as box boundaries, and the 10/90 % quantiles as whiskers. Sample sizes are indicated within the figure.

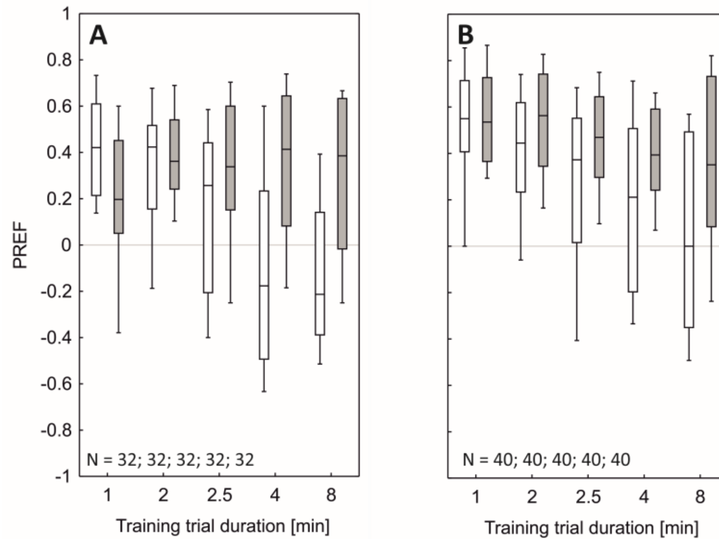

Figure S2: Preference scores underlying the performance indices from Figure 5. Documentation of preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the indicated training trial durations and the respective aversive reinforcer **(A)** high-concentration salt and **(B)** quinine. Box plots show the median as the middle line, the 25/75 % quantiles as box boundaries, and the 10/90 % quantiles as whiskers. Sample sizes are indicated within the figure.

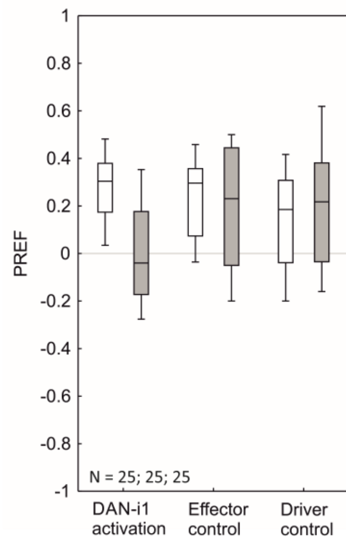

Figure S3: Preference scores underlying the performance indices from Figure 6A. Documentation of preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the experimental genotype (EXP) and both genetic controls (DRI, EFF). Box plots show the median as the middle line, the 25/75 % quantiles as box boundaries, and the 10/90 % quantiles as whiskers. Sample sizes are indicated within the figure.

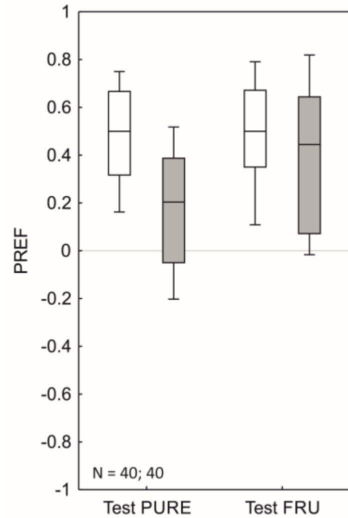

Figure S4: Preference scores underlying the performance indices from Figure 7. Documentation of preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the two groups which were identically trained but tested either on a pure or a fructose substrate. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

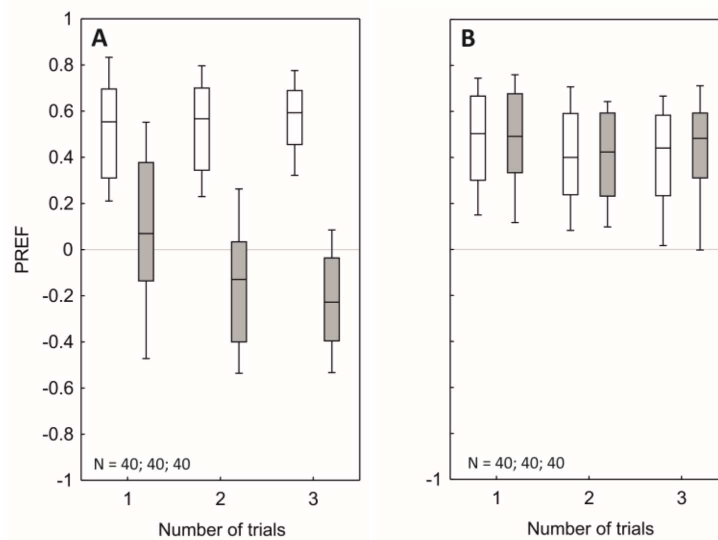

Figure S5: Preference scores underlying the performance indices from Figure 8. Documentation of preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the two groups which were identically trained but tested either on **(A)** a pure or **(B)** a fructose substrate. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

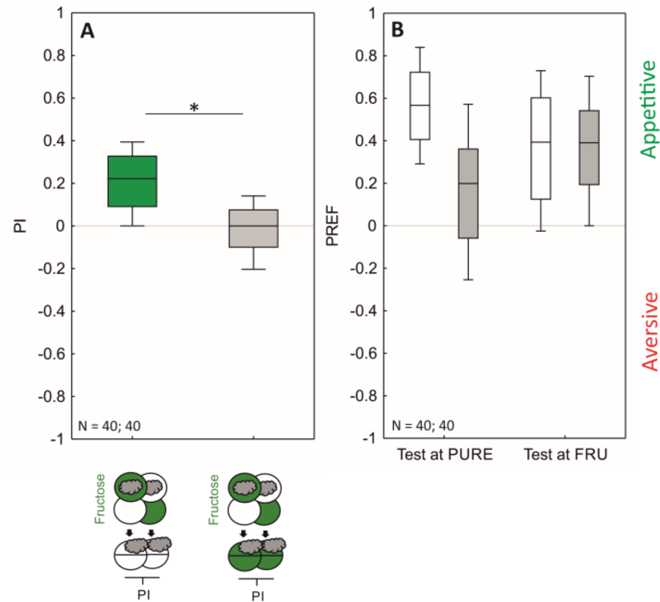

Figure S6: Performance indices and preference scores related to Figure 9C, D. (A, B) Documentation of the Performance indices (PI) (A) and counted preference scores (PREF) (B) for the experiment shown in Figure 9C, D. Preference scores are separated according to paired (open boxes) and unpaired (boxes with grey fill) training, and for testing either on a pure or a fructose substrate. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

