Changing expectations:
Cognitive flexibility and reward processing in larval *Drosophila*

Thesis

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Summary

How brains function to generate appropriate behaviours is a key question for neuroscientists. The larva of *Drosophila melanogaster* is emerging as a powerful study case for investigating the neural circuits underlying behaviour. Critically, the numerical simplicity of the larval nervous system has recently allowed its complete synapse connectome to be reconstructed, revealing unexpected circuit complexity. Examining the functions of the newly discovered circuit motifs through learning tasks more complex than hitherto employed now represents a major step. The present thesis deals with this challenge. In the first part, I establish a reversal learning paradigm, a non-elemental learning task where larvae are trained to reverse their behaviour according to previously learned contingencies. Reversal learning is demonstrated in a surprisingly quick, one-trial contingency adjustment, both in the appetitive and aversive domain, providing a simple and genetically easily accessible study case of cognitive flexibility. In the second part, I study one of the most complex neurons in the larval brain, the GABAergic anterior paired lateral (APL) neuron. Although APL function has been extensively studied in adults, major differences in APL connectivity between larvae and adults may suggest different functions between developmental stages, calling for a detailed look into APL function in larvae. Using a combination of behavioural analysis, optogenetics, pharmacology, and connectomics, I aim to understand how APL modulates associative olfactory memory. I first provide a detailed account of the structure and connectivity of APL. I further reveal that, surprisingly, activating APL optogenetically has a rewarding effect. Specifically, driving APL together with the presentation of an odour can establish an odour-specific, short-term associative memory. Systemic pharmacological inhibition of dopamine signalling impairs this memory, suggesting the involvement of downstream dopaminergic neurons. These findings thus provide a study case of unexpected behavioural and circuit complexity in an animal as seemingly simple as the *Drosophila* larva.

General introduction

"the present is full of future and filled with past."
Gottfried Wilhelm Leibniz

How brains use past experience to predict the future and organize behaviour is a fundamental question in modern neuroscience. Many attempts have been made to understand how and where specific memory traces (or engrams) are processed by the brain to generate appropriate behavioural actions. However, despite considerable progress in characterizing the underlying molecular and cellular mechanisms, as well as psychological corollaries, a clear understanding of how these processes are integrated and interact within neural circuits to control behaviour is still lacking. The fact that the engrams involve many parts of the brain makes the mapping of memory circuits experimentally challenging — especially in the case of the complex mammalian brain (for a detailed discussion, see Menzel & Benjamin 2013). If anything can, therefore, the relatively simple and tractable nervous systems of invertebrates, combined with a large panel of cutting-edge approaches, might allow these endeavours to be successfully pursued.

Using simple, tractable study cases to relate neural circuits to behaviour

Invertebrate research has contributed substantially to the current knowledge of fundamental neural principles underpinning behaviour. Pioneering studies using invertebrates such as the squid, the crayfish, or the sea slug *Aplysia californica*, led to the discovery of basic principles — further extended to vertebrates — of the ionic bases of action potentials, neurotransmission, neuromodulation, and motor control (for review, see Clarac 2013). These model organisms were considered at the time above all for their limited number of large, identifiable, and experimentally accessible neurons.

Moreover, invertebrates are suitable study cases for learning and memory research. Despite their small brains, many invertebrates possess a large repertoire of behaviours and behavioural flexibility allowing them to adapt to environmental changes. Notably, their learning skills and arguably cognitive abilities have been demonstrated using well-established paradigms. Early studies in *Aplysia* showed that successive tactile stimulations of the siphon decrease the gill-withdrawal response (a defensive reflex) through a non-associative learning process called habituation (Pinsker et al 1970). In contrast, sensitization can be observed when applying a

noxious stimulus (e.g. electric shock) to the *Aplysia*'s tail, leading to a higher gill-withdrawal response upon even mild tactile stimulation of the siphon (Pinsker et al 1970). Invertebrates can also establish predictive links between two stimuli (or events), a form of learning thus qualified as "associative". A common case of associative learning is classical (or Pavlovian) conditioning, where a neutral stimulus (called conditioned stimulus; CS), which usually does not elicit a behavioural response, becomes linked to a biologically pertinent stimulus (called unconditioned stimulus; US). In Pavlov's famous dog experiment, a neutral tone (CS) precedes the presentation of food (US), which naturally triggers a salivary response in the dog; repeated pairing of these two stimuli makes the tone alone, now a predictor of food, sufficient to induce salivation in expectation of food (Pavlov 1927). Whereas such classical conditioning involves two external events, operant (or instrumental) conditioning — another associative type of learning — occurs when a subject associates its own behavioural action with the result of this action (Skinner 1963). The investigation of the neurobiological mechanisms underpinning these various types of learning, pioneered by the reductionist approach of the Nobel Laureate Eric Kandel in *Aplysia*, and further extended to several invertebrate species (e.g. the nematode *Caenorhabditis elegans*, the honey bee *Apis mellifera*, the fruit fly *Drosophila melanogaster*), has yielded major discoveries such as the role of the synaptic plasticity implied by Hebbian learning theories, or the requirement for specific molecular components and signalling cascades for memory consolidation (Markram et al 1997; Bi & Poo 1998). Notably, these mechanisms are conserved across many phyla (for reviews, see Markram et al 2011; Kandel et al 2014; Abraham et al 2019).

Maybe more surprisingly, some invertebrates can perform advanced cognitive tasks formerly believed to be restricted to vertebrates and humans. For instance, adult flies exhibit attentional capacities, and outcome expectation has been demonstrated in larvae (Gerber & Hendel 2006; Sareen et al 2011). Honey bees can rapidly learn concepts such as "sameness" or solve numerical problems such as "more than/less than", providing a particularly enticing entry point for understanding the neuronal basis of cognition in a miniature brain (Giurfa et al 2001; Avarguès-Weber et al 2011; Avarguès-Weber & Giurfa 2013; Avarguès-Weber et al 2014; Howard et al 2018; Buatois et al 2020). As the relevant neuronal processes are not yet fully understood, however, it is uncertain whether the underlying circuit motifs are indeed shared across species.

Thus, invertebrate research has great potential and opens fascinating perspectives for understanding the general principles of how brains work and organize learned behaviour. Invertebrates have been and continue to be inspiring model organisms, mainly due on the one

hand to their experimentally accessible nervous system, and on the other to their behavioural faculties which are complex enough for conceptual comparisons with vertebrates. In particular, transgenic models such as *C. elegans* and *Drosophila*, for which the genome has been fully sequenced (*C. elegans* Sequencing Consortium 1998; Adams et al 2000), offer a wealth of possibilities for studying the processes of learning and memory at genetic, physiological, behavioural, and computational levels, and can thus contribute to an understanding of how these processes function in more complex brains. Having said this, it is worth noting that the advantages associated with the small number of neurons in these seemingly simple animals have not yet even been fully considered.

Drosophila as a powerful study case

Over the past few decades, investigations with the fruit fly *Drosophila melanogaster*, pioneered by the Nobel Prize laureate Thomas Hunt Morgan with his work on heredity, have led to the identification of widely conserved processes (e.g. circadian rhythms, drug dependence, or reward system) (Morgan 1910; Bargiello et al 1984; Zehring et al 1984; Kaun et al 2012; Dvořáček & Kodrík 2021). In addition to being practical to use in the laboratory, the multitude of genetic tools allowing spatiotemporally precise transgene expression, in combination with the large set of available behavioural paradigms, make the fruit fly an invaluable study case for studying biochemical and physiological mechanisms that mediate behaviour or different cognitive processes (Pitman et al 2009; Jennings 2011; Thum & Gerber 2019; Dissel 2020).

More recently, the larva of *Drosophila* – the principal feeding stage of the fly (**Figure 1**) – has proven to be an attractive model, mainly due to its simpler nervous system, which consists of only 10,000 neurons i.e. ten times fewer neurons than in adult flies but sharing a similar architecture (Cobb 1999; Gerber & Stocker 2007; Gerber et al 2009). The slower speed of movement exhibited by the larva, together with a range of behavioural features (e.g. taxes, kineses, or learned goal-directed behaviour) make it a model that balances simplicity and complexity (Cobb 1999; Gomez-Marin et al 2011; Schleyer et al 2015a,b; Thum & Gerber 2019). This is particularly relevant for the present work in the context of learning and memory. Following the pioneering work of Aceves-Pina and Quinn (1979), robust associative learning in larval *Drosophila* has been described since the early 2000s, in particular regarding Pavlovian conditioning using odours as the CS and taste reinforcers as the US (e.g. sugar as reward or high-concentration salt as punishment) (Scherer et al 2003; Gerber & Hendel 2006; Niewalda et al 2008; Saumweber et al 2011; Rohwedder et al 2012; Apostolopoulou et al 2013; Schleyer et al 2015a,b; Widmann et al 2018). Together with a large panel of associative learning

paradigms and the possibilities of *Drosophila* for transgenic manipulation, a single-cell resolution anatomical atlas of the complete larval nervous system is at hand (for more details, see section "The major memory centre: the mushroom bodies"; Li et al 2014; Almeida-Carvalho et al 2017; Eichler et al 2017; Saumweber et al 2018; Eschbach et al 2020a,b). These features establish the larva as an experimentally amenable, yet mnemonically competent study case for an integrative understanding of the circuit principles underlying learned behaviour (Gerber & Stocker 2007; Gerber et al 2009; Widmann et al 2018; Thum & Gerber 2019).

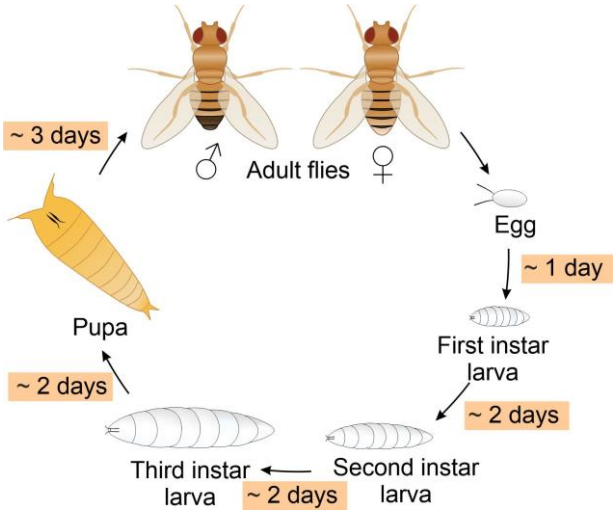

Figure 1. The life cycle of *Drosophila melanogaster*. The life cycle of the fly is about 10 days long at 25°C and comprises the embryonic, larval, pupal, and the adult phase. First-instar larvae hatch approximately one day after the eggs are laid. During the next four days larvae go through the second- and third-instar larval stages. Further, "wandering" third-instar larvae stop feeding and enter pupariation during which they undergo metamorphosis before emerging as adult flies. Adapted from Perveen (2018).

A rich genetic toolbox for dissecting neural circuits

Various genetic tools available for *Drosophila* allow single or small groups of cells to be manipulated with high temporal resolution. A famous approach is the GAL4 - upstream activating sequence (UAS) system, allowing transgene expression in a cell-specific manner (Brand & Perrimon 1993). The GAL4 protein is a yeast transcription factor (originally not expressed in *Drosophila*) and the GAL4 gene can be placed under the control of a cell-specific promoter. In the cells where it is expressed, the GAL4 protein can bind specifically to the UAS sequence as its recognition site. This UAS sequence can be combined with a given gene of interest cloned downstream to it. The GAL4- and the UAS-transgene constructs are kept separate in two distinct parental fly strains: the driver and the effector strains, respectively (**Figure 2A**). Crossing flies from these two lines produces double transgenic progeny, in which the GAL4 protein binds to its UAS promoter and drives transgene expression exclusively in those cells where the GAL4 gene is transcribed (**Figure 2A**). This approach has been further optimized to achieve higher spatiotemporal resolution through, for example, the co-expression of the thermosensitive GAL4 repressor $Gal80^{ts}$ which allows controlled GAL4 expression by heat induction (McGuire et al 2003). In addition, refined cell-type-specific expression is

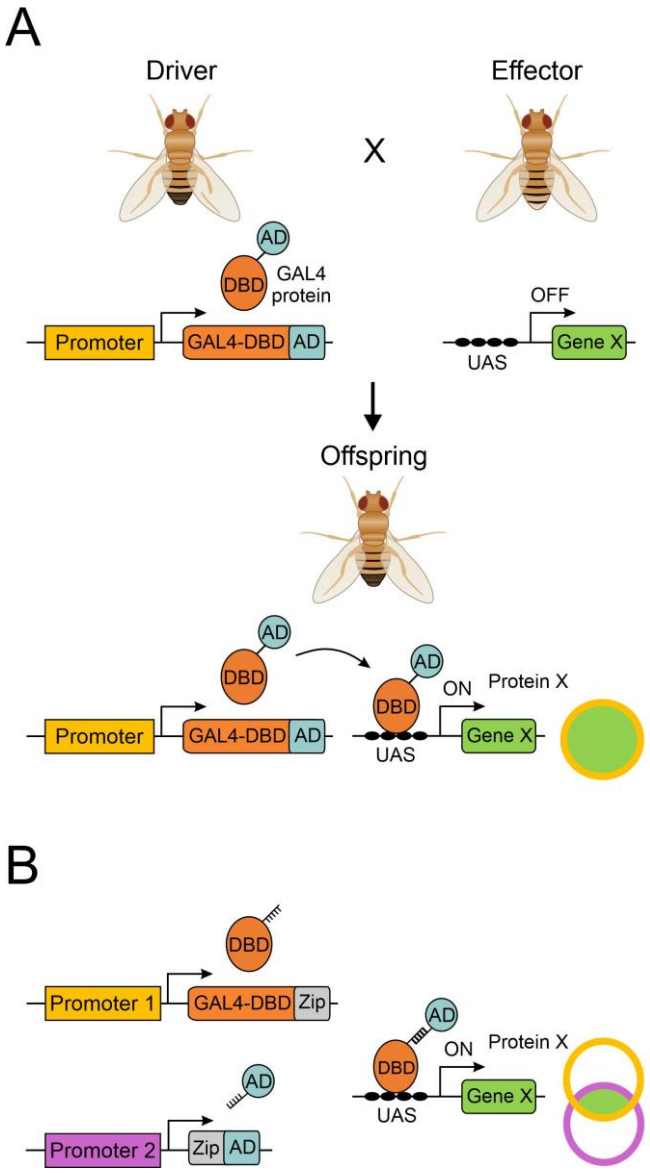

Figure 2. The binary GAL4-UAS system allows transgenes to be expressed in genetically defined sets of cells. (A) The original GAL4-UAS system consists of two separate fly strains: the driver and the effector. In the driver, the yeast GAL4 gene is placed under the control of a cell-specific promoter (yellow); the GAL4 is composed of its DNA-binding domain (DBD) and its activation domain (AD). In the effector, the upstream activation sequence (UAS) promoter can be combined with a particular gene X (green) cloned downstream to it. In the offspring of these two strains, the GAL4-DBD recognizes its UAS while the AD recruits the transcriptional machinery to drive transgene expression in a cell-specific manner, that is, only where the GAL4 is expressed (green filled yellow circle). **(B)** The method described in (A) has been refined by using the split-GAL4 system. In this approach, the coding region of the GAL4 is "split" such that its DBD is under the control of one promoter (yellow) and its AD is under the control of a second promoter (purple). The DBD and the AD are fused to Zip+ leucine zippers and are not able to promote gene expression alone. Only in cells where both promoters are active does the association of Zip+ adapters reconstitute the functional GAL4, thus limiting transgene expression to a few or even single cells (green-filled intersection between yellow and purple circles). Adapted from Luan et al (2020).

permitted through the split-GAL4 method, in which the DNA-binding and transcription-activation domains of the GAL4 gene are under control of two independent enhancers (Luan et al 2006; Pfeiffer et al 2010; Jenett et al 2012). As a result, a functional GAL4 protein is produced only in the cells where both enhancers are active, thus restricting transgene expression to a few or even to single neurons (**Figure 2B**).

A large collection of stable and specific driver strains has been generated, making it possible to express a variety of reporter and effector transgenes to monitor and manipulate the activity of single neurons or groups of neurons (**Box 1** provides few examples; for more details, see Owald et al 2015b). Notably, optogenetics provides tools for manipulating neuronal activity with light in a precise and non-invasive manner (reviewed in Ehmann & Pauls 2020). Larval *Drosophila* allowed for an early use of optogenetics in this model; in particular, the transparent cuticle of the larva is well-suited to controlling neural responses by shining light on awake,

freely behaving animals (for a pioneering study in larvae, see Schroll et al 2006). Various light-sensitive ion channels allow genetically defined cells to be activated or silenced. For instance, neuronal depolarization is permitted by expressing Channelrhodopsin 2 (ChR2), an algal blue-light-activated cation channel, or its variant CsChrimson, a red-light-gated channel (Boyden et al 2005; Nagel et al 2005; Klapoetke et al 2014). Recent improved versions of ChR2 (ChR2-XXM, ChR2-XXL) have been engineered to confer a higher expression level, a longer open state, and not to require additional feeding of the *all-trans* retinal co-factor (Dawydow et al 2014). Light-triggered neural silencing is also possible via the expression of chloride channels, such as Halorhodopsin, a yellow-light- activated pump, or more recently blue or green-light-sensitive GtACR channels (Inada et al 2011; Mauss et al 2017; Mohammad et al 2017).

Box 1. Examples of reporter and effector transgenes allowing to monitor and manipulate neuronal activity

- GCaMP: calcium-sensitive reporter allowing neuronal activity to be monitored (Wang et al 2003; Chen et al 2013).
- RNAi: RNA interference method that allows gene expression to be suppressed (Dietzl et al 2007).
- Kir2.1: inward-rectifier potassium channel that prevents neuronal action potentials, allowing to neuronal activity to be altered (Hodge 2009).
- *shibire*^{ts1}: transiently blocks vesicle endocytosis at restrictive temperature, preventing synaptic transmission with high temporal resolution (Kosaka & Ikeda 1983).
- dTrpA1: temperature-gated calcium channel that triggers depolarization of neurons at restrictive temperature (Rosenzweig et al 2005).
- ChR2: light-gated channel that allows the depolarization of neurons by shining blue light (Dawydow et al 2014).

Furthermore, combining these different approaches makes it possible to functionally interrogate neural circuits. For example, optogenetics or thermogenetics can be coupled with live-cell imaging to induce neuronal activity pre-synaptically and simultaneously visualize neuronal activity post-synaptically (Owald et al 2015b; Ehmann & Pauls 2020). To this end, the UAS-GAL4 approach can be combined with other binary systems following the same principle, including the LexA/lexAop or the QF/QUAS systems (Lai and Lee 2006; Potter et al 2010).

The larval chemosensory system

The capacity to locate food and avoid potentially noxious substances is primordial for survival. As described in Gerber et al (2009), olfaction and taste systems differ in their organization to elicit appropriate behaviour. Whereas olfaction allows animals to track down different resources from which the odours emanate (e.g. food, nest mates), taste intervenes upon physical contact with the targeted source. The variety of odours implies a high profile of discrimination and flexibility, including the capability to attribute a "meaning" to olfactory cues through learning (Gerber et al 2009). In contrast, taste is more hard-wired and gustatory cues are classified into relatively few categories, in the simplest scenario "good/bad" (Gerber et al 2009). Here, both modalities will be of particular interest regarding chemosensory associative learning. The following section therefore briefly describes the chemosensory system in larval *Drosophila* (based on the more exhaustive accounts of Cobb 1999; Gerber & Stocker 2007; Vosshall & Stocker 2007; Gerber et al 2009; Berck et al 2016; Thum & Gerber 2019; Miroschnikow et al 2020).

The overall organization of the larval olfactory system is comparable to that of insects and mammals, but with fewer cells (Cobb 1999; Gerber & Stocker 2007; Vosshall & Stocker 2007; Stocker 2008; Eichler et al 2017; **Figure 3**). Odorants are detected at the level of the larval antenna, the dorsal organ; specifically, its olfactory "dome" sensillum receives the dendritic arbours of 21 cholinergic olfactory receptor neurons[1] (Fishilevich et al 2005; Kreher et al 2008). Notably, the dome is encircled by six further taste sensilla, making the dorsal organ a mixed organ for olfaction and taste (Gerber et al 2009). Each olfactory receptor neuron expresses one type of olfactory receptor, together with the co-receptor ORCO – also called OR83b (Benton 2006; Vosshall & Hansson 2011). The olfactory receptor neuron cell bodies are located within the dorsal organ ganglion, from where the olfactory information is further conveyed to the primary olfactory centre, the antennal lobe (Python & Stocker 2002; Ramaekers et al 2005). The antennal lobe, structurally and functionally analogous to the mammalian olfactory bulb, is compartmentalized into 21 spherical functional units, the glomeruli (Ramaekers et al 2005; Kay & Stopfer 2006; for a detailed account in adult flies, see Grabe et al 2015). Each olfactory receptor neuron projects to a single antennal lobe glomerulus, where it synapses onto local neurons and projection neurons, which are respectively similar to the granule/periglomerular cells and mitral/tufted cells in vertebrates (for review, see Kay & Stopfer 2006). The 14 identified local neurons, which are either GABAergic or cholinergic, establish lateral connections between glomeruli and generate odour-evoked activity patterns within the antennal

[1] All cell numbers are per body side throughout unless mentioned otherwise

lobe (Olsen & Wilson 2008; Chou et al 2010). A recent electron-microscopy reconstruction of the antennal lobe revealed glutamatergic local neurons with unknown function (Berck et al 2016). The projection neurons innervate either only one or several antennal lobe glomeruli and are thus classified as uni- or multi-glomerular, respectively (larvae: Berck et al 2016; adult flies: Bates et al 2020b; Schlegel et al 2021). The 21 larval cholinergic uni-glomerular projection neurons convey the olfactory signal from the antennal lobe to two higher order neuropils: the lateral horn and the mushroom body (Ramaekers et al 2005; Berck et al 2016; adult flies: Lerner et al 2020; preprint by Zheng et al 2020). The 14 multi-glomerular projection neurons identified in larvae project to the lateral horn as well as to various regions within and around the mushroom body (Berck et al 2016). Analogous to the mammalian cortical amygdala and piriform cortex, the lateral horn and the mushroom body have been associated with innate and learned olfactory behaviour, respectively (Heimbeck et al 2001; Heisenberg 2003; Sacco & Sacchetti 2010; Parnas et al 2013; Root et al 2014; Schultzhaus et al 2017; but see Dolan et al 2018; Dolan et al 2019; Chakraborty & Sachse 2021). The mushroom body input region, called the calyx, consists of ~ 34 discrete glomeruli formed by synaptic contacts between the projection neurons and the cholinergic intrinsic mushroom body Kenyon cells (KCs) (Masuda-Nakagawa et al 2005, 2009; Baltruschat et al 2020; Puñal et al 2021). In the calyx, the convergent-divergent connectivity between the projection neurons and the KCs allows combinatorial, specific odour coding and enhances the odour space (Gerber & Stocker 2007; Eichler et al 2017). In addition, reciprocal connections between the KCs and the giant, GABAergic anterior paired lateral (APL) neuron within the calyx preserve the sparse representation of olfactory cues (larvae: Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018; adult flies: Liu & Davis 2009; Lei et al 2013; Lin et al 2014; Amin et al 2020; Puñal et al 2021; see also Chapter II). As mentioned above, these features are largely shared between the larval and adult olfactory system, suggesting an optimal strategy for processing olfactory information (Farris 2015).

Apart from its reduced number and non-redundancy of neurons, the larval olfactory circuit displays specificities as compared to adults, too: maybe most strikingly, almost all olfactory projections remain ipsilateral (Ramaekers et al 2005; Berck et al 2016). Moreover, the larval antennal lobe lacks a convergent-divergent connectivity and a small set of early-born, single-claw KCs establish one-to-one connections with the projection neurons, ensuring a coarse yet complete representation of external cues (Eichler et al 2017). Later during development, the remaining multi-claw KCs get randomly wired with the projection neurons, allowing for combinatorial coding of sensory inputs (Eichler et al 2017). In the mushroom body the

serotonergic dorsal paired median (DPM) interneuron, which is reciprocally connected with the KCs and intermingled with APL in adults, is absent at the larval stage (Eichler et al 2017; Saumweber et al 2018; see also section "The major memory centre: the mushroom body" and Chapter II). Whether indeed these features make the larva relatively less efficient in odour discrimination as compared with adults remains to be tested systematically. Even if this were so, however, its chemosensory system does allow the larva to perform simple olfactory learning tasks, discrimination and generalization (indeed already as first-instar), as well as even more complex tasks, as the present thesis will show (Mishra et al 2010; Pauls et al 2010b; Eschbach et al 2011b; Almeida-Carvalho et al 2017; Mancini et al 2019/Chapter I).

The larval gustatory system is also organized in a numerically reduced manner (Python & Stocker 2002; Apostolopoulou et al 2015; Miroschnikow et al 2018; Miroschnikow et al 2020). Gustatory processing starts from three external and four internal sense organs (**Figure 3A**). As mentioned earlier, at least one of these (the dorsal organ) has a mixed olfactory-taste function but additional thermosensory, mechanosensory and hygrosensory properties of the other structures cannot be ruled out (Klein et al 2015; Apostolopoulou et al 2015; Miroschnikow et al 2018; Miroschnikow et al 2020). The gustatory receptor neurons send their axonal projections from the respective sense organs to multiple compartments of the primary taste centre, the suboesophageal zone (Singh & Singh 1984; Kwon et al 2011). From there, taste information is further conveyed to pre-motor centres to trigger innate gustatory behaviour, and by a – despite recent major advances – yet to be determined wiring logic to modulatory neurons projecting to higher brain centres, including the mushroom body (Colomb et al 2007; Melcher & Pankratz 2005; Masek & Keene 2016; Miroschnikow et al 2018, 2020; **Figure 3A**).

Despite the lower total number of larval gustatory receptor neurons as compared to adults, larvae can detect different gustatory cues (Niewalda et al 2008; Schipanski et al 2008; Cameron et al 2010; El-Keredy et al 2012; Rohwedder et al 2012; Apostolopoulou et al 2014b; Apostolopoulou et al 2015; Schleyer et al 2015a; Toshima et al 2019; Toshima & Schleyer 2019). Critically, cue detection depends on the kind of receptor expressed (reviewed in Apostolopoulou et al 2015) and the mechanisms underlying different aspects of larval feeding behaviour have recently started to be unravelled (Choi et al 2020). In addition, single gustatory receptor neurons can be tuned to different taste modalities and possibly even detect oppositely valenced tastants, a feature not reported in mammals so far van (van Giesen et al 2016; preprint by Maier et al 2020). Although the taste system differs anatomically between insects and mammals, in both phyla gustatory receptor neurons are tuned to classify different taste

substances as either attractive (e.g. sweet or low-salt concentrations) or repellent (e.g. bitter or high-salt concentrations) (for review, see Yarmolinsky et al 2009).

In order to track-down odours larvae exhibits a range of well-described locomotor features (Cobb 1999; Gomez-Marin et al 2012; Gershow et al 2012; Wystrach et al 2016; Tastekin et al 2018; Loveless et al 2019; preprint by Sakagiannis et al 2021). The development of high-resolution tracking systems has allowed to describe how these "microbehaviours" are modulated by olfactory and gustatory cues (Schleyer et al 2015b; Paisios et al 2017; Thane et al 2019). Odorants impact on the frequency and direction of turning manoeuvres, but not the crawling speed; differently, the presence of tastants affects the run speed and probably the rate of turning, but not its direction (Schleyer et al 2015b; Paisios et al 2017) – also note that learnt, but not innate, larval chemotaxis can be modulated by gustatory stimuli (Schleyer et al 2011). Thus, olfactory behaviour seems to be directed towards the odour-source, whereas gustatory behaviour is rather directed to a site-on-body and involves co-mechanosensory processing. Recently, innate olfactory preference (i.e. experimentally naïve) has been shown to be dependent on the feeding state of the animals, illustrating the flexibility of the olfactory system (Vogt et al 2021). Moreover, recent studies suggest that larval foraging behaviour can be modulated by the availability of resources in the environment (Ringo et al 2018; preprint by Wosniack et al 2021).

Overall, the larval chemosensory system is drastically lower in cell number and for the most part without cellular redundancy as compared to its adult counterpart. Moreover, the segregation between olfactory and taste systems seems less strict in the larvae and may suggest different biological needs as larvae live *in* their substrate (Vosshall & Stocker 2007). Also, the larva is characterized by its inexhaustible feeding motivation (and lack of sexual motivation, as far as one can tell), consistent with a higher number of gustatory than olfactory afferents – a situation that is reversed in adults (Vosshall & Stocker 2007; Gerber et al 2009). The reduced speed of locomotion in the larva thus makes it a non-redundant, slower and noisier (i.e. integrated over longer times) model system.

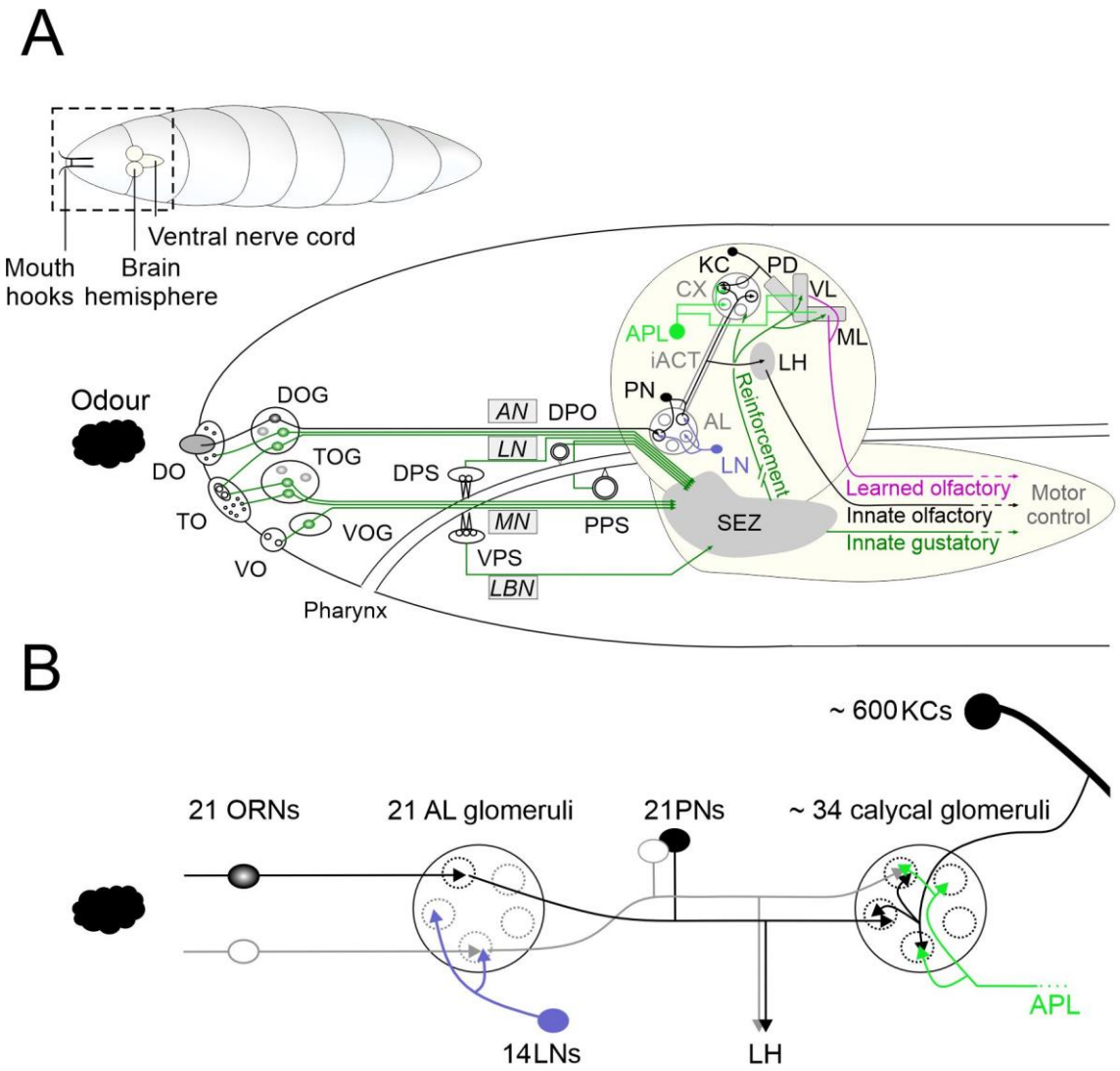

Figure 3. The larval chemosensory system. (A) Schematic overview of the larval body (dorsal view), including the mouth hooks, the brain hemispheres and the ventral nerve cord; the stippled box indicates the region described below. The larval chemosensory system (lateral view) exhibits three external organs (DO, TO and VO) and four internal organs that are located along the pharynx (DPS, VPS, PPS and DPO). Somas of sensory neurons are found in ganglia (DOG, TOG and VOG). A given odour (dark cloud) is detected at the level of the DO, which is composed of its olfactory dome (grey-filled oval) and additional gustatory sensilla (white-filled circles). Olfactory receptor neurons (dark) extend their axonal projections through the antennal nerve (AN) within the antennal lobe (AL). In the AL, lateral neurons (LN; purple) interconnect the AL glomeruli to shape odour-evoked activity. Projection neurons (PN; dark) convey the olfactory information through the inner antennocerebral tract (iACT) from the AL to the mushroom body calyx (CX) and the lateral horn (LH). The LH gives direct inputs to pre-motor centres to promote innate olfactory behaviour, whereas the bypass via the mushroom body is required for learned behaviour. The mushroom body intrinsic Kenyon cells (KC; grey) have their dendrites in the MB calyx (CX) and extend their axons through the peduncle (PD) to constitute the vertical and medial lobes (VL and ML, respectively). The GABAergic anterior paired lateral neuron (APL; light green) broadly innervates the mushroom body and sparsens KC odour-evoked activity. Gustatory afferents (dark green) from the DO, TO, VO, and the pharyngeal organs project via several nerves to the suboesophageal zone (SEZ): the antennal, the labral, the maxillary, and the labial nerve (AN, LN, MN, LBN, respectively). From there, taste information is further conveyed to pre-motor centres to trigger innate gustatory behaviour, and to aminergic modulatory neurons to deliver internal reinforcement signals within the mushroom body. Adapted from Gerber & Stocker (2007). **(B)** The larval olfactory pathway exhibits a similar organization as compared to adult flies, but with fewer neurons. Olfactory receptor neurons (ORNs) project into AL glomeruli (stippled circles). Further, uni-glomerular PNs convey the odour signal to the LH and the CX. Green arrows indicate projections from APL. Filled ovals and circles indicate active neurons; empty ovals and circles indicate inactivity. For clarity, projections from multi-glomerular PNs are omitted. Adapted from Ramaekers et al (2005).