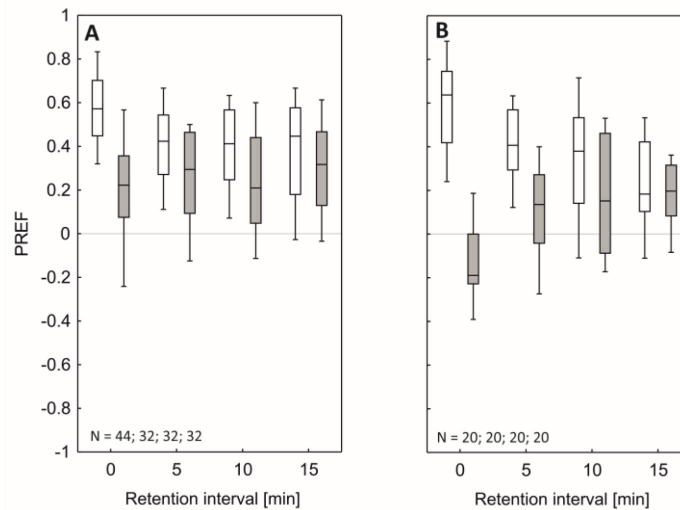

Figure S7: Preference scores underlying the performance indices from Figure 10. Documentation of preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the indicated retention intervals for animals trained **(A)** once, or **(B)** three times. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

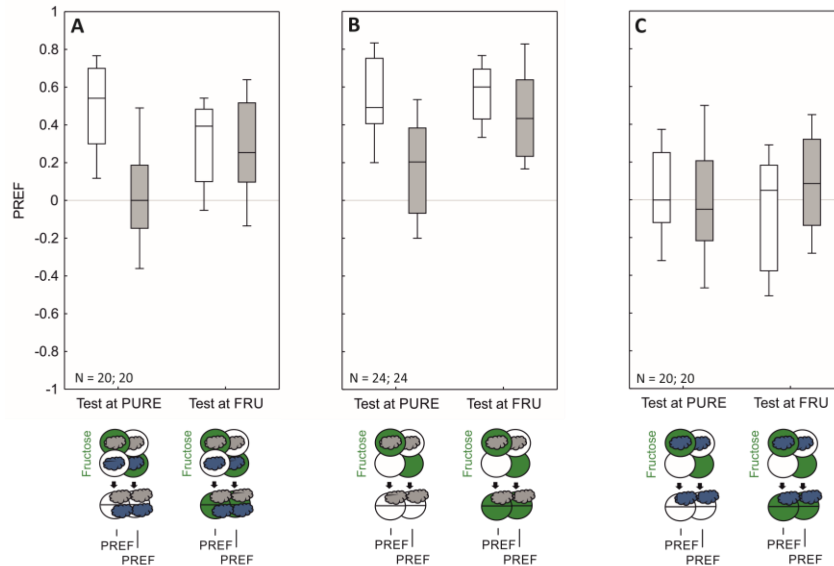

Figure S8: Preference scores underlying the performance indices from Figure 11. Documentation of preference scores (PREF) for the sketched training and testing conditions in **(A)** a two-odor paradigm with n-amylacetate and 1-octanol as odors (grey and blue clouds, respectively), **(B)** a one-odor paradigm with n-amylacetate as odor, or **(C)** a one-odor paradigm with 1-octanol as odor. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. In (A), positive preference scores reflect a relative preference for n-amylacetate rather than 1-octanol as the choice alternative, in (B) they reflect preference for n-amylacetate, and in (C) preference for 1-octanol. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

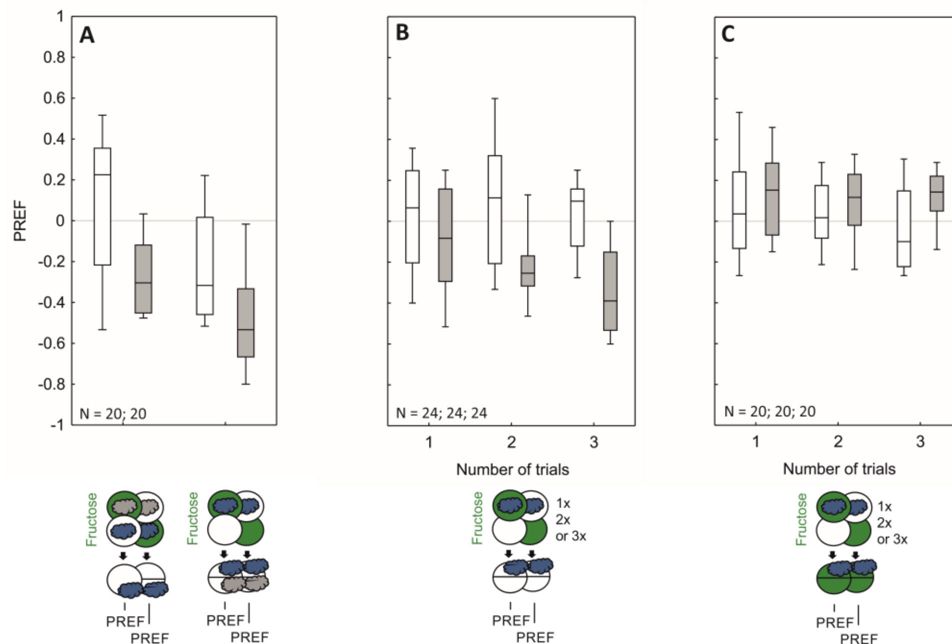

Figure S9: Preference scores underlying the performance indices from Figure 12. Documentation of preference scores (PREF) for the sketched training and testing conditions, namely **(A)** for either differential training or differential testing using n-amylacetate and 1-octanol as odors (grey and blue clouds, respectively) with the testing carried out on a pure substrate, **(B)** for the indicated number of training trials using 1-octanol for training and testing, with testing carried out on a pure substrate, or after the same training regimen but tested on **(C)** a fructose substrate. Green Petri dishes indicate fructose; dishes without fill indicate Petri dishes with only the substrate, i.e. pure agarose, but without any tastant added. In the two left-most plots in (A) positive scores reflect avoidance of 1-octanol; in all other cases positive scores reflect approach towards 1-octanol. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