A rich behavioural toolbox for studying associative learning and memory

Beyond smelling and tasting, larvae can associate sensory cues from both modalities. Associative learning is adaptive in that it allows, for example, the anticipation of rewarding or aversive events on the basis of previous experiences. Larval associative learning was first reported using odour-shock conditioning, a Pavlovian paradigm pioneered a few years earlier in adult flies (larvae: Aceves-Pina & Quinn 1979; adult flies: Quinn et al 1974; Tully & Quinn 1985). Animals formerly exposed to an odour (CS) together with punitive electric shock (US) exhibit conditioned avoidance of the punished odour, which now predicts the aversive event. An appetitive odour-sugar version of this paradigm was further developed, requiring starvation before training in the case of adult flies (Tempel et al 1983; Schwaerzel et al 2003; Thum et al 2007). The availability of these paradigms in *Drosophila*, combined with large-scale genetic screens for "learning mutants", has provided insights into the mechanisms underlying associative learning and memory (Zars 2000; Davis 2005; McGuire et al 2005; Keene & Waddell 2007; Pitman et al 2009). During this period, however, the potential of the larva as a suitable model system for learning and memory research received considerably less attention than adult flies did. It is only over the past decade that the larva has been reintroduced as a study case for chemosensory learning (Scherer et al 2003; Neuser et al 2005; Gerber & Stocker 2007; Gerber et al 2009). Through extensive parametric analysis, Gerber and co-workers established quick, simple, and robust learning paradigms that do not require high-cost equipment (Scherer et al 2003; Gerber et al 2004a; Saumweber et al 2011; Michels et al 2017). Larvae are typically trained on agarose-filled Petri dishes that feature odours as the predictive cues (CS) and taste reinforcers (US) added to the agarose substrate (Scherer et al 2003; Saumweber et al 2011; Widmann et al 2018). As in adults, larval learning experiments consist of a two-group reciprocal design, allowing non-associative effects (e.g. handling, odour and taste exposure) to be averaged out (Gerber & Stocker 2007; Widmann et al 2018; adult flies: Tully & Quinn 1985). Whereas some studies initially used individually assayed larvae, current procedures are typically performed *en masse* (~ 30 larvae per group) which reduces the scatter of the data, but has no influence on memory scores in comparison to individual animals (Scherer et al 2003; Neuser et al 2005; Saumweber et al 2011). This is consistent with the apparent lack of task-related social interaction in larvae (Niewalda et al 2014). Larval associative learning was demonstrated using two-odour, differential conditioning in which one odour is reinforced whereas a second, different odour is not (Scherer et al 2003; Neuser et al 2005). A one-odour, absolute version of the paradigm was further introduced, consisting of paired and unpaired presentations of odour and reinforcement (Saumweber et al 2011; Saumweber et al 2018;

Schleyer et al 2018). Various stimuli have been implemented as ecologically valid appetitive and aversive reinforcers (**Box 2** provides some examples; for more details, see Widmann et al 2018), thus showing the capacities of larvae to form associative memories in either valence domain. As for odour-taste memories, those are not only specific to the trained odour identity and intensity, but also rely both on the value and the quality of the reinforcement, and are expressed if their resulting consequences are beneficial to the animals (Craig 1917; Gerber & Hendel 2006; Chen et al 2011; Mishra et al 2013; Schleyer et al 2011; Schleyer et al 2015a,b; for more details, see Schleyer et al 2018). Further analysis of the larval mnemonic capacities has revealed long-term memory and cold-shock-anaesthesia-resistant memory (larvae: Widmann et al 2016; Brünner et al 2020; Eschment et al 2020; adult flies: Quinn & Dudai 1976; Folkers et al 1993; Shyu et al 2017; Scheunemann et al 2019; Siegenthaler et al 2019; Jacob & Waddell 2020; Inami et al 2020; for review, see Roselli et al 2021). Larvae can also form associations after a single training cycle only (one-trial learning: Weiglein et al 2019) and reverse previously learned contingencies (reversal learning: Mancini et al 2019/Chapter I). Whether larvae can perform higher forms of learning (e.g. sensory-preconditioning: Brogden 1939; second-order conditioning: Rescorla 1980; negative patterning: Deisig et al 2001; Durrieu et al 2020; for review see Young et al 2011) remains to be tested. Notably, behavioural analyses are mainly performed using third-instar larvae, which are easier to handle and observe due to their larger size, allowing for high-resolution behavioural tracking and semi-automated conditioning (Schleyer et al 2015b; Paisos et al 2017; Tomasiunaite et al 2018; Thane et al 2019). However, a recent electron microscopy reconstruction of the complete nervous system has been made available for a first-instar larva only, this being much smaller than a third-instar larva and thus relatively quicker to reconstruct (Ohyama et al 2015; Berck et al 2016; Eichler et al 2017; Eschbach et al 2020a,b). To reconcile behavioural and connectomics data, the behavioural faculties observed in third-instar larvae have been tested and confirmed in first-instar larvae as well (Almeida-Carvalho et al 2017).

Thus, the large panel of behavioural paradigms in larvae across development stages, together with the recent connectomics dataset and the plethora of genetic tools, provide unprecedented opportunities for mapping behaviour onto defined circuitry. In particular, well-defined brain structures where smell-taste associations take place have received considerable attention over the last decade. The next section highlights the brain structure that turned out essential in this respect, the mushroom body.

Box 2. Examples of appetitive and aversive reinforcers used in larval associative learning experiments

- Sugars, low concentrations of salt, amino acids, or ethanol can be used as the reward in associative learning (Scherer et al 2003; Hendel et al 2005; Michels et al 2005; Gerber & Hendel 2006; Niewalda et al 2008; Schipanski et al 2008; Rohwedder et al 2012; Schleyer et al 2015a,b; Kudow et al 2017; Toshima et al 2019; Toshima & Schleyer 2019; Schumann et al 2021).

- Bitter-tasting quinine and caffeine, as well as high salt concentrations function as different punishments (Gerber & Hendel 2006; Niewalda et al 2008; Schleyer et al 2011; El-Keredy et al 2012; Apostolopoulou et al 2014b; König et al 2014; Apostolopoulou et al 2016; Widmann et al 2016).

- Aversive memory can also be studied by using non-tastant reinforcers such as electric shock, light, heat, or substrate vibration (Khurana et al 2009; Pauls et al 2010a; Eschbach et al 2011a; Essen et al 2011; Khurana et al 2012; Saumweber et al 2014).

- Different concentrations of the agarose substrate, which can confer a reinforcing effect (Apostolopoulou et al 2014a; Kudow et al 2019)

The major "memory centre": the mushroom body

The mushroom bodies, or *corpora pedunculata*, are paired central brain structures in insects and other arthropods except crustaceans (Strausfeld et al 1998; Heisenberg 2003; Fahrbach 2006; Modi et al 2020). More than 100 years of work in insects has shown the involvement of the mushroom bodies in associative learning and memory; they are thus considered as a "memory centre" of the insect brain and often compared to mammalian brain structures such as the hippocampus, the cerebellum or the piriform cortex (Campbell & Turner 2010; Farris 2011). Remarkably, a role in "intelligent behaviour" was already attributed to the mushroom bodies in 1850 by Felix Dujardin (1801-1860), who discovered and described them for the first time in honey bees and ants. Recent decades have seen an explosion in research on the mushroom bodies, pioneered in honey bees and the fruit fly *Drosophila* (Menzel 1974; Heisenberg 1980; see also Heisenberg & Gerber 2002). In *Drosophila*, the mushroom bodies are essential for associative learning and memory, beyond other biological processes such as sleep, locomotion, decision making, or social behaviour (Davis 1993; Martin et al 1998; Tang & Guo 2001; Heisenberg 2003; Joiner et al 2006; Fiala 2007; Zhang et al 2007; Vogt et al 2014; Vogt et al 2016; Sun et al 2020). Mutationally or chemically ablating of the mushroom bodies impairs learning in adult flies, and expression of proteins that are necessary for associative learning occurs predominantly in these structures (Heisenberg et al 1985; Nighorn et al 1991; Han et al 1992; de Belle & Heisenberg 1994; Zars et al 2000; Qin et al 2012). Furthermore, blockade of

mushroom body output synapses prevents retrieval but not acquisition of associative memory, making the mushroom body the major site for associative memory traces (Dubnau et al 2001; McGuire et al 2001; Schwärzel et al 2003; Gerber et al 2004a; Séjourné et al 2011; for a more recent discussion see Gerber & Aso 2017; but see Ueoka et al 2017; Yamazaki et al 2018).

The mushroom bodies are constituted by tightly packed, cholinergic intrinsic neurons called the Kenyon cells (KCs) (Technau 1984; Aso et al 2009; Barnstedt et al 2016). During development, the proliferation of four mushroom body neuroblasts per hemisphere produces ~ 73 mature embryonic-born KCs in first-instar larvae, and further yields to ~ 2200-2500 KCs in adults (Technau & Heisenberg 1982; Aso et al 2009; Eichler et al 2017; Aso & Rubin 2020; Li et al 2020). Interestingly, the embryonic-born KCs are required for larval appetitive olfactory learning, whereas the larval-born KCs are not likewise necessary and may have no particular role during the larva's life (Pauls et al 2010b). The dendritic branches of the KCs form the mushroom body input region called the calyx and receive olfactory information from the projection neurons (**Figure 3A**, **Figure 4A**). The parallel axonal projections of the KCs extend as a bundle to form the peduncle, and further bifurcate to shape the medial and the vertical lobes (Eichler et al 2017; in adults the organization into the lobes is more complex: Lee et al 1999; Aso et al 2014a; Li et al 2020). The KC axonal fibres are tiled by axonal terminals of modulatory mushroom body input neurons (MBINs) and dendrites of mushroom body output neurons (MBONs), thus establishing distinct compartments (larvae: Eichler et al 2017; Saumweber et al 2018; Eschbach et al 2020a,b; adult flies: Tanaka et al 2008; Mao & Davis 2009; Aso et al 2014a,b; Takemura et al 2017; Li et al 2020). Each of the 11 anatomically defined compartments in larvae typically display 1-3 MBINs and 1-5 MBONs intersecting the axonal projections of the KCs (Eichler et al 2017; Saumweber et al 2018; **Figure 4A**). In both larvae and adults, the MBINs include octopaminergic and dopaminergic neurons (OANs and DANs, respectively), as well as neurons with unknown transmitters (Eichler et al 2017; Saumweber et al 2018; Thum & Gerber 2019). The MBONs have been identified as being either GABAergic, glutamatergic or cholinergic (larvae: Eichler et al 2017; Saumweber et al 2018; Eschbach et al 2020a,b; adult flies: Aso et al 2014b; Li et al 2020). Sensory coding at the KC level is sparsened by the hemispherically single GABAergic anterior paired lateral (APL) neuron, which receives excitatory inputs from, and sends back inhibitory projections to, the KCs (larvae: Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018; adult flies: Lin et al 2014; Amin et al 2020; **Figure 4B, C**). In adults, APL is functionally coupled via gap junctions with the dorsal paired median (DPM) neuron, another interneuron reciprocally connected with the KCs (Pitman et al 2011; Wu et al 2011; Wu et al 2013; Zhang et al 2013). In contrast, DPM is

absent in the larva, suggesting potential differences in APL function between the two development stages (Eichler et al 2017; Saumweber et al 2018). A more detailed description of the structure, connectivity and functions of the larval APL neuron will be provided in Chapter II.

Modulatory OANs and DANs have been shown to mediate reinforcement signals in insect learning (Menzel 2001; Cognigni et al 2018; Adel & Griffith 2020). In honey bees, a single identified OAN called VUMmx1 (for "ventral unpaired median neuron of maxillary neuromere 1") conveys the reward signal to olfactory neuropiles in the bee brain (Hammer 1993). The VUMmx1 neuron receives its input in the suboesophageal zone and projects to the antennal lobe, the mushroom body calyx, and the lateral horn (Hammer 1993). Presenting an odour together with electrical activation of VUMmx1, or with micro-injection of octopamine in the antennal lobe or the calyx, is sufficient to establish an associative appetitive memory for that odour (Hammer 1993; Hammer & Menzel 1998). In *Drosophila*, the homologue of VUMmx1 has been identified in both larvae (sVUMmx1, also called OAN-a2: Eichler et al 2017) and adults (OA-VUMa2: Busch et al 2009). Mutant flies lacking tyramine β-hydroxylase (TβH), the enzyme responsible for octopamine synthesis, are impaired in odour-sugar learning while odour-shock learning remains unaffected (Schwaerzel et al 2003). In contrast, blocking synaptic output from a relatively large set of DANs covered by the TH-GAL4 driver impairs aversive learning, but leaves appetitive learning intact (Schwaerzel et al 2003). Consistently, optogenetic activation of OANs (using TDC-GAL4) or DANs (using TH-GAL4) can confer positive or negative reinforcement, respectively (larvae: Schroll et al 2006; Honda et al 2014; adult flies: Claridge-Chang et al 2009, Burke et al 2012) (note that as yet there is no demonstration of a rewarding effect of the mentioned VUMmx1-homologue in *Drosophila*). These findings suggested that TDC-OANs were therefore only involved in appetitive reinforcement, whereas TH-DANs were only responsible for aversive signalling, in line with reports in other insects (Unoki et al 2005; Unoki et al 2006; Vergoz et al 2007; Tedjakumala & Giurfa 2013; Mizunami & Matsumoto 2017; but see Mancini et al 2018; Vieira et al 2018). However, this seemingly strict division of labour needed to be reconsidered when it turned out that a mutation in the dopaminergic DopR1 receptor (also known as dDA1) alters both appetitive and aversive learning (larvae: Selcho et al 2009; adult flies: Kim et al 2007; Qin et al 2012). Furthermore, a specific set of DANs that is not completely covered by TH-GAL4 was found to mediate reward signalling in both larval and adult *Drosophila* (larvae: Rohwedder et al 2016; adult flies: Burke et al 2012; Liu et al 2012). In larvae, this subset consists of four identified DANs from the primary protocerebral anterior medial (pPAM) cluster innervating the medial lobes of the

mushroom bodies (Rohwedder et al 2016; Eichler et al 2017; Saumweber et al 2018; only one of these pPAM neurons is covered in TH-GAL4: Selcho et al 2009; M. Schleyer: personal communication). The pPAM neurons are necessary for larval appetitive but not aversive learning (Rohwedder et al 2016). Moreover, within the pPAM cluster some DANs integrate the nutritive value of sugars, whereas others act downstream of OANs to convey the sweet taste of sugars (larvae: Selcho et al 2014; Rohwedder et al 2016; adult flies: Burke & Waddell 2011; Burke et al 2012; Ichinose et al 2015; Musso et al 2015; Huetteroth et al 2015; Yamagata et al 2015). More recently, refined transgene expression provided by split-GAL4 drivers revealed two of these pPAM neurons (DAN-i1 and DAN-h1) to be individually reward-inducing upon their optogenetic activation in larvae (Saumweber et al 2018; Weiglein et al 2019; Schleyer et al 2020; Eschbach et al 2020a; Weiglein et al 2021). In contrast, three DANs outside the pPAM cluster and innervating the peduncle and the vertical lobe (DAN-d1, DAN-f1 and DAN-g1) were shown to act individually as a punishment (Eschbach et al 2020a; Weiglein et al 2021). This segregation between non-overlapping subsets of rewarding and punishing DANs innervating different compartments along the mushroom body lobes parallels the situation in adults (Claridge-Chang et al 2009; Mao & Davis 2009; Aso et al 2010; Burke et al 2012; Liu et al 2012; Galili et al 2014; Yamagata et al 2015; Boto et al 2019; Adel & Griffith 2020; Boto et al 2020; but see Aso et al 2012; Yamagata et al 2016); strikingly, however, the number of putative rewarding DANs is massively lower in the larva than in its adult counterpart: 4 pPAMs and 83 PAMs, respectively (larvae: Eichler et al 2017; Saumweber et al 2018; adult flies: Aso et al 2014a; Li et al 2020). Thus, distinct sets of DANs provide reinforcement signals of opposite valence to specific target areas in the mushroom body. Analogously, in mammals aversive DANs project to the medial prefrontal cortex and the rostromedial tegmental area, whereas rewarding DANs project to the nucleus accumbens (Lammel et al 2011; Lammel et al 2012; Lammel et al 2014). Therefore, the twofold function of dopamine in *Drosophila* mirrors the situation in mammals and may indeed reflect cross-species principles (Waddell 2013; Groessl et al 2018; Menegas et al 2018; de Jong et al 2019; Adel & Griffith 2020).

As discussed in more detail below, the current working model, which has been built largely upon studies in adult flies, suggests that the coincidence of the odour signal and the valence signal takes place in the specific set of odour-activated KCs, and within the pertinent mushroom body compartment. Such coincidence induces synaptic changes (or a memory trace) at the KC-to-MBON synapses (Séjourné et al 2011; Placais et al 2013; Bouzaiane et al 2015; Cohn et al 2015; Hige et al 2015; Perisse et al 2016; Hattori et al 2017; Berry et al 2018; Handler et al 2019; Zhang et al 2019). The MBONs are connected to the pre-motor system and classified as

either approach- or avoidance-promoting; an alteration in the synaptic weight of the KCs onto the MBON network (initially balanced in untrained animals) skews the animal's behaviour towards approach or avoidance, depending on previous experiences (Aso et al 2014b; Owald et al 2015a; Owald & Waddell 2015; Perisse et al 2016; Eschbach et al 2020b). For instance, odour-shock learning depresses synapses between odour-specific KCs and approach-promoting MBONs, such that a subsequent presentation of the trained odour triggers appropriate odour-driven avoidance (Owald & Waddell 2015; Hige et al 2015; Berry et al 2018). The same rationale applies for appetitive learning, yet in separate mushroom body compartments: alterations in KC-to-MBON pathways that mediate avoidance shift the balance towards conditioned approach (Aso et al 2014b; Hige et al 2015; Owald et al 2015a; Felsenberg et al 2018; Felsenberg 2021; **Figure 4C**). Thus, individual mushroom body compartments can be seen as functional units integrating (i) memory traces localized in the KCs, (ii) different value signals conveyed via OANs/DANs, and (iii) adaptive pre-motor commands across the MBON network (Heisenberg 2003; Davis 2011; Cognigni et al 2018; Thum & Gerber 2019; Aso & Rubin 2020; Eschbach et al 2020a,b; **Figure 4C**). It is precisely this triadic integration of past (KC-MBON synapse strength), present (input to KCs, OANs and DANs), and future (output from MBONs) that makes the mushroom body an attractive study case for brain research – echoing the introductory quote from Leibniz.

Recent electron-microscopy reconstructions of the central nervous system of larval and adult *Drosophila* have allowed the mapping of full, annotated, synaptic-resolution connectomes of several brain structures, including the mushroom body (larvae: Berck et al 2016; Eichler et al 2017; Eschbach et al 2020a,b; adult flies: Takemura et al 2017; Franconville et al 2018; Zheng et al 2018; Li et al 2020; Hulse et al 2020; Scheffer et al 2020; Schlegel et al 2021).

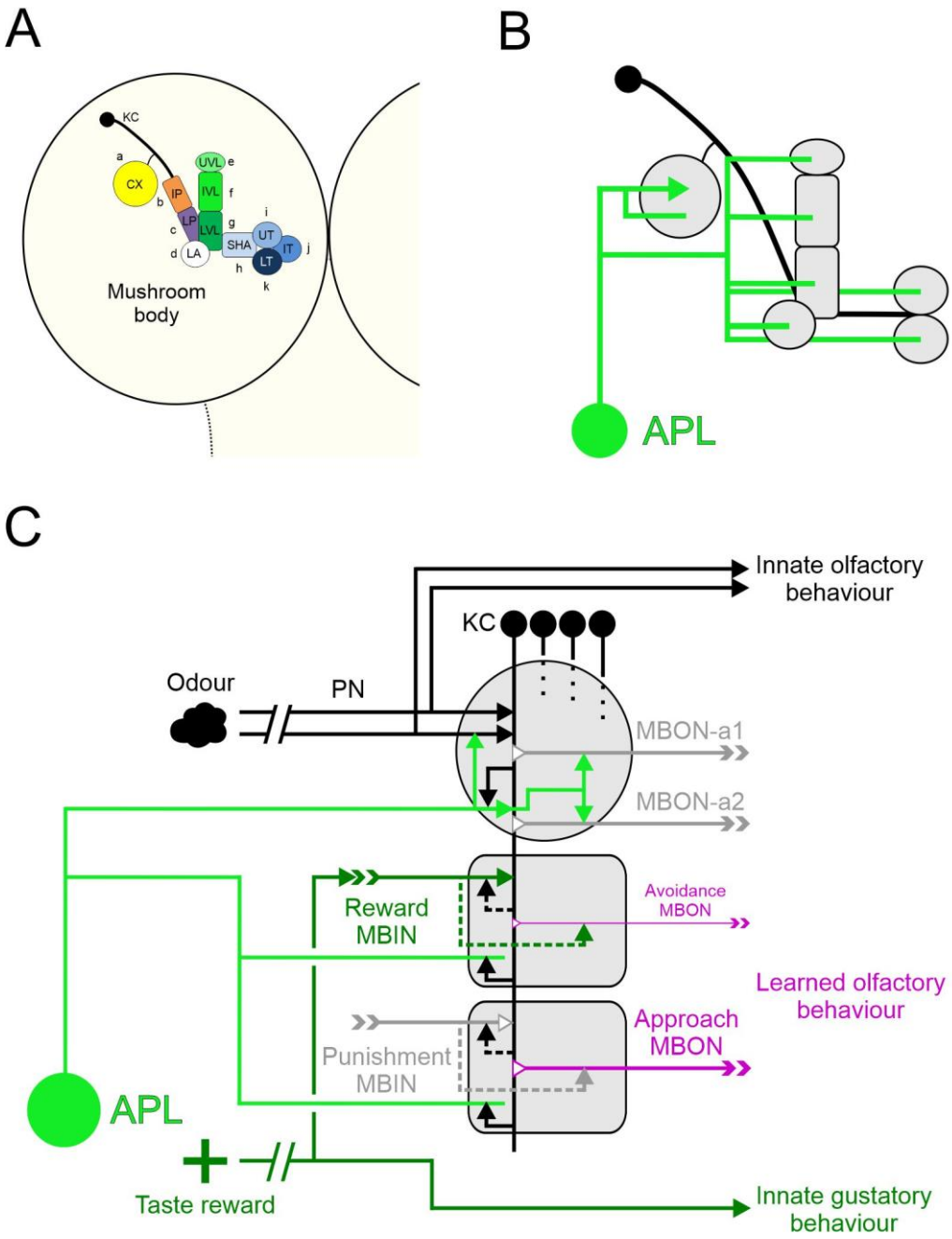

Figure 4. The major "memory centre": the mushroom body. (A) Schematic overview of one larval brain hemisphere with the mushroom body. The larval mushroom body is composed of its intrinsic Kenyon cells (KCs) and is organized in 11 compartments (a-k indicate compartment innervation by the mushroom body extrinsic neurons), namely "CX: calyx; IP and LP: intermediate and lower peduncle; LA: lateral appendix; UVL, IVL and LVL: upper, intermediate, and lower vertical lobe; SHA, UT, IT, LT: shaft as well as upper, intermediate and lower toe of the medial lobe". Adapted from Saumweber et al (2018). **(B)** The larval anterior paired lateral (APL) neuron collects input from the KCs in the calyx and in a subset of the compartments in the lobes (green bars), but delivers output almost exclusively in the calyx (arrow). Adapted from Eichler et al (2017); Saumweber et al (2018). **(C)** Simplified working hypothesis for associative odour-reward learning, featuring the APL neuron. Within the calyx (grey-filled circle), a given odour (dark cloud) leads to the activation of a specific pattern of KCs (dark-filled circles) via a set of projection neurons (PN; dark). Within the lobes (grey-filled rectangles), modulatory mushroom body input neurons (reward MBIN; dark green) carry taste reward signals to the KCs, which send their axonal projection on towards mushroom body output neurons (MBONs; magenta). The APL neuron (bright green) shapes KC odour-evoked activity and establishes some synaptic contacts with the calyx MBONs (MBON-a1 and -a2) as well as selected PNs (please note that additional connections between APL and mushroom body extrinsic neurons are not displayed; for more details

see Figures 3, 4). The recent electron microscopy reconstruction of a first-instar larval nervous system additionally revealed unexpected KC-to-MBIN, MBIN-to-MBON connections (stippled arrow lines; note that newly discovered KC-to-KC and MBON-to-MBIN synapses are not displayed). During associative learning, the coincidence detection between the odour and the reward signal depresses synapses from odour-activated KCs to avoidance-promoting MBONs, but leaves the activity of approach-promoting MBONs from other compartments intact. Presenting the learned odour alone after conditioning thus promotes appropriate odour-driven approach. The same rationale applies for odour-punishment learning, occurring at the synapses between the KCs and the respective MBONs within distinct compartments. Note that PN-to-KC and most of APL-to-KC synapses are only found in the calyx.

Unexpectedly, these electron-microscopy reconstructions have revealed three novel motifs in each mushroom body compartment: (i) KC-to-DAN, (ii) DAN-to-MBON, and (iii) KC-to-KC connections (Eichler et al 2017; Thum & Gerber 2019; **Figure 4C**). These newly discovered types of connection thus represent more than half of the types of connection in the mushroom bodies – i.e. more than half of the synaptic classes have been previously overlooked (larvae: Eichler et al 2017; Thum & Gerber 2019; adult flies: Takemura et al 2017; Li et al 2020). Although much progress has been made, the role of these circuit motifs in behaviour remains largely unknown, calling for follow-up functional and modelling studies (Felsenberg et al 2018; König et al 2019; Lyutova et al 2019; Eschbach et al 2020a,b; Springer & Nawrot 2020; Jacob & Waddell 2020: Jacob et al 2021; McCurdy et al 2021). Additional features have been revealed by connectomics and light-microscopy data, including an inter-hemispheric network provided by MBINs and MBONs, a multi-layered MBON connectivity, and recurrent feedbacks from MBONs onto MBINs via direct or indirect synaptic steps (larvae: Eichler et al 2017; Eschbach et al 2020a,b; adult flies: Takemura et al 2017; Li et al 2020; Otto et al 2020; Ichinose et al 2021). These wiring diagrams of the mushroom bodies and their efferent pathways now provide unprecedented opportunities for relating these circuit motifs, which are likely shared across insects, to neuronal function and learned behaviour. The unexpected complexity of this circuitry, indeed, calls for mushroom body function to be probed with correspondingly complex learning tasks (Mancini et al 2019/Chapter I).

From neural circuits to molecular mechanisms

As mentioned in the previous section, associative olfactory learning leads to the formation of a memory trace in those KCs that are activated by the odour and that receive the aminergic valuation signal. This memory trace is reflected by synaptic plasticity occurring at the KC-to-MBON synapses and underlies conditioned behaviour. But what are the molecular components and pathways involved? The associative coincidence of odour and reinforcement in the KCs is detected by the *rutabaga* type 1 adenylyl cyclase (Crittenden et al 1998). Specifically, *rutabaga* loss-of-function mutations lead to learning defects, which can be rescued by restoring *rutabaga*

in the mushroom bodies (Duerr & Quinn 1982; Tempel et al 1983; Zars et al 2000; McGuire et al 2003; Mao et al 2004; Liu et al 2006; Perisse et al 2007; Tumkaya et al 2018). During associative conditioning, an odour-evoked presynaptic calcium (Ca^{2+}) influx activates the Ca^{2+}/calmodulin-dependent protein kinase; at the same time, the aminergic reinforcement is signalled through G-protein-coupled receptors (GPCRs) (Livingstone et al 1984; Levin et al 1992; Riemensperger et al 2005; Widmann et al 2018). Simultaneous activation of the Ca^{2+}/calmodulin and the GPCR pathways significantly activates the adenylyl cyclase, which boosts its production of cyclic adenosine monophosphate (cAMP) (Dudai et al 1976; Dudai et al 1988; *Aplysia*: Abrams & Kandel 1988); in turn, cAMP activates protein kinase A, which further phosphorylates various downstream proteins (Taylor et al 1990; Tomchik & Davis 2009; Gervasi et al 2010; Boto et al 2014; Louis et al 2018; *Aplysia*: Hawkins 1984). One of them possibly included is Synapsin (Klagges et al 1996; Godenschwege et al 2004; larvae: Michels et al 2005; Michels et al 2011; Diegelmann et al 2013; Kleber et al 2016; Widmann et al 2016; adult flies: Godenschwege et al 2004; Knapek et al 2010; for further aspects on the molecular mechanisms of memory trace formation, see Widmann et al 2018; Thum & Gerber 2019). By and large, and as far has been tested, the molecular mechanisms are conserved between the larval and the adult stages (adult flies: Quinn & Dudai 1976; Folkers et al 1993; Tully et al 1994; Perazzona et al 2004; Honjo & Furukubo-Tokunaga 2005; larvae: Widmann et al 2016; Widmann et al 2018; Thum & Gerber 2019).

Objectives of the thesis

As the above sections make clear, the larva of *Drosophila* is emerging as a useful model for studying learning and memory at genetic, behavioural, cellular, and molecular levels. Given the relative simplicity of the larval nervous system, the availability of learning paradigms allows for an integrative understanding of how neural circuits orchestrate behaviour. Previous studies in larvae have mainly used elemental learning tasks in which the conditioned and unconditioned stimuli are linked unambiguously with each other. However, the ability of larvae to achieve more complex forms of learning remains unknown. Here, I assess cognitive flexibility in larval *Drosophila* by developing a reversal learning paradigm, a non-elemental-learning task where animals are trained to reverse previously learned contingencies. Reversal learning is demonstrated both in the appetitive and aversive domain, providing a genetically tractable study case of cognitive flexibility. The respective results are part of Mancini et al (2019) and shown in Chapter I. Next, I focus on one of the largest and most complex neurons in the larval brain: the APL neuron. Identified both in larval and adult *Drosophila*, this hemispherically single,

GABAergic neuron mediates sparse odour coding within the mushroom body and is involved in various learning and memory processes, including reversal learning in adult flies (Ren et al 2012; Wu et al 2012). However, the larval APL neuron exhibits a number of distinct features as compared to its adult equivalent, which may suggest different functions between stages. I specifically investigate how APL modulates memory acquisition and retrieval in larval *Drosophila*. Using a combination of connectomics, optogenetics and pharmacology, I provide a detailed account of the structure and connectivity of the larval APL neuron, and further reveal its unexpected rewarding properties upon optogenetic activation. These results are presented in Chapter II.

Chapter I

Cognitive flexibility in larval *Drosophila*?

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Introduction

Change is one of the few constancies in nature. For higher animals at least, attuning functional connectivity to changed environmental contingencies can be intrinsically rewarding, a 'quenching of informational thirst' that has been formalized as a minimization of prediction error (Kaplan & Oudeyer 2007; Marvin & Shohamy 2016). Obviously, however, the result of such an adaptation needs to be adaptable itself: what has just been learned to be right can turn out to be wrong shortly afterwards. For the analysis of such cognitive flexibility and its distortions, reversal learning is a particularly fruitful paradigm (Pavlov 1927; Stalnaker et al 2009; Brigman et al 2010; Izquierdo & Jentsch 2012; Gruner & Pittenger 2017; Goarin et al 2018). A reversal learning paradigm can consist of a first training phase during which the animals learn that a cue A predicts a reward to occur whereas a cue B predicts that the reward will not occur (A+/B), followed by a second training phase during which these contingencies are reversed (A/B+). In a simplified version of such a differential conditioning paradigm, reversal learning can also be studied by omitting cue B, in what is called absolute conditioning. Indeed, various forms of reversal learning paradigm have been used across sensory modalities and valence domains in vertebrates and invertebrates, including worms, molluscs and insects such as the honey bee *Apis mellifera* (Young 1962; Jacobson 1963; Rajalakshmi & Jeeves 1965; Giurfa 2003; Izquierdo et al 2017) and the fruit fly *Drosophila melanogaster* (Tully & Quinn 1985; Tully et al 1990; Ren et al 2012; Wu et al 2012; Chouhan et al 2015; Foley et al 2017; McCurdy et al 2021).

Here, I develop an experimental strategy to study olfactory reversal learning in the *Drosophila* larva, an emerging study case for neurobiology and the behavioural sciences (Mayford & Kandel 1999; Helfrich-Förster 2004; Cobb et al 2008; Reaume and Sokolowski 2011; Gomez-Marin and Louis 2012; Diegelmann et al 2013; Clark et al 2018; Almeida-Carvalho et al 2017; Kohsaka et al 2017; Widmann et al 2018; Thum & Gerber 2019; Eschbach & Zlatic 2020; Vogt 2020). Reversal learning was implemented using odours as predictive cues and tastants as ecologically valid appetitive and aversive reinforcers (Gerber & Hendel 2006; Schleyer et al 2015a; Widmann et al 2016). Given the numerical simplicity of the larval nervous system, the present paradigm will be useful for mapping reversal learning to identified circuitry and revealing genetic modulators of cognitive flexibility (Rohwedder et al 2016; Eichler et al 2017; Saumweber et al 2018).

Materials and Methods

Flies and Materials

Fruit flies *Drosophila melanogaster* of the Canton-S wild-type strain were kept in mass culture, maintained at 25˚C, 60 %–70 % humidity, and a 12/12 h light/dark cycle. We used third-instar larvae aged 5 days (120 h) after egg laying. Petri dishes of 85 mm inner diameter (Sarstedt, Nümbrecht, Germany) were used, filled either with 1 % agarose only (CAS: 9012-36-6; Roth, Karlsruhe, Germany) or with 1 % agarose with fructose added (99 % purity; 2 M; CAS: 57-48-7; Roth, Karlsruhe, Germany) as the reward (+) or with 2.5 % agarose with sodium chloride added (99.9 % purity; 1.5 M; CAS: 7647-14-5; Sigma Aldrich) as the punishment (-). Once solidified, the dishes were covered with their lids and left at 4°C until the experiment started, and for a maximum of two weeks.

As the odours, we used n-amyl acetate (AM, CAS: 628-63-7; Merck) diluted 1:20 or 1:250 (the latter only for differential, two-odour aversive learning) in paraffin oil (CAS: 8042-47-5; AppliChem), 1-octanol (OCT, undiluted, CAS: 111-87-5; Sigma–Aldrich) and benzaldehyde (BA, undiluted; CAS: 100-52-7; Sigma-Aldrich). Paraffin oil is without behavioural effect as an odour (Saumweber et al 2011). Before experiments, 10 µl of the respective odour was added to custom-made odour containers made of Teflon (5 mm inner diameter) covered by perforated lids (5-10 holes of 0.5 mm diameter each). All experiments were performed under a fume hood, at 23-25 °C.

Behavioural experiments

The reversal learning procedure is described as follows (**Figure 5A**). Any changes of the standard procedure are mentioned in the respective part of the Results section.

The reversal learning procedure consisted of a first training phase, followed by a first test, and a subsequent second training phase followed by a second test. In both training phases a standard two-group reciprocal conditioning paradigm was used (Scherer et al 2003; Neuser et al 2005; Gerber & Hendel 2006; Saumweber et al 2011; for a detailed manual: Michels et al 2017). In one of the groups the larvae were trained such that the odour was paired with the reward, whereas the other group received unpaired training, that is, separate presentations of odour-alone and reward-alone. To equate the groups for handling, the total number of training events and the total duration of training, a blank was introduced for the paired group during which no reward and two empty odour containers (EM) were presented. After such reciprocal training (AM+/EM in the paired group and AM/EM+ in the unpaired group), the preference for the odour was assessed in a first test. During the second training phase, the procedure was the same except that the contingencies between the odour and the reward were reversed, and two cycles of the above-mentioned training were given. The experiment concluded with a second test for odour preferences in both groups.

Specifically, before starting an experiment, a cohort of 30 larvae was collected from a food vial and briefly washed in tap water. During paired training (AM+/EM), the larvae were placed by using a wet, soft brush in the middle of a Petri dish with fructose added, in the presence of two containers filled with AM. Then, the lid was closed and the larvae were allowed to move freely for 2.5 min. The larvae were then removed and placed on a fresh, pure agarose Petri dish in the presence of two empty containers, the lid was closed, and the larvae could again move freely for 2.5 min. This cycle was performed once. The sequence of training events within a cycle was alternated across repetitions of the experiments, i.e. for half of the cases we started with AM+, and for the other half with EM. After this training phase, the larvae were tested for their odour preference (Test 1). During the test, the larvae were placed in the centre of a fresh, pure agarose Petri dish containing one AM container on one side, one EM container on the other side, and the lid was closed. After 3 min, the number of larvae on the AM side (#AM), the EM side (#EM), as well as in the neutral middle zone (10 mm) was counted and olfactory preference (PREF) was calculated as:

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

Larvae that had moved onto the lid or onto the odour containers during the test (typically < 5 %) were discarded from the analysis.

As for the unpaired case (AM/EM+), the procedure was the same except that the odour and the reward were presented separately to the animals. The larvae were placed onto a pure agarose Petri dish in the presence of AM. Then, they were transferred to an agarose Petri dish with fructose added, together with empty odour containers. The larvae were then tested for their odour preference (Test 1) and the olfactory preference score was calculated according to Equation (1).

Appetitive associative memory is indicated by a relatively higher preference for AM after AM+/EM training in the first training phase compared to the reciprocal AM/EM+ training during that phase. These differences in AM preference were quantified by the associative memory score:

$$(2)\ Memory\ score = \frac{PREF\ paired\ in\ phase\ 1 - PREF\ unpaired\ in\ phase\ 1}{2}$$

Thus, positive score values indicate appetitive associative memory related to the first training phase, whereas negative score values would indicate aversive associative memory related to it.

After the first test, both groups of larvae were transferred to a subsequent second training phase, using the same procedure as for the first training phase, except that the contingencies between the odour and the reward were reversed. In other words, the group that had initially received paired training was now trained by two cycles of unpaired presentations of odour and reward (Phase 1: AM+/EM; Phase 2: AM/EM+); the group initially trained unpaired now received two cycles of paired training (Phase 1: AM/EM+; Phase 2: AM+/EM) (please note that an extinction procedure would involve the presentation of only the odour, but not the reward). Then, a second test (Test 2) was performed and the olfactory preference score was calculated according to Equation (1). The associative memory score was calculated as:

$$(3)\ Memory\ score = \frac{PREF\ unpaired\ in\ phase\ 2 - PREF\ paired\ in\ phase\ 2}{2}$$

Thus, appetitive associative memory related to the second training phase is indicated by negative score values, whereas positive score values would indicate aversive associative memory related to it.

Note that whenever the second training phase was omitted, score values were calculated according to Equation (2); whenever the first training phase was omitted, score values were calculated according to Equation (3).