Supplemental data

II - Timing-dependent valence reversal in the appetitive domain

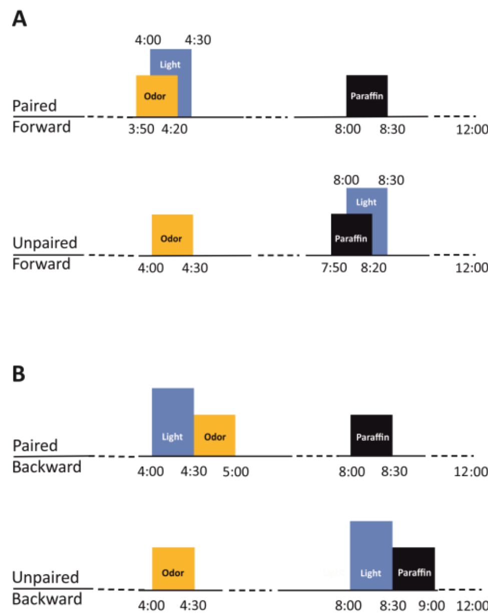

Figure S10. Training procedure. Larvae were trained to associate the odor with the optogenetic activation of the respective DAN (paired), or received odor and DAN activation separately (unpaired). Both odor presentation and DAN activation lasted 30 s. In the paired case larvae received odor presentation and DAN activation at different relative timings (inter-stimulus interval, ISI, defined as the time interval from the onset of DAN activation to the onset of odor presentation). **(A)** Example time-lines for forward conditioning at an ISI of -10 s with the paired presentation (odor-DAN activation) followed by paraffin as the solvent (top row), and for different groups of animals for unpaired training. Of note, the sequence of events during the training trials, i.e. odor-DAN activation or paraffin for the paired case, or odor and paraffin-light for the unpaired case, was reverse in half of the cases. **(B)** As in (A), for backward conditioning at an ISI of 30 s. Yellow rectangles indicate the odor *n*-amylacetate, black rectangles paraffin as the solvent and blue rectangles optogenetic DAN activation. Unless mentioned otherwise, three such 8-min training trials were performed, followed by a test for odor preference.

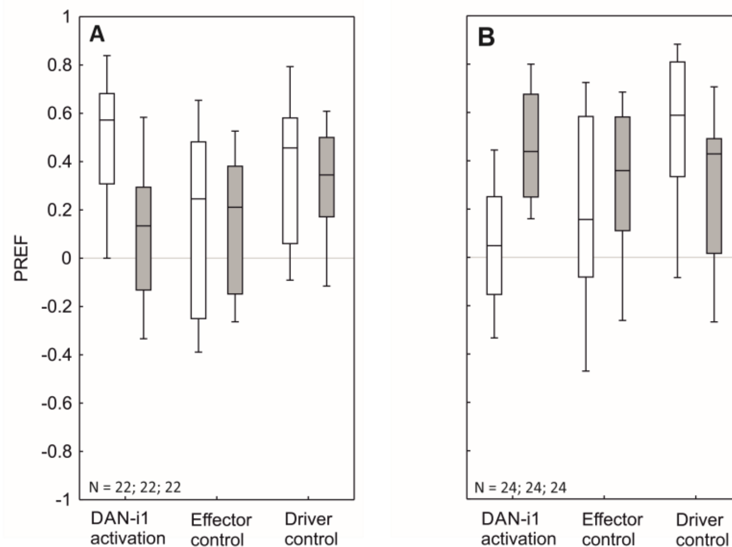

Figure S11. Preference scores for the reciprocally trained groups underlying the performance indices from Figure 13. Preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training for the experimental genotype (EXP) and both genetic controls (DRI, EFF) after **(A)** forward and **(B)** backward conditioning. Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Sample sizes are indicated within the figure.

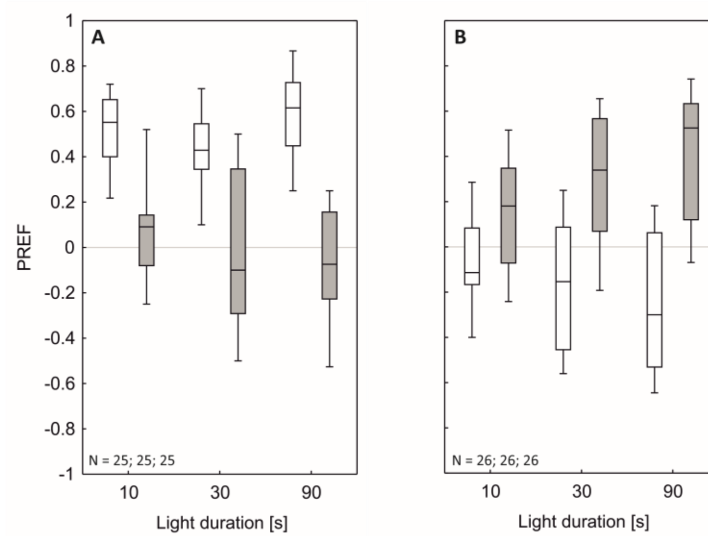

Figure S12. Preference scores for the reciprocally trained groups underlying the performance indices from Figure 14. Preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training after one trial forward **(A)** and backward **(B)** conditioning. Blue light was turned on for either 10, 30 or 90sec. Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Sample sizes are indicated within the figure.