Statistics

Non-parametric statistical tests were applied throughout. For comparisons to chance levels (i.e. to zero) one-sample sign tests (OSS) were used. For between-group comparisons, Kruskal-Wallis tests (KW) and Mann-Whitney U-tests (MWU) were used for multiple and two-group comparisons, respectively. For within-group comparisons, Wilcoxon matched-pairs tests were used. The Bonferroni-Holm correction was applied to maintain an error rate below 5 %. Statistical analyses were performed with Statistica 12 (StatSoft, Tulsa, OK, USA) and R (Development Core Team 2016). Sample sizes (i.e. biological replications) were chosen based on previous studies that reveal moderate to mild effect sizes (Paisios et al 2017; Saumweber et al 2018) and are indicated in the figure legends. A sample size of N = 1 included ~ 30 animals of both sexes for each reciprocal trained group. Data were displayed as box plots, the middle line showing the median, the box boundaries the 25 and 75 % quantiles, and the whiskers the 10 and 90 % quantiles. Graphs, figures, and scketches were generated with Statistica 13 (SCR_014213, StatSoft Inc, Tulsa) and Corel Draw 2019 (SCR_013674, Corel Corporation); references are documented in **Supplemental Table 1**.

Results

Drosophila larvae adapt to changes in odour-reward contingency

The initial procedure consisted of a first training phase and a first test, followed by a second training phase with reversed contingencies and a second test (**Figure 5**).

During the first training phase, one cohort of approximately 30 larvae received paired odour-reward training. This involved placing them in the middle of a Petri dish with fructose added as a reward to the agarose substrate (+), and presenting the odour n-amyl acetate (AM, evaporating from custom-made perforated Teflon containers). After 2.5 min the larvae were transferred to a fresh agarose Petri dish with plain agarose but no reward and empty (EM) odour containers (AM+/EM training). Another cohort of larvae was trained reciprocally, i.e with unpaired presentations of the odour and the reward (AM/EM+ training). As reviewed by Schleyer et al (2018), paired and unpaired training establish memories of opposite "sign": paired training establishes AM as a predictor of the occurrence of a reward, whereas unpaired training

establishes AM as a predictor of the non-occurrence of the reward. After one such training cycle, the first test of odour preference was performed. To this end, the larvae were placed in the centre of a fresh, plain agarose Petri dish with an AM odour container on one

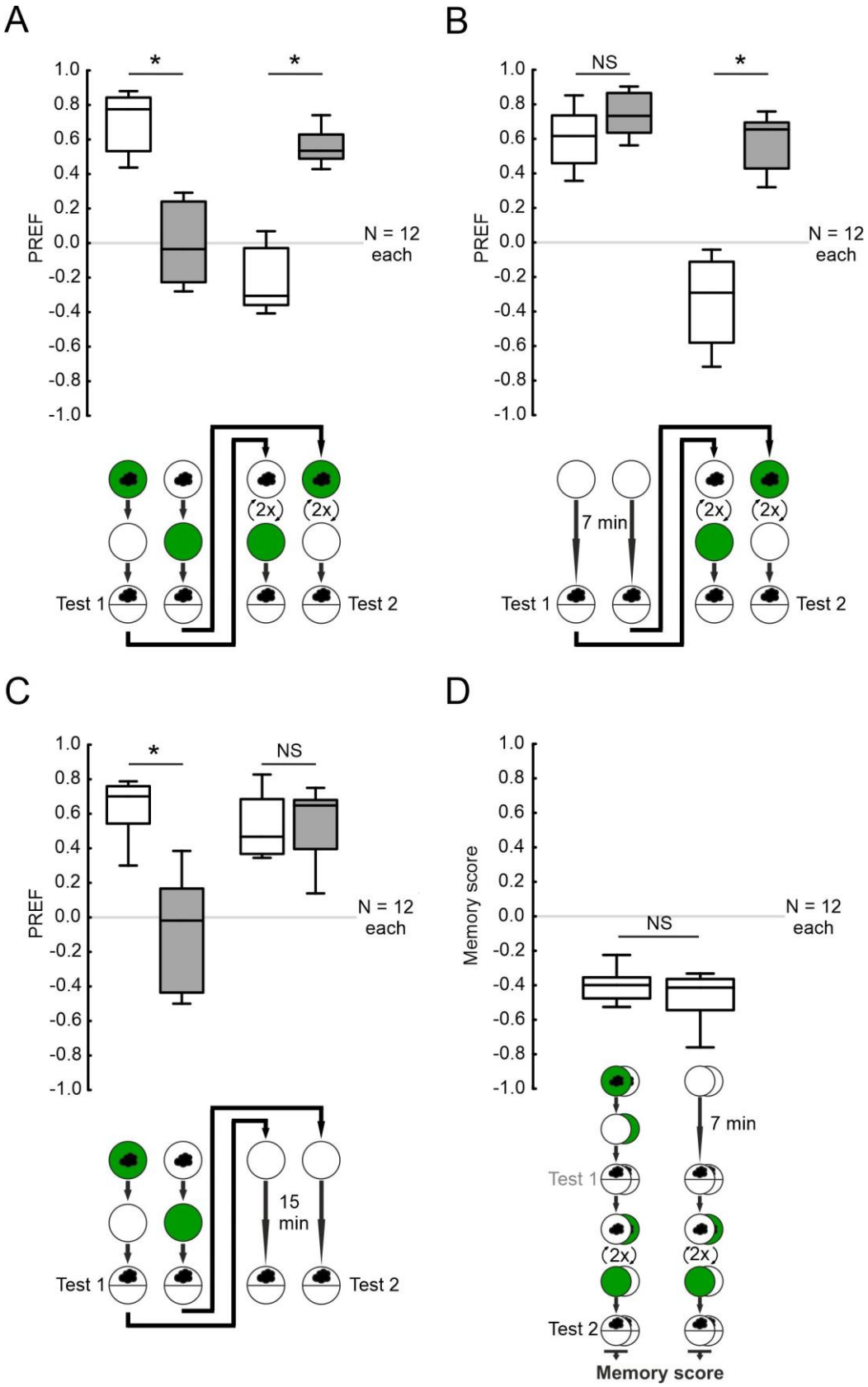


Figure 5. *Drosophila* larvae adapt to changes in odour–reward contingency. **(A)** One group of third-instar larvae received paired odour–reward training; that is, they were exposed to the odour n-amyl acetate (dark cloud) on a Petri dish with an agarose substrate with a fructose reward added (green fill of Petri dish), and then transferred to a Petri dish with just the agarose substrate (white fill of Petri dish) and no odour. The second group of larvae was trained reciprocally, that is, with unpaired presentations of the odour and the reward. After one such training cycle there followed the first test of odour preference (Test 1), and a second training phase with two cycles of training with reversed contingencies of the odour and the reward. Then, the larvae were tested again for their odour preference (Test 2). In the first test, the larvae showed higher levels of odour preference after paired than after unpaired training. This pattern of results was reversed during the second test. Thus, after the first training phase, the larvae behaved according to the odour–reward contingencies during the first training phase, whereas after the second training phase their behaviour was largely in accordance with the reversed contingencies in the second training phase. **(B)** As in (A), except that the first training phase was replaced by a waiting period of 7 min on a Petri dish with just the agarose substrate. The larvae showed equal levels of odour preference in the first test. In the second test, they behaved according to the odour–reward contingencies during the second training phase. **(C)** As in (A), except that the second training phase was replaced by a waiting period of 15 min on a Petri dish with just the agarose substrate. In the first test, the larvae behaved according to the odour–reward contingencies during the first training phase; this effect had vanished by the time of the second test. **(D)** Associative memory scores calculated from the difference in preference scores between paired versus unpaired training, for the second test of the experiments shown in (A) (left plot) and (B) (right plot), respectively. Negative memory scores indicate appetitive associative memory in accordance with the second training phase. Memory scores are equal regardless of whether or not there had been odour–reward training during the first training phase. This conforms to the conclusion from (C) that there was no measurable impact of the first training phase on larval behaviour during the second test. Data are displayed as box plots, the middle line showing the median, the box boundaries the 25% and 75% quantiles, and the whiskers the 10% and 90% quantiles. Sample sizes are given within the figure. * and NS refer to MWU comparisons between groups (* $p < 0.05$ corrected according to Bonferroni-Holm and NS $p > 0.05$).

side, and an empty EM container on the other side. After 3 min the number of larvae on the AM side, the EM side, as well as in a neutral middle zone (10 mm) was counted and the preference for AM was calculated (PREF) (Equation 1; Materials and Methods section). Appetitive associative memory is indicated by a relatively higher preference for AM after AM+/EM training compared to the reciprocal AM/EM+ training. These differences in AM preference were then quantified by the associative memory score (Equation 2; Materials and Methods section). Note that, according to convention, appetitive associative memory for the first training phase is revealed by positive score values.

Immediately following the first test, a second training phase was performed in which the contingencies were reversed, such that animals that had received AM+/EM training in the first phase were now trained AM/EM+, and those initially trained AM/EM+ were now trained AM+/EM. This second training phase, consisting of two training cycles, was followed by a second odour preference test. Importantly, according to convention and to emphasize the opposite effects of the first and the second training phase, appetitive associative memory for the second training phase is revealed by negative score values (Equation 3; Materials and Methods).

In the first test, the larvae showed higher levels of odour preference after paired than after unpaired training (**Figure 5A, left-two boxes**), confirming that one cycle of such training is sufficient to establish associative odour memory (Widmann et al 2016; Weiglein et al 2019).

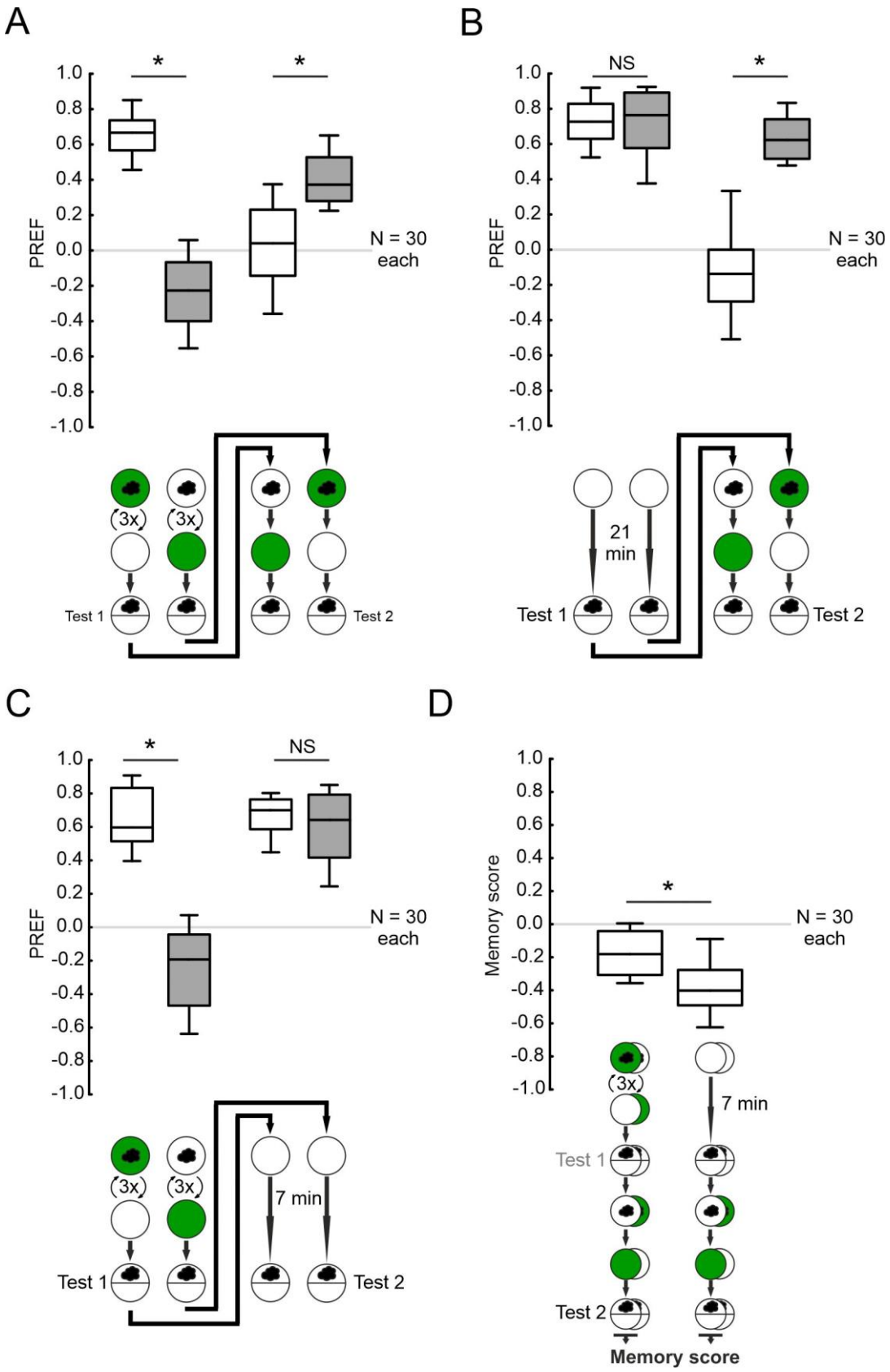

A
B
C
D
PREF
Memory score
N = 30 each
*
NS
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
3x
21 min
7 min
Test 1
Test 2
Memory score

Figure 6. Modification of the procedure allows memory for the first training phase to be detected. The larvae were trained and tested as in Figure 5, except that three cycles of training were given in the first training phase, and only a single cycle of training in the second training phase. **(A)** The larvae received three cycles of either paired odour–reward training or unpaired presentations of the odour and the reward, followed by the first test of odour preference (Test 1); then they received a second training phase with one cycle of training with reversed contingencies of the odour and the reward, followed by a second test of odour preference (Test 2). After the first training phase, the larvae behaved according to the odour–reward contingencies during the first training phase, whereas after the second training phase their behaviour was largely in accordance with the reversed contingencies in the second training phase. **(B)** As in (A), except that the first training phase was replaced by a 21 min waiting period. The larvae showed equal odour preference in the first test. In the second test, they behaved according to the odour–reward contingencies during the second training phase. **(C)** As in (A), except that the second training phase was replaced by a 7 min waiting period. In the first test the larvae behaved according to the odour–reward contingencies during the first training phase; this effect had vanished by the time of the second test. **(D)** Memory scores calculated from the difference in preference scores between paired versus unpaired training, for the second test of the experiments shown in (A) (left plot) and (B) (right plot), respectively. Negative memory scores indicate appetitive associative memory in accordance with the second training phase. Memory scores are less negative for larvae that had received odour–reward training during the first training phase, showing a residual associative effect of the training during that phase. Sample sizes are given within the figure. * and NS refer to MWU comparisons between groups (* $p < 0.05$ corrected according to Bonferroni-Holm and NS $p > 0.05$). Other details as in Figure 5.

This pattern was reversed after the second training phase. That is, in the second test the larvae behaved according to the odour-reward contingency during the second training phase (**Figure 5A, right-two boxes**). The ease of this reversal was striking, compared with what has been observed, for example, in experiments with honey bees (Ben-Shahar et al 2000; Komischke et al 2002; Hadar & Menzel 2010; Mota & Giurfa 2010; Boitard et al 2015; Cabirol et al 2018). I therefore wondered whether memory for the first training phase persists until the second test. To address this question, the first training phase was omitted and the larvae were merely placed onto agarose Petri dishes with neither odour nor reward. As expected, the larvae showed equal levels of odour preference in the first test, as they had not yet received any differential treatment during the experiment (**Figure 5B**). Also as expected, in the second test the larvae behaved according to the odour-reward contingency during the second training phase (**Figure 5B**). Critically, the performance indices calculated from the preference scores during the second test are equal regardless of whether or not there had been odour-reward training during the first training phase (**Figure 5D**). In a further experimental condition, the second training phase was omitted and substituted with a waiting period during which the larvae were placed onto agarose Petri dishes with neither odour nor reward (**Figure 5C**). As expected, in the first test the larvae behaved according to the first training phase, an effect that did not persist until the second test, however **(Figure 5C)**.

These results suggest that the experience during the first training phase no longer had a measurable impact on larval behaviour during the second test. Rather, the results indicate that larvae adapt to changed environmental contingencies through rapid memory decay and/or extinction, combined with rapid learning of the new contingency. I therefore explored whether

increasing the amount of training in the first training phase while decreasing it in the second training phase could uncover 'true' reversal learning, i.e. evidence for a process in which a persisting effect from the first training phase confronts the animals with a contingency contradictory to what they experience during the second training phase.