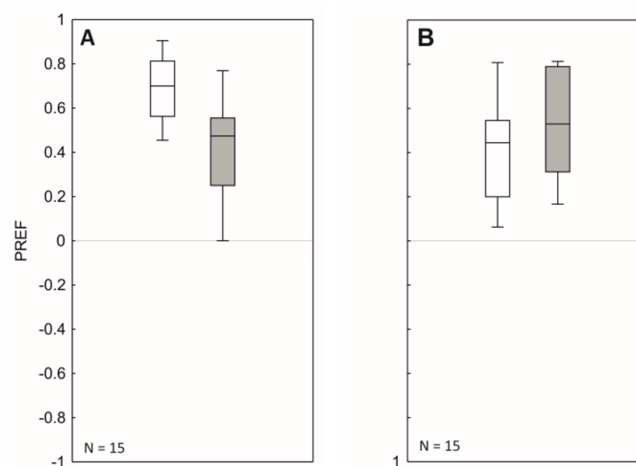

Figure S13. Preference scores for the reciprocally trained groups underlying the performance indices from Figure 15. Preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training after one trial of forward **(A)** and backward **(B)** conditioning. Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Sample sizes are indicated within the figure.

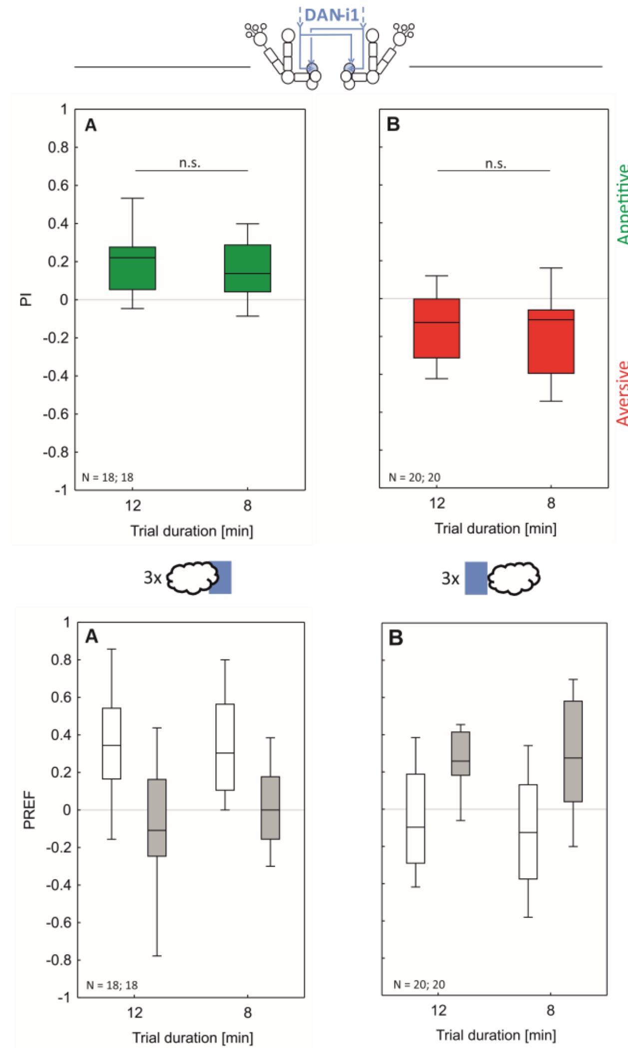

Figure S14. Trial duration has no effect on memory expression after forward and backward conditioning. Larval offspring of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL as the effector strain underwent training with the odor n-amylacetate and optogenetic activation of DAN-f1 by blue light as in Figure 13. One training trial lasted either 8 or 12 min. For the shorter trial duration 2 min at the beginning and end of the trial were cut off, such that the relative intervals between presentations remained unchanged. **(A)** Appetitive reward memory was confirmed regardless of trial duration. **(B)** Similarly, aversive frustration memory was detected after both trial durations, suggesting that a shortened version of the paradigm can be used to safe experimental time **(C,D)** Underlying Preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training after three trial forward (C) and backward (D) conditioning. . Red fill indicates aversive frustration memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$); green fill correspondingly indicates appetitive reward memory. n.s. refers to non-significant Bonferroni-Holm-corrected pairwise comparisons with Mann-Whitney U-tests ($p > 0.05$). Other details as in the legend of Figure 13.

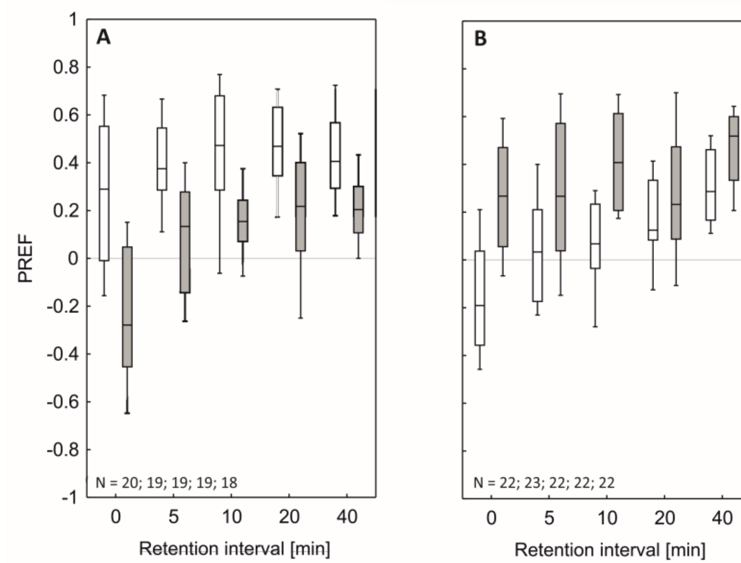

Figure S15. Preference scores for the reciprocally trained groups underlying the performance indices from Figure 16. Preference scores (PREF) after paired (open boxes) and unpaired (boxes with grey fill) training after one trial forward **(A)** and backward **(B)** conditioning for either 10, 30 or 90 s of DAN-activaion. Box plots represent the median as the middle line and 25%/75% and 10%/90% as box boundaries and whiskers, respectively. Sample sizes are indicated within the figure.

Supplemental data

III - Timing-dependent valence reversal in the aversive domain

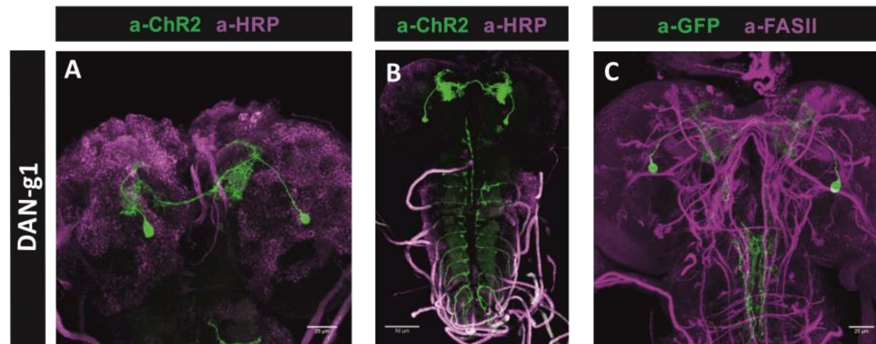

Figure S16. Characterization of the driver strain covering DAN-g1. (A) Whole-mount larval brains were prepared from the offspring of the driver strain covering DAN-g1 (SS01716) crossed to the effector strain UAS-ChR2-XXL. Antibody staining with a primary mouse α-ChR2 antibody and a secondary Cy3 goat α-mouse antibody (green) visualizes the expression pattern of DAN-g1 against a reference background from Alexa 488 α-HRP antibody staining (magenta) in the central brain **(B)** Preparation as in (A) showing the central brain and the ventral nerve cord. Given the strong expression in the ventral nerve cord, this driver strain was not further investigated in behavioral paradigms. **(C)** Whole mounts were prepared from the offspring of the same driver strain crossed to pJFRC2-10xUAS-IVS-mCD8::GFP, combined with a primary rabbit α-GFP antibody and a secondary goat α-rabbit Alexa 488 antibody (green); to discern better the innervation of the respective DANs in the mushroom body a primary mouse α-FAS II antibody and a secondary CY3 goat α-mouse antibody were used. Data were acquired under a confocal microscope with a 63x glycerol objective (A, C), or a 20x objective (B). Scale bars indicate 25 µm (A, C) or 50 µm (B).

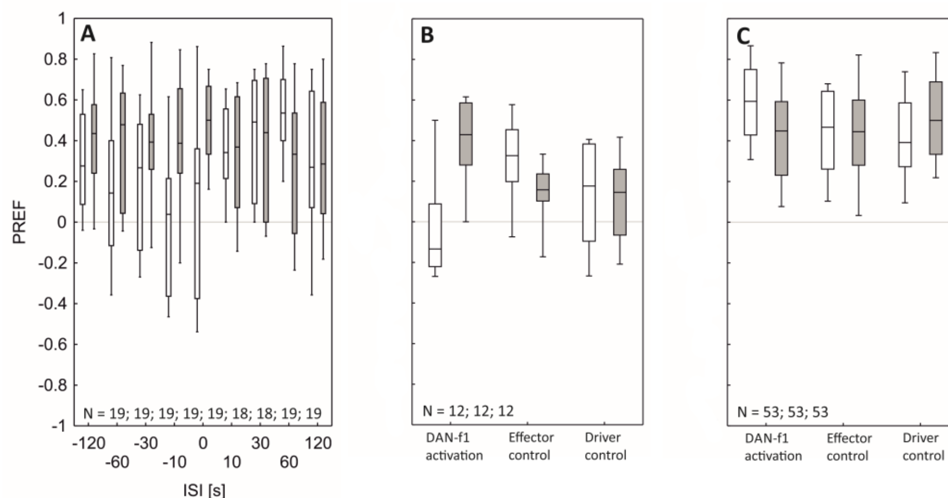

Figure S17. **Preference scores underlying the performance indices (PIs) from Figure 21.** Documentation of preference scores (PREF) after paired training with odor and optogenetic DAN-f1 activation (open boxes) or unpaired training for reference (boxes with grey fill) for the PIs from Figure 21A at the indicated inter-stimulus-interval (ISI) **(A)**, and for the indicated genotypes at ISI= -10 s from Figure 21B **(B)**, and at ISI= 60 s from Figure 21C **(C)**. Data are displayed as box plots, with the median indicated by the middle line, the box boundaries indicating 25 and 75 % quantiles, and the whiskers 10 and 90 % quantiles. Sample sizes are indicated within the figure.