Modification of the procedure for better detectability of first training phase memory

The larvae were trained as before, except that three cycles of training were applied in the first training phase and only a single cycle of training in the second training phase (**Figure 6**). As in the previous experiment, the larvae behaved according to the second training phase during the second test (**Figure 6A, B**). In addition, this experiment provided mixed evidence for a persisting effect from the first training phase during the second test. On the one hand, odour

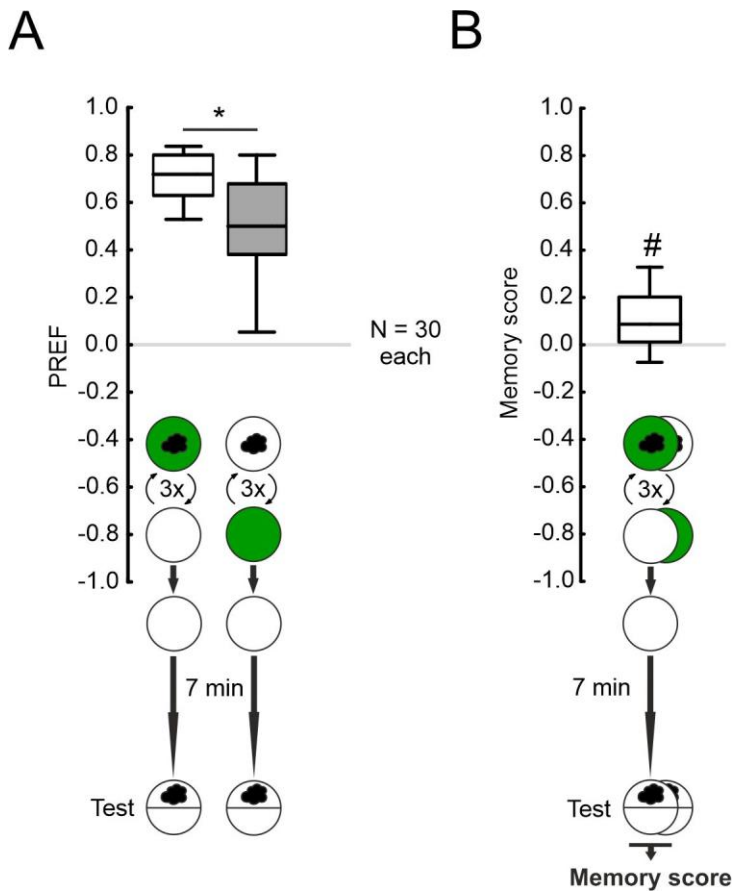


Figure 7. Omitting the first test improves the detectability of memory for the first training phase. The larvae were trained and tested as in Figure 6C, except that the first test was omitted. **(A, B)** The larvae received three cycles of either paired odour–reward training or unpaired presentations of the odour and the reward, followed without testing by a 7 min waiting period and then a test for their odour preference, which for these animals is their first test (Test). Appetitive associative memory was revealed both by higher odour preferences in the paired than in the unpaired group (A), and by significantly positive memory scores (B). Sample sizes are given within the figure. * refers to MWU comparisons between groups in (A), # refers to OSS comparisons to chance levels, that is, to zero in (B) (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5, 6.

preferences during the second test were indistinguishable (**Figure 6C, Figure S1A**) or were only slightly higher (**Figure S1B**) after the larvae had received paired versus unpaired training in the first training phase. Thus, this suggested that there is little if any memory left from the first training phase at this time point. On the other hand, the performance indices during the

second test suggest a persisting effect from the first training phase (**Figure 6D**): the performance indices were less negative when the larvae had received first and second training with opposite contingencies as compared to an omission of the first training phase. Given this mixed evidence, I next asked whether omitting the first test would make the effects of the first

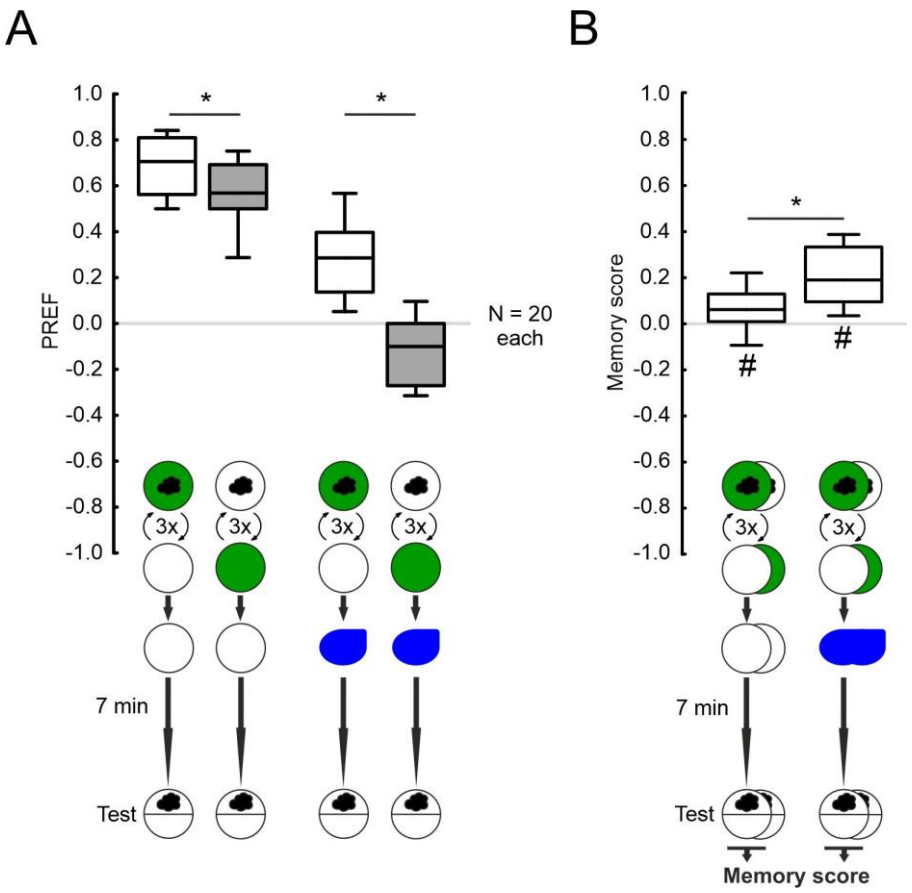


Figure 8. The context of the waiting period influences the detectability of memory for the first training phase. The larvae were trained and tested as in Figure 7, with a further experimental group for which the context during the waiting period between training and testing was implemented in a different way. **(A, B)** The larvae received three cycles of either paired odour–reward training or unpaired presentations of the odour and the reward, followed by a 7 min waiting period and then a test for their odour preference, which for these animals is their first test (Test). The groups differed according to how the waiting period was implemented. The larvae were either put in a Petri dish with an agarose substrate (left plots; this corresponds to the procedures used in Figures 5–7) or in a water droplet on a Petri dish lid (right plots). For either implementation of the waiting period, appetitive associative memory was revealed both by higher odour preferences in the paired than in the unpaired group (A) and by significantly positive memory scores (B). Critically, a comparison of memory scores revealed stronger memory when the animals were kept in the water droplet (B). Sample sizes are given within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–7.

training phase more easily detectable, since the first test can be regarded as an extinction trial for the first training phase. Under these conditions, memory from the first training phase was revealed both by higher odour preferences in the paired than in the unpaired group during the single test (**Figure 7A**), and by significantly positive performance indices (**Figure 7B**). In the

follow-up experiments, the first test was thus omitted throughout. Nonetheless, we judged the remaining memory for the first training phase to be rather weak (**Figure 7B**). I suspected that this may be because maintaining the animals on an agarose Petri dish between the end of training and the test provides a very similar context to that during training, such that interference with the target memories might have taken place. For the following experiment, the larvae were therefore kept either on an agarose Petri dish or in a water droplet on an empty Petri dish lid, a context that is more distinct from the one during training. Odour preferences were higher for the paired-trained than for the unpaired-trained groups in either case (**Figure 8A**). Importantly, the performance indices revealed stronger memory when the animals were kept in the water droplet (**Figure 8B**). This procedure was therefore used for the following appetitive reversal learning experiments.

Appetitive reversal learning in larval *Drosophila*

Larvae were tested either (i) after one-phase training, (ii) after two-phase training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase (**Figure 9**). Performance indices after reversed-contingency training were less negative than when the first training phase was omitted, suggesting a persisting impact from the first training phase (**Figure 9B**). In turn, after reversed-contingency training the performance indices were more negative than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase (**Figure 9B**). The fact that the performance indices after reversed-contingency training were significantly negative and that the performance indices were significantly positive when the second training phase was omitted confirms these conclusions (**Figure 9B**). Thus, performance indices after reversed-contingency training reflect the effects of both the first and the second training phase (for a conceptual replication with shortened training trial durations see **Figure S2**). I next tested reversal learning using a differential, two-odour version of our paradigm. The same procedure as in Figure 9 was used except that 1-octanol (OCT, undiluted) was applied as the second odour (i.e. training was either AM+/OCT or AM/OCT+). Then, the larvae were tested for their choice between AM and OCT and the data were analysed as detailed in the Methods section. In this two-odour, differential conditioning paradigm, too, the larvae showed reversal learning (**Figure 10A, B**).

Together, these results demonstrate reversal learning of larval *Drosophila* in the appetitive domain.

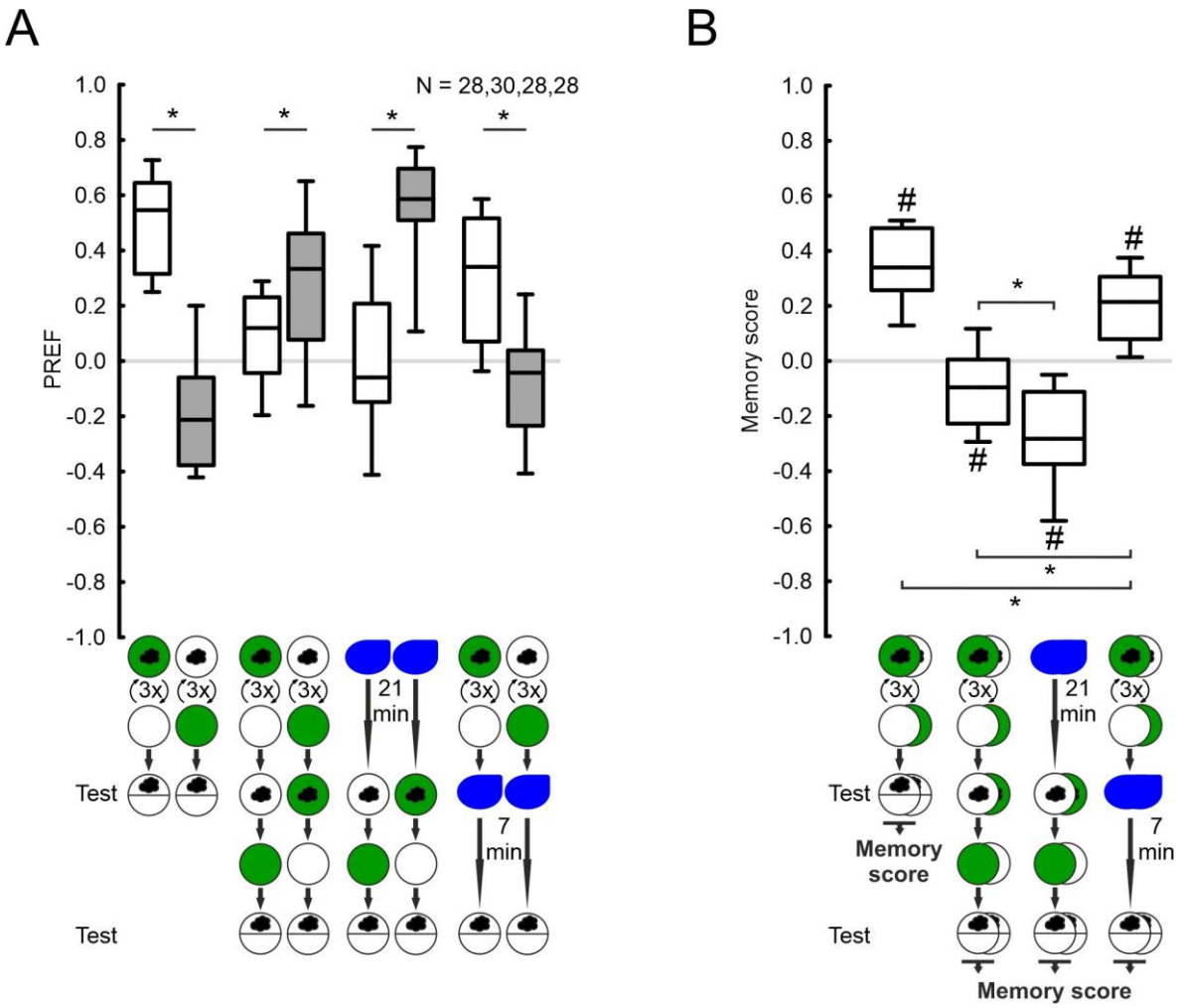


Figure 9. Absolute appetitive reversal learning paradigm in larval *Drosophila*. (A) The larvae were tested for their odour preference either (i) immediately after a one-phase training, (ii) after training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase. **(B)** Memory scores calculated from the preference scores in (A). Positive and negative scores indicate appetitive memory related to the first and the second training phase, respectively. The memory scores after reversed-contingency training were less negative than when the first training phase was omitted, suggesting a persisting impact from the first training phase. In turn, after reversed-contingency training, memory scores were more negative than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase. The fact that the PIs after reversed-contingency training were significantly negative and that memory scores were significantly positive when the second training phase was omitted confirms these respective conclusions. Sample sizes are given within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–8.

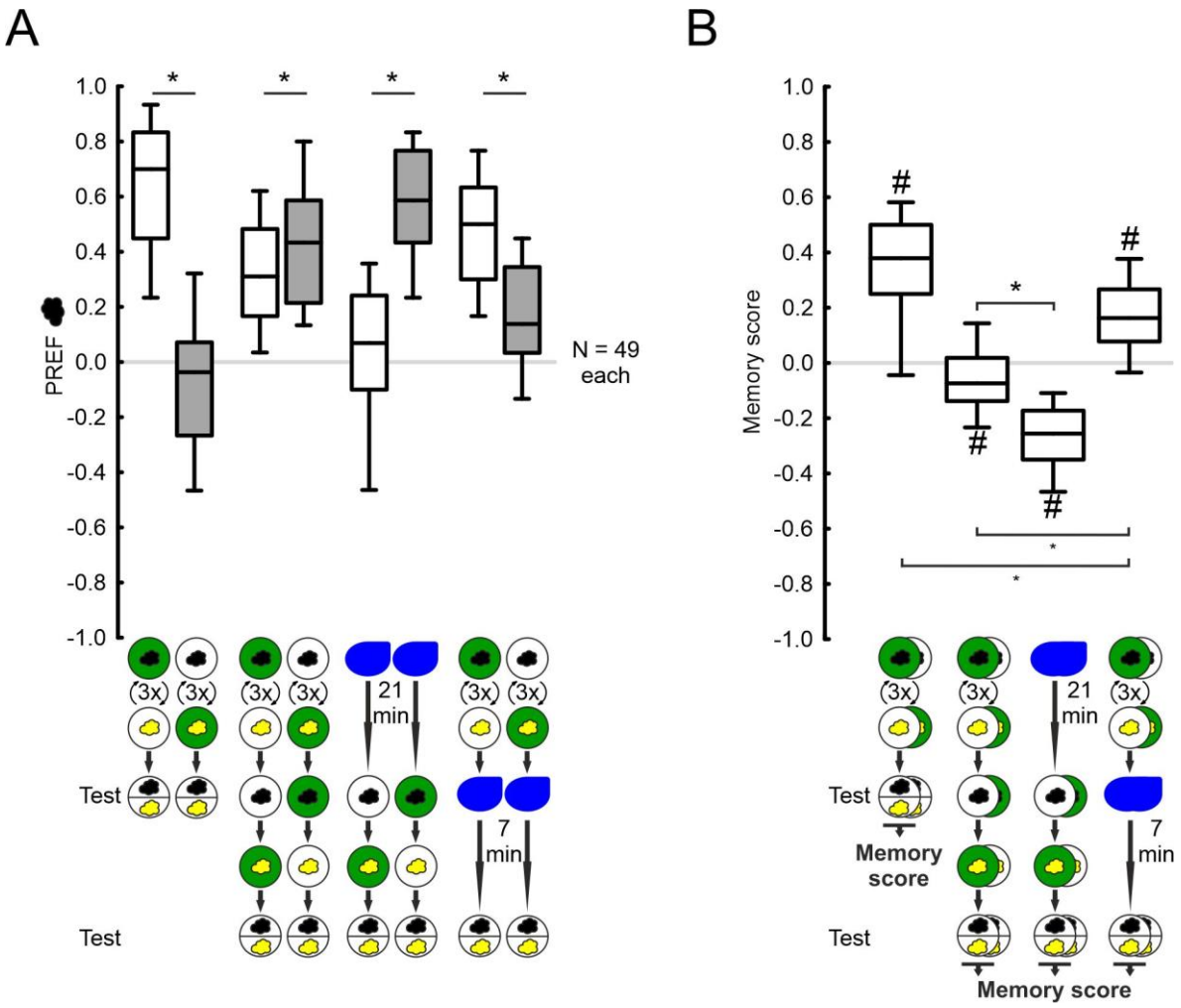


Figure 10. **Differential appetitive reversal learning paradigm in larval *Drosophila***. The larvae were trained and tested as in Figure 9, except that 1-octanol was used as the second odour (yellow cloud) in all training trials in which n-amyl acetate (dark cloud) was not presented. **(A)** The larvae were tested either (i) immediately after one-phase training, (ii) after training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase. Preference scores (PREF) reflect preference for n-amyl acetate (dark cloud). **(B)** Memory scores calculated from the preference scores in (A). Positive and negative scores indicate appetitive memory related to the first and the second training phase, respectively. Memory scores after reversed-contingency training were less negative than when the first training phase was omitted, suggesting a persisting impact from the first training phase. In turn, after reversed-contingency training, memory scores were more negative than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase. The fact that memory scores after reversed-contingency training were significantly negative and that scores were significantly positive when the second training phase was omitted confirms these respective conclusions. Sample sizes are given within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–9.

Aversive reversal learning in larval *Drosophila*

Finally, I enquired into reversal learning in the aversive domain. As in the appetitive case, both absolute conditioning and differential conditioning paradigms were performed, this time using highly concentrated salt (sodium chloride, NaCl, 1.5 M) as the punishment (Gerber & Hendel 2006). In the one-odour aversive paradigm (**Figure 11A, B**), the larvae were trained as in Figure 9 except that (i) one rather than three training cycles was applied in the first training

phase, and (ii) the duration of the individual training trials and the duration of the test were 5 min each rather than 2.5 min and 3 min, respectively. Also, (iii) rather than maintaining the larvae in a water droplet, fresh agarose Petri dishes with neither odour nor punishment were used for maintaining the larvae when omitting the first or the second training phase. In the case of salt as the punishment, these parameters seemed suitable in the light of Widmann et al (2016). Further, (iv) the test was performed on salt-containing Petri dishes, as punishment memories in larvae are only behaviourally expressed as part of learned escape behaviour, i.e. if the presence of the punishment warrants escape (Gerber & Hendel 2006; Schleyer et al. 2011; Schleyer et al 2015a; Widmann et al 2016).

For the two-odour aversive paradigm, the procedure was as described in the preceding paragraph, using AM as one of the odours (diluted 1:250 in paraffin oil) and benzaldehyde (BA, undiluted) as the second odour (**Figure 12A, B**). Please note that according to convention, aversive memory related to the first training phase is indicated by negative performance indices (Equation 2; Materials and Methods), whereas aversive memory for the second training phase is shown by positive performance indices (Equation 3; Materials and Methods section). For both absolute and differential conditioning, the performance indices were more positive after reversed-contingency training than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase (**Figure 11B, 12B**).

This conclusion is confirmed by the observation that the performance indices after reversed-contingency training were significantly positive (**Figure 11B, 12B**). Memory for the first training phase is detectable as well, as shown by the significantly negative performance indices when the second training phase was omitted (**Figure 11B, 12B**). However, the performance indices are statistically indistinguishable after reversed-contingency training and when the first training phase is omitted, so the comparison of these conditions does not provide evidence for a behavioural effect of the memory for the first training phase (**Figure 11B, 12B**). Of note, two independent replications of these two experimental conditions, in differential conditioning, do reveal a persisting impact of the first training phase (**Figure 13, Figure S3A, B**). In any event, and as expected, increasing the number of training cycles in the first training phase outweighs the impact of the second training phase (**Figure S3C, D**).