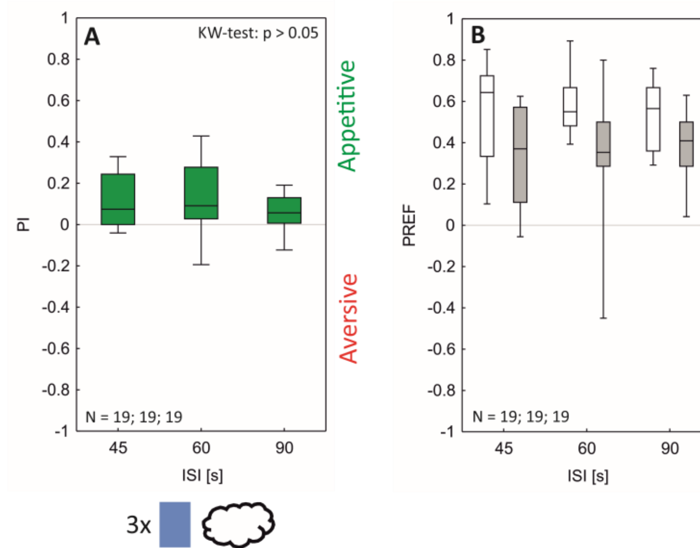

Figure S18. Confirmation of relief memory through backward conditioning with DAN-f1. (A) Larval offspring of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL as the effector strain underwent three training trials comprising backward conditioning of the odor *n*-amylacetate with optogenetic activation of DAN-f1 by blue light, at the indicated inter-stimulus-interval (ISI) of either 45, 60 or 90 s. In all cases, reference groups of larvae received light activation unpaired from the odor. The performance index (PI), as a measure of associative memory, reflects the difference in odor preference after paired versus unpaired training. Positive memory scores reflect appetitive memory. A Kruskal-Wallis test across groups was not significant ($p > 0.05$). Green fill indicates appetitive relief memory relative to chance levels (PI = 0) with Bonferroni-Holm-corrected one-sample sign tests ($p < 0.05$). The training procedure is indicated in the sketch at the bottom of (A): the blue rectangle indicates blue light for optogenetic activation of DAN-f1; white clouds indicate the odor *n*-amylacetate. **(B)** Documentation of preference scores (PREF) after paired training (open boxes) and unpaired training (boxes with grey fill) underlying the PI scores in (A). Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

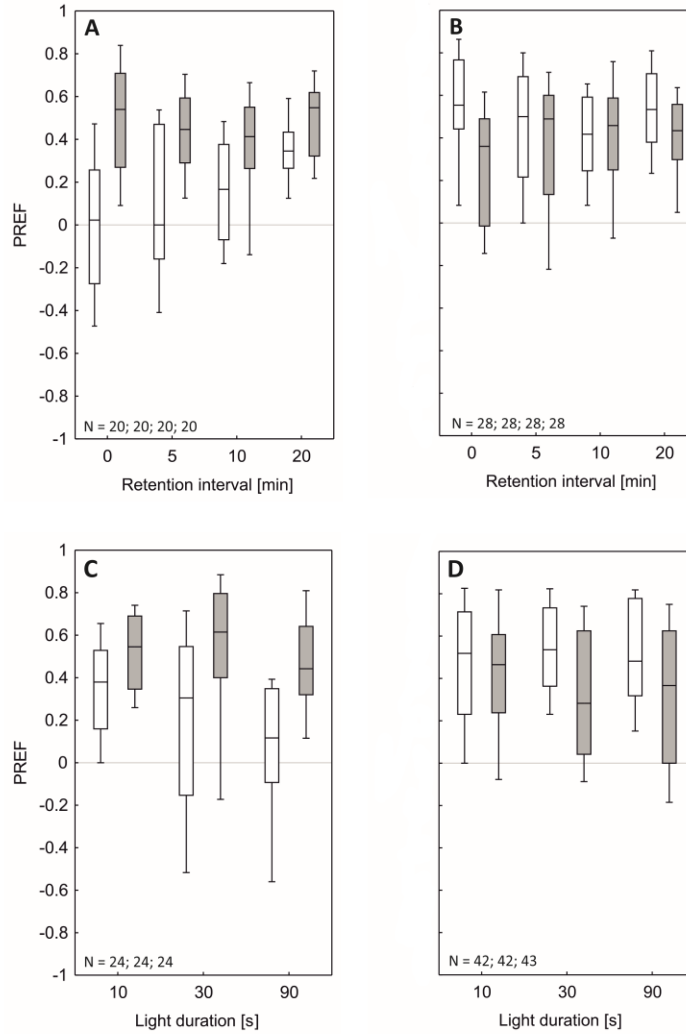

Figure S19. Preference scores underlying the performance indices (PIs) from Figure 22. Documentation of preference scores (PREF) after paired training with odor and optogenetic DAN-f1 activation (open boxes) and unpaired training for reference (boxes with grey fill) underlying the PIs from Figure 22A-D **(A-D)**. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

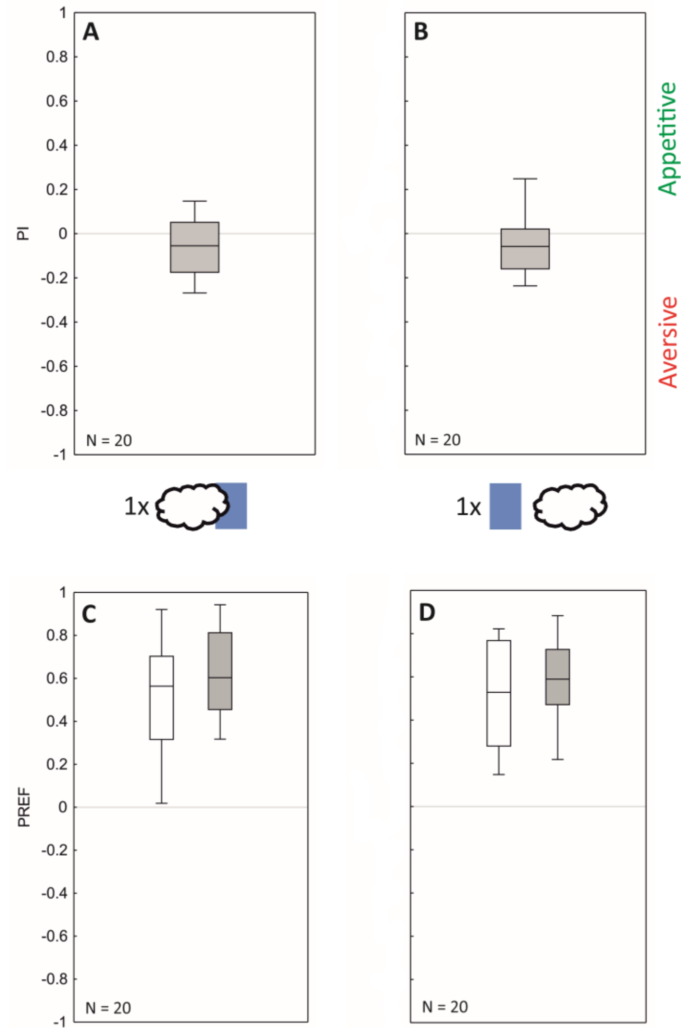

Figure S20. No one-trial conditioning through DAN-f1 activation. Larval offspring of the driver strain covering DAN-f1 crossed to UAS-ChR2-XXL as the effector strain underwent one training trial pairing the odor *n*-amylacetate with optogenetic activation of DAN-f1 by blue light, either at an ISI of -10 s (forward) or 60 s (backward). In all cases, reference groups of larvae received light activation unpaired from the odor. The performance index (PI), as a measure for associative memory, reflects the difference in odor preference after paired versus unpaired training. Positive PIs reflect appetitive memory, whereas negative PIs reflect aversive memory. **(A)** No aversive punishment memory was detectable after one-trial forward conditioning, **(B)** and also no appetitive relief memory was observed after one-trial backward conditioning. The training procedure is indicated in sketches at the bottom of the figures. Blue rectangles indicate the blue light for optogenetic activation, white clouds the odor *n*-amylacetate. **(C, D)** Documentation of preference scores (PREF) after paired (open boxes) and unpaired training (boxes with grey fill) underlying the PIs from (A, B). Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

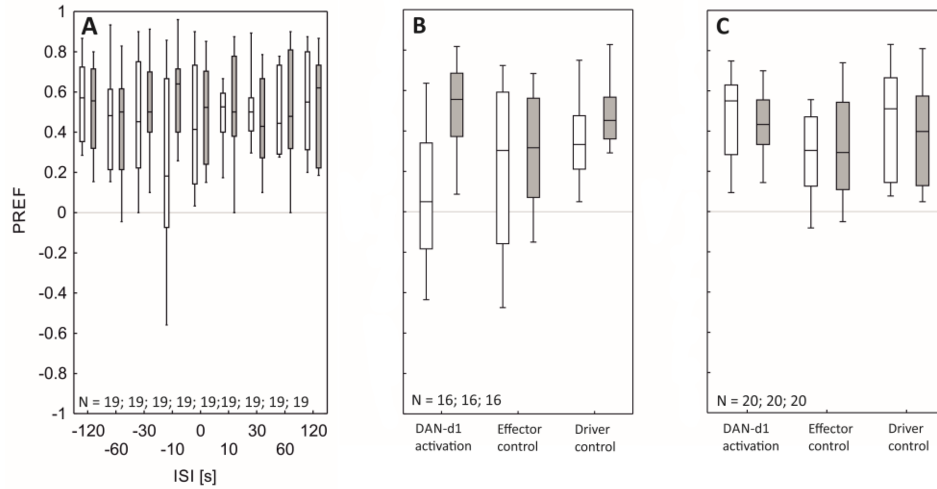

Figure S21. Preference scores underlying the performance indices (PIs) from Figure 23. Documentation of preference scores (PREF) after paired training with odor and optogenetic DAN-d1 activation (open boxes) or unpaired training for reference (boxes with grey fill) for the PIs from Figure 23A at the indicated inter-stimulus-interval (ISI) **(A)**, and for the indicated genotypes at ISI= -10 s from Figure 23B **(B)**, and at ISI= 30 s from Figure 5C **(C)**. Data are displayed as box plots, with the median indicated by the middle line, the box boundaries indicating 25 and 75 % quantiles, and the whiskers 10 and 90 % quantiles. Sample sizes are indicated within the figure.

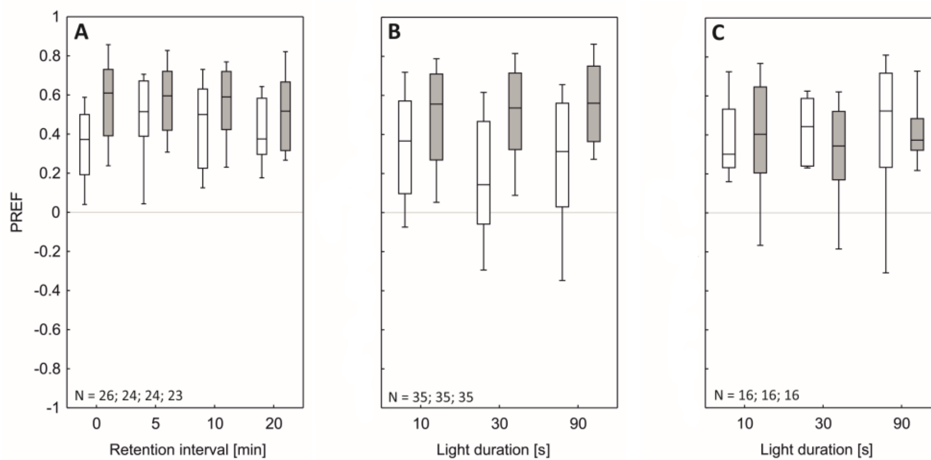

Figure S22. Preference scores underlying the performance indices from Figure 24. Documentation of preference scores (PREF) after paired training with odor and optogenetic DAN-d1 activation (open boxes) and unpaired training for reference (boxes with grey fill) underlying the PIs from Figure 24A-C **(A-C)**. Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figure.