Overall, these results demonstrate reversal learning of larval *Drosophila* in the aversive domain as well.

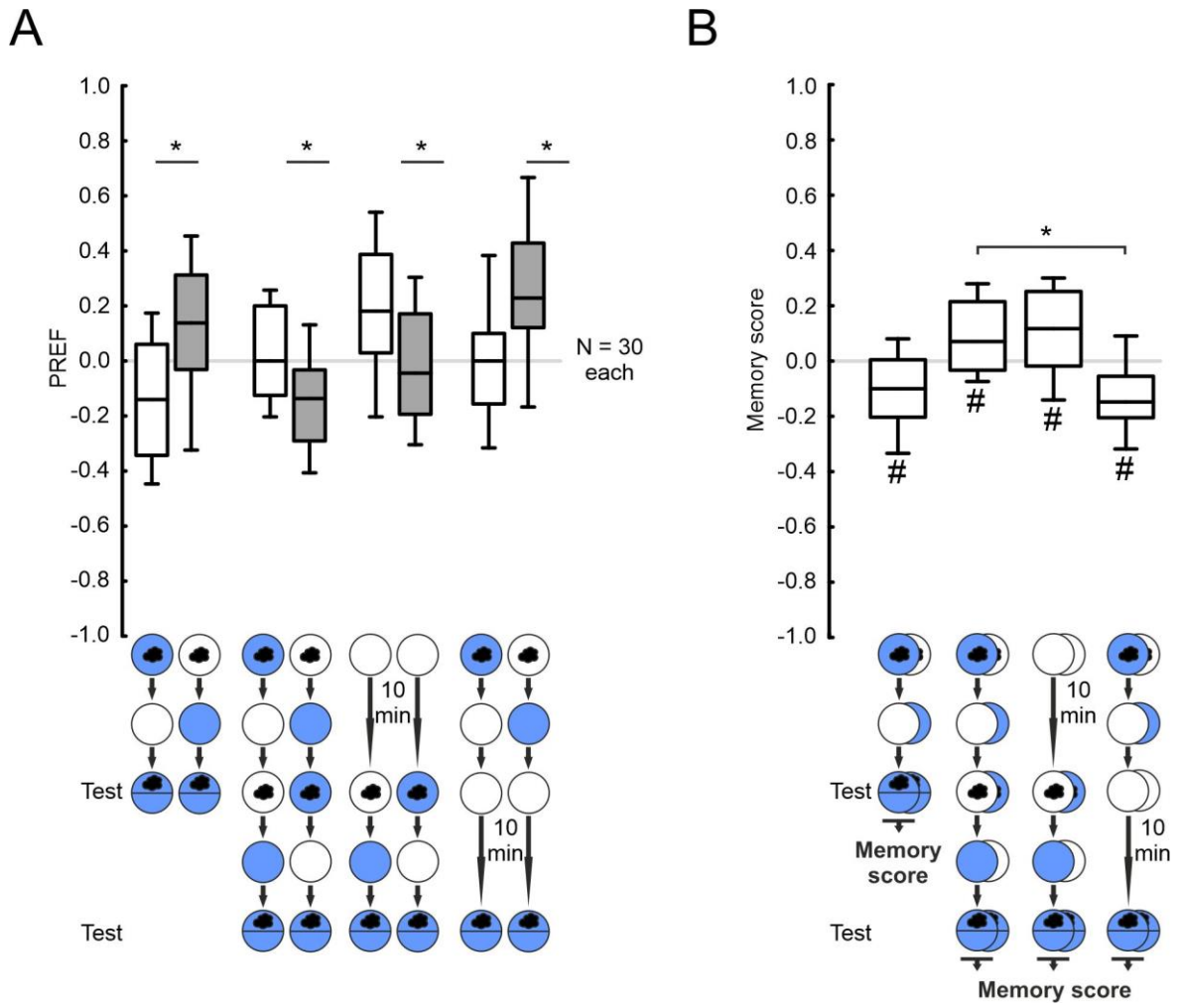


Figure 11. Absolute aversive reversal learning paradigm in larval *Drosophila*. The larvae were trained as in Figure 9 with modifications for measuring reversal learning in the aversive domain. Specifically, (i) a high concentration of salt (sodium chloride) was used as the punishment (blue fill of Petri dish); (ii) only one cycle of training was given in the first training phase; (iii) the duration of individual training trials and the duration of the test were 5 min each; (iv) agarose Petri dishes with neither odour nor punishment were used for maintaining the larvae when omitting the first or the second training phase; and (v) the test was performed on salt-containing Petri dishes. **(A)** The larvae were tested either (i) immediately after one-phase training, (ii) after training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase. **(B)** Memory scores calculated from the preference scores in (A). Negative and positive scores indicate aversive memory related to the first and the second training phase, respectively. Memory scores after reversed-contingency training were more positive than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase. In addition, memory scores after reversed-contingency training were significantly positive, confirming this conclusion. The fact that memory scores were significantly negative when the second training phase was omitted suggests behaviour in accordance with the first training phase. However, memory scores were no less positive after reversed-contingency training than when the first training phase was omitted, offering no evidence for a behavioural effect of the memory for the first training phase. Sample sizes are given within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–10

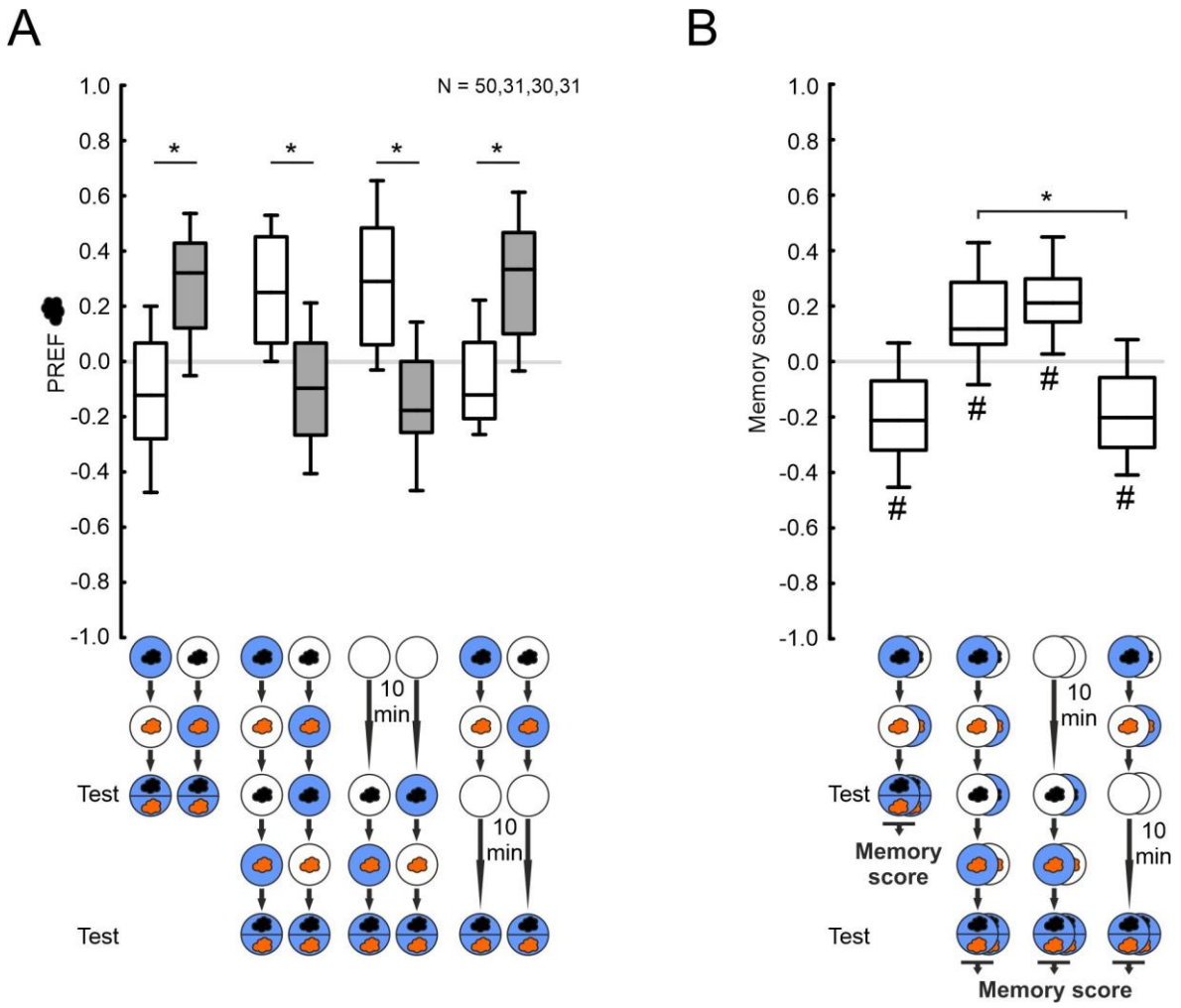


Figure 12. Differential aversive reversal learning paradigm in larval *Drosophila*. The larvae were trained and tested as in Figure 11, except that benzaldehyde was used as the second odour (orange cloud) in all training trials in which n-amyl acetate (dark cloud) was not presented. **(A)** The larvae were tested either (i) immediately after one-phase training, (ii) after training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase. Preference scores (PREF) reflect preference for n-amyl acetate (dark cloud). **(B)** Memory scores calculated from the preference scores in (A). Negative and positive scores indicate aversive memory related to the first and the second training phase, respectively. Memory scores after reversed-contingency training were more positive than when the second training phase was omitted, suggesting behaviour in accordance with the second training phase. In addition, memory scores after reversed-contingency training were significantly positive, confirming this conclusion. The fact that memory scores were significantly negative when the second training phase was omitted suggests behaviour in accordance with the first training phase. However, memory scores are no less positive after reversed-contingency training than when the first training phase is omitted, offering no evidence for a behavioural effect of the memory for the first training phase (see also Figure 13). Sample sizes are given within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*,# $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–11.

Discussion

Strategies for contingency adjustment

These results demonstrate reversal learning in larval *Drosophila*. After first associating an odour with the presence or the absence of a reinforcer (i.e. reward or punishment), the animals

adapted their behaviour to the reversal of these contingencies. I regard this as 'true' reversal learning because after reversed-contingency training the effects of both the first and the second training phase remain behaviourally detectable (**Figures 9-13**). Notably, the present data do not reveal whether this comes about by individual animals having distinct, opposing associative memories for the two training phases, or whether subsets of animals have such memories for only one or the other of the training phases, or whether all animals operate using a trial-by-trial update of their experiences. Recent data on the spatially segregated co-existence of opposing physiological memory traces in adult flies – specifically the co-existence of an acquisition-memory trace and an extinction-memory trace for one and the same odour – are consistent with the first scenario (Felsenberg et al 2018; Otto et al 2020). Nonetheless, at least for the appetitive domain, the extinction/decay of memory appears to be rather rapid in larvae; fittingly I observe 'true' reversal learning only in a paradigm with relatively intense training for the initial association (3 cycles) and relatively little training for the second (1 cycle). This is in contrast with results, for example, in the honey bee, where appetitive reversal learning paradigms include several reversed-contingency training trials and often use a 1:1 ratio of trial numbers in the first and the second training phase (Ben-Shahar et al 2000; Komischke et al 2002; Hadar & Menzel 2010; Mota & Giurfa 2010; Boitard et al 2015; Cabirol et al 2018).

Specifically, during the second phase, bees typically persist in responding to the cue that was originally reinforced, meaning that the effects of training from the first phase persist and need to be overcome during the second, reversed-contingency training phase (Hadar & Menzel 2010; Mota & Giurfa 2010). This might indicate two different cognitive strategies which can both bring about contingency adjustment: during reversed-contingency training, bees might take advantage of a higher mnemonic capacity and be able to maintain memory for the initial association more easily despite the conflicting memories to be established during the reversal phase. Such a strategy would come at the cost of relatively slow and incomplete contingency adjustment. In contrast, the larvae might more easily discard old and establish new memories for the sake of quick contingency adjustment, coming at the cost of only having a narrow temporal window for their mnemonic record – consistent with the relatively quick memory decay in these animals (Neuser et al 2005; Kleber et al 2016; Weiglein et al 2019). Conceivably, this is adaptive for bees because the initial contingencies might re-emerge during their relatively long lives as foragers, whereas larvae might have already pupariated before this is the case.

Generality of reversal learning across valence domains

Reversal learning was found in both the appetitive domain (**Figures 9-10**) and the aversive domain (**Figures 11-13**), corresponding to what has been reported for adult flies (Tully & Quinn 1985; Tully et al 1990; Ren et al 2012; Wu et al 2012; Chouhan et al 2015), bees (Giurfa 2003; Mota & Giurfa 2010; Claudio et al 2018) and vertebrates (Rajalakshmi & Jeeves 1965; O'Malley & Bruning 1969; Morris & Dolan 2004; Bissonette et al 2008; Costa et al 2015; Izquierdo et al 2017; Atlas & Phelps 2018). This suggests reversal learning as a general faculty widespread in the animal kingdom, even where the nervous system is numerically as simple as is the case for the larva with its approximately 10,000 neurons (Dumstrei et al 2003; Nassif et al 2003).

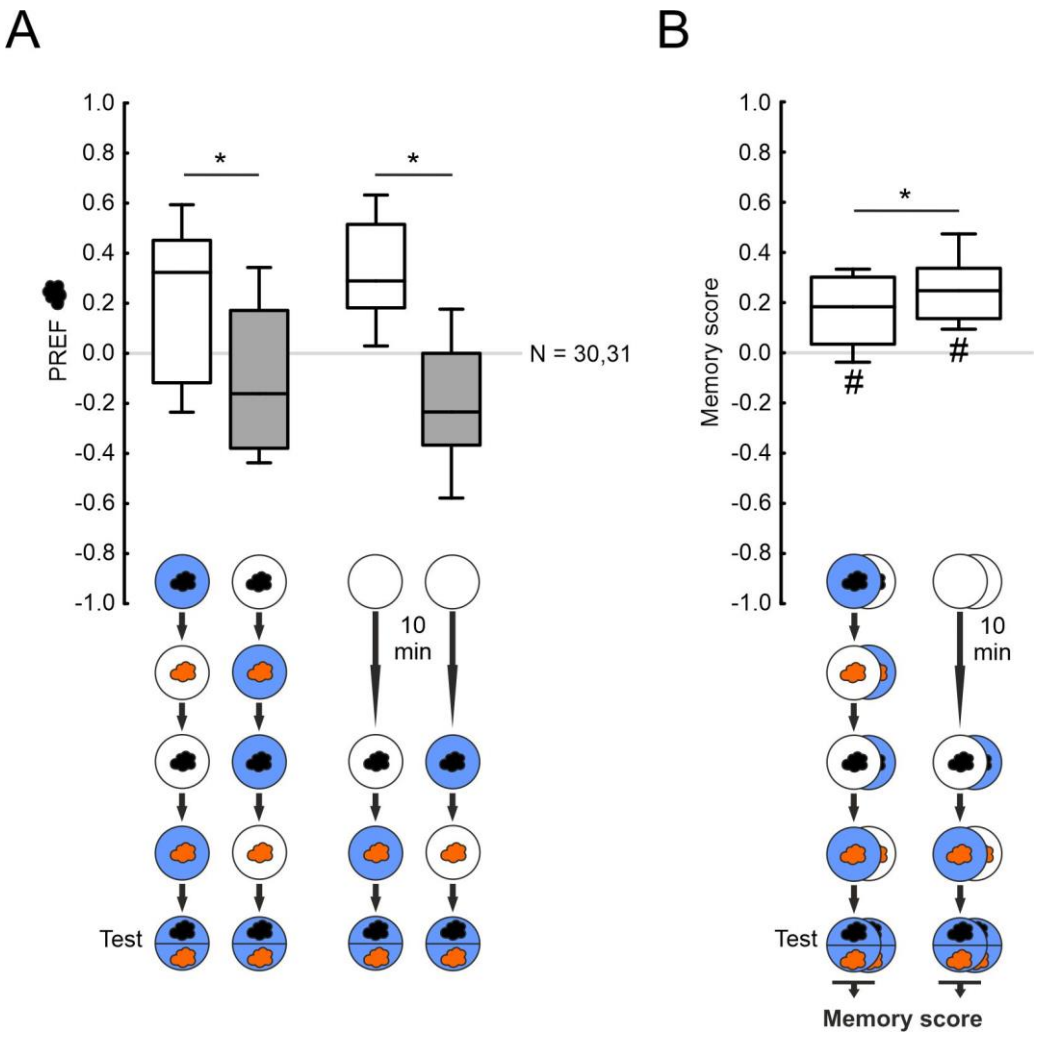


Figure 13. Influence of the first training phase in the differential aversive reversal learning paradigm, revisited. **(A)** Repetition of two experimental conditions from Figure 12. The larvae were tested after training with reversed contingencies in the first and the second training phase, or after omitting the first training phase. Preference scores (PREF) reflect preference for n-amyl acetate (dark cloud). **(B)** Memory scores calculated from the preference scores in (A). Positive scores indicate aversive memory related to the second training phase. Memory scores after reversed-contingency training were less positive than when the first training phase was omitted, suggesting a small yet significant persisting impact from the first training phase. Sample sizes are given

within the figure. * refers to MWU comparisons between groups, # refers to OSS comparisons to chance levels, that is, to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm). Other details as in Figures 5–12.

It should be noted that, for the present experiments, quantitative comparisons of the ease of reversal learning across valence domains are not informative. The reason is that the initial experiments suggested that different experimental parameters need to be chosen to detect reversal learning in the appetitive and the aversive case. Procedural differences include the number of cycles in the first training phase (1 cycle versus 3 cycles), the duration of individual training trials (2.5 min versus 5 min), test duration (3 min versus 5 min), the identity of the second odour for differential conditioning (OCT versus BA) and the circumstances in which the animals are maintained when omitting either the first or the second training phase (water droplet versus pure agarose Petri dish). Bearing these caveats in mind, I note that, as previously reported, aversive memories appear more stable over time than appetitive ones (Widmann et al 2016; compare the respective leftmost and rightmost plots in **Figure 9B** and **Figure 10B** to the corresponding results in **Figure 11B** and **Figure 12B**).