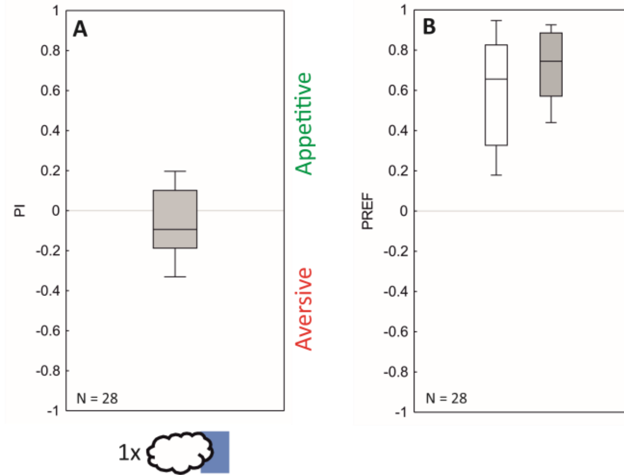

Figure S23. No one-trial conditioning through DAN-d1 activation. Larval offspring of the driver strain covering DAN-d1 crossed to UAS-ChR2-XXL as the effector strain underwent one training trial pairing the odor *n*-amylacetate with optogenetic activation of DAN-d1 by blue light at an ISI of -10 s (forward), whereas reference groups of larvae received light activation unpaired from the odor. The performance index (PI), as a measure for associative memory, reflects the difference in odor preference after paired versus unpaired training. Positive PIs reflect appetitive memory, whereas negative PIs reflect aversive memory. **(A)** No aversive punishment memory was detectable after one training trial. The training procedure is indicated at the bottom of the figure. The blue rectangle indicates the blue light for optogenetic activation, white clouds the odor *n*-amylacetate. **(B)** Documentation of preference scores (PREF) after paired (open boxes) and unpaired training (boxes with grey fill) underlying the PIs from (A). Data are displayed as box plots, with the median as the middle line, the box boundaries as 25 and 75 % quantiles, and the whiskers as 10 and 90 % quantiles. Sample sizes are indicated within the figures.

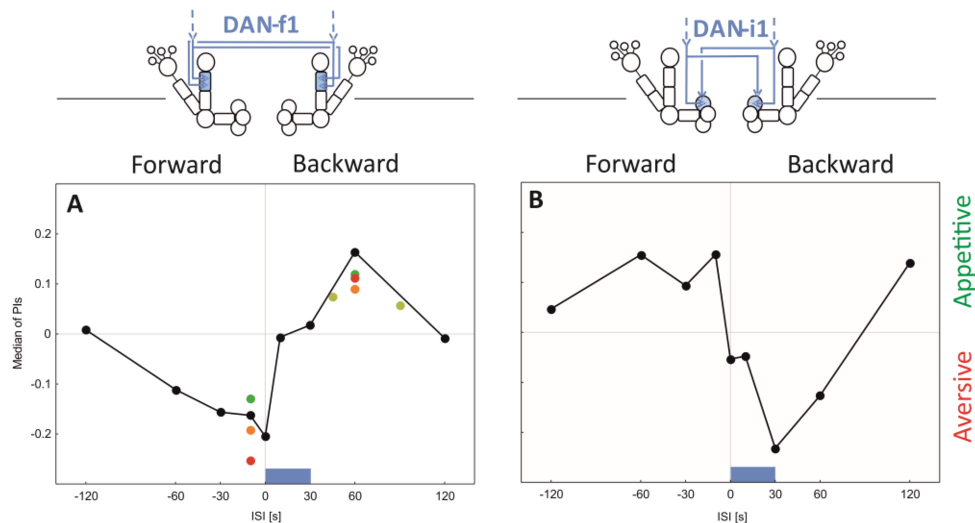

Figure S24. The temporal fingerprints of DAN-f1 and DAN-i1 teaching signals. (A) Same as in Figure 26A. That is, for optogenetic activation of DAN-f1 (sketched at the top) the median PI scores obtained in this study are plotted against the timing of this activation in relation to odor presentation (the inter-stimulus-interval, ISI) (black Figure 21A; orange Figure 21B, C; red Figure 22A, B; green Figure 22C, D; beige Figure S18A, in all cases of the experimental genotype and the standard training procedure). **(B)** As in (A), for DAN-i1 (Saumweber et al. 2018; loc. cit. Fig. 6A).

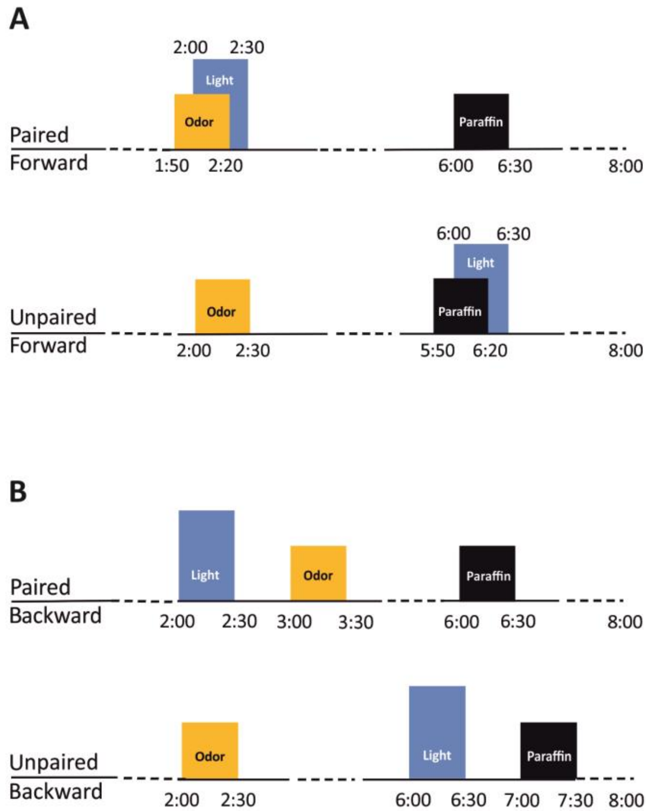

Figure S25. Training procedure. Larvae were trained to associate the odor with the optogenetic activation of the respective DAN (paired), or received odor and DAN activation separately (unpaired). Both odor presentation and DAN activation lasted 30 s. In the paired case larvae received odor presentation and DAN activation at different relative timings (inter-stimulus interval, ISI, defined as the time interval from the onset of DAN activation to the onset of odor presentation). **(A)** Example time-lines for forward conditioning at an ISI of -10 s with the paired presentation (odor-DAN activation) followed by paraffin as the solvent (top row), and for different groups of animals for unpaired training. Of note, the sequence of events during the training trials, i.e. odor-DAN activation or paraffin for the paired case, or odor and paraffin-light for the unpaired case, was reverse in half of the cases. **(B)** As in (A), for backward conditioning at an ISI of 60 s. Yellow rectangles indicate the odor *n*-amylacetate, black rectangles paraffin as the solvent and blue rectangles optogenetic DAN activation. Unless mentioned otherwise, three such 8-min training trials were performed, followed by a test for odor preference.

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