Utility of a larval reversal learning paradigm

The availability of a reversal paradigm enriches the behavioural toolbox for studying associative learning in the larva. As argued by Thum & Gerber (2019), such an extension is important because the newly discovered synaptic connections in the mushroom bodies, the brain centre for associative learning in insects, now call for functional interpretation (Eichler et al 2017; Takemura et al 2017; Eschbach et al 2020a,b; Li et al 2020). Indeed, these connections suggest a richer mnemonic functionality than previously acknowledged on the basis of the typically rather simple tasks used to investigate them (Heisenberg 1998; Menzel & Giurfa 2001; Heisenberg 2003). In this context, the present reversal learning paradigm might become useful (for pioneering work on the cellular basis of reversal learning in adult flies: Ren et al 2012; Wu et al 2012; for related work in bees: Devaud et al 2007; Boitard et al 2015; Cabirol et al 2018). In addition, the possible co-existence of i) memory extinction/decay with ii) memory for the first training phase and iii) memory for the second training phase might imply a complexity of memory ‘content’ that also defines critical demands for computational models of adaptive behaviour in these animals (Felsenberg et al 2018; Otto et al 2020; McCurdy et al 2021; Springer & Nawrot 2021).

In psychological terms, reversal learning may serve as an indicator of cognitive flexibility when an animal is confronted with environmental variations (Izquierdo et al 2017). Such a

measure could be useful for seeing what the full range of effects of drugs affecting memory is, or for delineating the scope of mutant phenotypes (Michels et al 2018).

Chapter II

Rewarding properties of the larval APL neuron

Introduction

Larvae of the fruit fly *Drosophila*, which naturally live on overripe fruit, provide a powerful study case for investigating the neurogenetic bases of learning and memory (Gerber & Stocker 2007; Widmann et al 2018; Eschbach & Zlatic 2020). Their small size and low number of neurons have allowed for an ongoing community effort to reconstruct their chemical synapse connectome, revealing unexpected complexity. In the mushroom body, a higher brain structure for sensory integration and memory in insects, more than half of the classes of synaptic connections had previously escaped attention (**Figure 4C**; Eichler et al 2017; Eschbach et al 2020a,b; adult flies: Takemura et al 2017; Li et al 2020). For instance, dopaminergic mushroom body input neurons (DANs) not only relay ascending information to local compartments along the elongated axonal fibres of the mushroom body intrinsic Kenyon cells (KCs), but also integrate local information from the KCs and recurrent signals originating from mushroom body output neurons (MBONs; these likewise respect compartmental boundaries) (Eschbach et al 2020a,b; Schleyer et al 2020). Similar complexity is observed for octopaminergic neurons (OANs) and input neurons using unidentified signalling (Eichler et al 2017; Saumweber et al 2018; Eschbach et al 2020a,b). Collectively, this should prepare us for surprises regarding mushroom body function. Here I study the most complex mushroom body interneuron, the anterior paired lateral (APL) neuron.

APL is a hemispherically unique local interneuron and can be identified from the earliest larval stage on (Eichler et al 2017). It receives most of its input from, and in turn provides GABAergic output to, the cholinergic KCs, suggesting a role in sparsening the sensory representation within the mushroom body (Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018; adults: Honegger et al 2011; Lin et al 2014; Inada et al 2017; Amin et al 2020; further insects: Homberg et al 1987; Grünewald 1999; Papadopoulou et al 2011). In contrast to most other aspects of mushroom body connectivity, however, there are major differences in APL connectivity between larvae and adults.

In adults APL innervates all 15 mushroom body compartments and the calyx, where the KCs receive input from sensory projection neurons (Tanaka et al 2008; Aso et al 2014a;

Mayseless et al 2018). In larvae APL also innervates the calyx, but only six of the 10 compartments (**Figure 4B**; Eichler et al 2017; Saumweber et al 2018).

In adults APL reciprocally connects with the KCs in the calyx and in all compartments, whereas in larvae such reciprocal connections exist only in the calyx and only KC-to-APL synapses are found otherwise (Wu et al 2013; Masuda-Nakagawa et al 2014; Zheng et al 2018; Eichler et al 2017; Takemura et al 2017; Saumweber et al 2018; Scheffer et al 2020).

In adults APL is electrically coupled to the dorsal paired median neuron (DPM), a local interneuron that innervates all compartments but not the calyx (Pitman et al 2011; Wu et al 2011). DPM is serotonergic, co-releases GABA, and can express the amnesiac peptide (Waddell et al 2000; Lee et al 2011; Haynes et al 2015; Turrel et al 2018). Strikingly, DPM and innervation by serotonergic neurons is absent in larvae (Huser et al 2012; Eichler et al 2017; Saumweber et al 2018).

These differences caution against extrapolations of findings about APL function between larvae and adults, and might suggest that APL does not 'only' regulate sparse KC activity and other functions depending on such sparsening (Liu et al 2007; Liu & Davis 2009; Ren et al 2012; Wu et al 2012; Lin et al 2014). In this context, I provide a comprehensive account of the structure of the larval APL neuron, its metamorphic development, and the spatial arrangement of its synapses. Investigating its role in Pavlovian conditioning, I discover that, surprisingly, activating APL optogenetically exerts a rewarding effect. These rewarding properties are scrutinized in detail and are shown to involve a downstream, dopamine-dependent process.

Materials & Methods

Drosophila strains

Drosophila melanogaster were kept and maintained as mentioned in Chapter I/ Mancini et al (2019). Randomly chosen third-instar, feeding-stage transgenic larvae aged 5 days were used, unless mentioned otherwise. The strains used in this study and their genotypes are listed in **Supplemental Table 1**.

Immunohistochemistry

All antibodies used in this study are listed in **Supplemental Table 1**.

Expression profile of the SS01671 driver strain

To validate specific expression in the larval APL neuron from the SS01671-GAL4 driver (abbreviated as APL-GAL4; Saumweber et al 2018), it was crossed to UAS-ChR2XXL::tdtomato to express a tomato-tagged version of ChR2XXL (FlyBase ID: FBtp0131815; Saumweber et al 2018). Double-heterozygous third-instar progeny (abbreviated as APL>ChR2XXL::tdtomato) were dissected in ice cold Ringer's solution and brains were fixed for 30 min in 10 % formaldehyde dissolved in phosphate buffered saline (PBS, pH 7.2, P4417, Sigma Aldrich) at room temperature. After consecutive washing steps (3 x 10 min each) in PBT (0.3 % Triton-X-100 [CAS: 9036-19-5, Roth] in PBS), brains were blocked in 5 % normal goat serum solution (NGS; 005-000-121, Jackson Immunoresearch Laboratories; in PBS) for 2 h at room temperature. To provide a reference staining of fibre tracts (including the mushroom bodies), tissues were incubated overnight at 4 °C with a primary monoclonal mouse anti-FASII antibody (AB_528235, DSHB) diluted 1:50 in blocking solution containing 4 % NGS in PBS. After six washes (10 min each) in PBS, tissues were treated overnight at 4 °C with a secondary polyclonal goat anti-mouse Alexa Fluor 488 antibody (A11001, Invitrogen) diluted 1:200 in PBS. Brains were then washed in PBS (6 x 10 min each) and mounted in Vectashield (Vector Laboratories Inc) on a cover slip. Signal detection from the tomato-tag of ChR2XXL (labelling the APL neuron) did not require antibodies; rather, the tomato fluorescence signal was detected directly under the microscope. Image z-stacks were acquired with a Leica TCS SP8 confocal microscope (Leica Mikrosysteme Vertriebs GmbH) at 1024 × 1024 pixel resolution. Image processing was performed using Imaris software (version 9.72, Bitplane).

To examine the inter-hemispheric symmetry in the morphology of APL, the APL-GAL4 driver was crossed to UAS-mCD8::GFP (Lee and Luo 1999; Bloomington Stock Centre no. 5137) as the effector. Third-instar larvae were put on ice and dissected in PBS. Brains were fixed in 4 % PFA for 20 min at room temperature. After successive washing steps (3 x brief; 1 x 5 min; 3 x 15 min; 1 x 90 min) in 3% PBT (3 % Triton-X-100 [CAS: 9002-93-1, Sigma Aldrich] in PBS) on ice, brains were blocked with 5% NGS (G9023, Sigma Aldrich) in PBT for 1 h at room temperature and incubated for 48 h with primary antibodies at 4°C. Brains were then washed (2 x brief; 3 x 15 min; 1 x 60 min; on ice; 1 x 30 min at room temperature) in 3% PBT before application of the secondary antibodies for at least 24 hours at 4°C. After final washing steps (3 x brief; 3 x 5 min; 2 x 15 min) in 3% PBT, brains were mounted on poly-L-lysin-coated coverslips (following Janelia FlyLight recipe), dehydrated through a series of increasing concentrations of ethanol (EtOH) (1x brief in distilled water; 1 x 10 min 30% EtOH; 1 x 10 min 50% EtOH; 1 x 10 min 75% EtOH; 1 x 10 min 95% EtOH; 3 x 10 min 100% EtOH)

and cleared (3 x 5 min) in xylene (247642, CAS:1330-20-7, Sigma Aldrich). Finally, brains were mounted in DPX mounting medium (dibutyl phthalate in xylene; 06522, Sigma Aldrich) and left in darkness for at least 24 h before imaging.

The primary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a polyclonal rabbit anti-GFP (A6455, Life Technologies) diluted 1:1000 in 3% PBT (for APL staining), (iii) a monoclonal mouse 4F3 anti-DLG (AB_528203, Developmental Studies Hybridoma Bank) diluted 1:200 in 3% PBT (for mushroom body staining), and (iv) a monoclonal rat anti-N-Cadherin antibody (DN-Ex #8-s, Developmental Studies Hybridoma Bank) diluted 1:50 in 3 % PBT (for neuropil staining).

The secondary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a polyclonal goat anti-rabbit Alexa Fluor 488 (A11008, Life Technologies), (iii) a polyclonal goat anti-mouse Alexa Fluor 568 (A10037, Life Technologies), and (iv) polyclonal goat anti-rat Alexa Fluor 647 (712-605-153, Jackson ImmunoResearch) all diluted 1:500 in 3% PBT. Confocal microscopy was conducted on a Zeiss LSM800 confocal laser scanning microscope with ZEN 2.3 software. Image z-stacks were acquired with a LSM800 confocal microscope (Zeiss) at 1024 × 1024 pixel resolution. Image processing was performed using Imaris software (version 9.72, Bitplane).

To analyse the coverage of the mushroom body compartments between left- and right-hemisphere APL neurons, mean pixel intensities were measured by using ImageJ (Fiji ImageJ, version 1.53c). Greyscale maximum intensity projections of the GFP-channel (labelling APL membranes) were created, whereas the DLG-channel (labelling the mushroom body) served as template for orientation. The mushroom body compartments were selected by using the ROI Manager.

To validate the expression of the ChR2XXL effector transgene used for activating APL, the APL-GAL4 driver was crossed to the UAS-ChR2XXL effector (Dawydow et al 2014; Bloomington Stock Centre no. 58374). The procedure followed Schleyer et al (2020). In brief, brains of third-instar larval progeny (abbreviated as APL>ChR2XXL) were dissected in ice cold Ca^{2+}-free saline solution and fixed in Bouin's solution (HT10132, Sigma-Aldrich) for 7 min at room temperature. After six successive washing steps (3 x brief; 3 x 15 min) in 0.2 % PBT, brains were incubated overnight at 4°C with a primary monoclonal mouse anti-ChR2 antibody (610180, ProGen Biotechnik) diluted 1:100 in 0.2 % PBT. Brains were then washed (3 x 10 min each) in 0.2 % PBT and incubated for 1 h at room temperature with a secondary polyclonal donkey anti-mouse Cy3 antibody (715-165-150, Jackson ImmunoResearch Laboratories) diluted 1:300 in 0.2 % PBT. Finally, samples were washed (3 x 10 min each) in

0.2 % PBT and mounted in Vectashield (Vector Laboratories Inc.) on a cover slip. Image z-stacks were acquired with a Leica TCS SP8 confocal microscope (Leica Mikrosysteme Vertriebs GmbH) at 1024 × 1024 pixel resolution. Image processing was performed using Imaris software (version 9.72, Bitplane).

GABA staining

To confirm that APL is GABAergic, the APL-GAL4 driver was crossed to a UAS-CsChrimson::mVenus effector (Klapoetke et al 2014; Bloomington Stock Centre no. 55135), and third-instar progeny (abbreviated as APL>Chrimson) were dissected in PBS. Brains were fixed for 20 min with 4 % PFA in 3 % PBT on ice. After successive washing steps (2 x brief; 1 x 5 min; 3 x 15 min; 1 x 2 h) in 3 % PBT, brains were blocked for 1-2 h in 2 % NGS solution (S-1000, Vector Laboratories Inc; in PBS) on ice. After two overnight incubations at 4 °C with the primary antibodies, brains were rinsed (2 x brief; 1 x 5 min; 3 x 15 min; 1 x 2 h) in 3 % PBT and incubated overnight with the secondary antibodies at 4 °C. Preparations were finally washed (2 x brief; 1 x 5 min; 5 x 15 min) in 3 % PBT, mounted in Vectashield (Vector Laboratories Inc) on a cover slip, and scanned under a LSM510 confocal microscope (Zeiss) at 1024 × 1024 pixel resolution. Image processing was performed using Imaris software (version 9.72, Bitplane).

The primary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a monoclonal rat anti-N-Cadherin antibody (DN-Ex #8-s, Developmental Studies Hybridoma Bank) diluted 1:50 in 3 % PBT (for neuropil staining), and (iii) a polyclonal rabbit anti-GABA antibody (A2052, Sigma Aldrich) diluted 1:500 in 3 % PBT.

The secondary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a polyclonal Cy3-conjugated goat anti-rat antibody (A10522, Life Technologies) diluted 1:200 in 3 % PBT, and (iii) a polyclonal Cy5-conjugated goat anti-rabbit antibody (A10523, Life Technologies) diluted 1:200 in 3 % PBT. Signal detection from the mVenus tag of the Chrimson transgene allows visualization of APL membranes without antibodies under the fluorescence microscope.

APL regional synaptic polarity

For analysing the regional synaptic polarity of the larval APL neuron, the APL-GAL4 driver was crossed to a double-effector with both UAS-Dsyd-1::GFP (Owald et al 2015a) and UAS-DenMark (Nicolai et al 2010; Bloomington Stock Centre no. 33062). Third-instar progeny (abbreviated as APL>Dsyd-1::GFP/DenMark) were dissected, fixated, dehydrated, and

mounted as described in the preceding section. Image processing was performed using Imaris software (version 9.72, Bitplane).

The primary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a monoclonal rat anti-N-Cadherin antibody (DN-Ex #8-s, Developmental Studies Hybridoma Bank) diluted 1:50 in 3 % PBT (for neuropil staining), (iii) a polyclonal FITC-conjugated goat anti-GFP antibody (ab 6662, Abcam) diluted 1:1000 in 3 % PBT (for visualization of the GFP-tag from Dsyd-1::GFP to label presynaptic regions), and iv) a polyclonal rabbit anti-DsRed antibody (632496, Clontech) diluted 1:200 in 3 % PBT (for detecting the DenMark signal to label postsynaptic regions).

The secondary antibody mixture consisted of (i) 2 % NGS diluted 1:25 in 3 % PBT, (ii) a polyclonal Cy3-conjugated goat anti-rat antibody (A10522, Life Technologies) diluted 1:200 in 3 % PBT, and (iii) a polyclonal Cy5-conjugated goat anti-rabbit antibody (A10523, Life Technologies) diluted 1:200 in 3 % PBT.

Chemical tagging for tracking APL development

Chemical tagging provides an alternative method to label specific cells and structures in tissues. The tag-based approach uses genetically-driven enzyme-based protein "tags" that are expressed in specific cells and that bind small fluorescent substrates, resulting in fast and specific tissue staining with low background signals (Kohl et al 2014; Sutcliffe et al 2017; Meissner et al 2018).

Tagging was used to track the regional synaptic polarity of APL during development. Specifically, the synaptic reporters synaptotagmin fused to the chemical tag SNAPm (Syt1-SNAPm) were used to label pre-synaptic regions, and telencephalin fused to CLIPm (TLN-CLIPm) to label post-synaptic regions (Kohl et al 2014). The effectors UAS-Syt1:SNAP (Kohl et al 2014; Bloomington Stock Centre no. 58379), UAS-TLN:CLIP (Kohl et al 2014; Bloomington Stock Centre no. 58382) and UAS-mCD8::GFP (for labelling APL) were used together with the intersectional driver APLi-GAL4 (NP2631-GAL4, GH146-FLP, tubP-FRT-GAL80-FRT) for specific expression in both larval and adult APL neurons (Lin et al 2014; Mayseless et al 2018).

The procedures followed Kohl et al (2014). In brief, brains of either third-instar larvae, or pupae (6 h or 12 h after puparium formation) or adults of the genotype APLi/Syt1:SNAP > mCD8::GFP/TLN:CLIP were dissected in ice-cold phosphate buffer (PB; 0.1 M) and fixed in 4 % PFA at room temperature for 20 min. Brains were permeabilized and washed 3 times in PBT (0.3 % Triton-X 100 in PBS) for 5-10 min. Then, chemical tag ligands were applied in a

300 µ L volume on a nutator for 15 min, at room temperature. Chemical substrates were SNAP-tag ligands (SNAP surface 549 - BG 549 [NEB, S9112S]) and CLIP-tag ligands (CLIP surface 647 - BC 647 [NEB, S9234S]) at final concentrations of 1 mM in 0.3 % PBT. To minimize cross-reactivity, SNAP-tag ligands were applied 10 min before CLIP-tag ligands. To label APL, brains were immunostained; after consecutive washing steps (3 x 10 min each) in 0.3 % PBT, preparations were blocked in 5 % NGS solution (005-000-121, Jackson Immunoresearch Laboratories; in PBT) for 30 min. Brains were then incubated overnight with a primary antibody mixture consisting of (i) 5 % NGS diluted in 0.3 % PBT and (ii) a polyclonal chicken anti-GFP antibody (AB_10000240, Aves Labs) diluted 1:500. After five consecutive washing steps (3 x brief; 2 x 20 min) in 0.3 % PBT, brains were incubated for 2 h at room temperature with a polyclonal secondary FITC-conjugated goat anti-chicken antibody (A16055, Invitrogen) diluted 1:300 in 0.3% PBT. Preparations were mounted in slowfade (Invitrogen) on slides, and analysed under a confocal microscope (Zeiss LSM 800). Image processing was performed using Imaris software (version 9.51, Bitplane).

Volume reconstruction of APL from an electron-microscopy dataset

Radial volume annotations were added to an existing skeleton reconstruction of the APL neuron in both hemispheres in an electron microscopy dataset of a 6 h-old stage 1 larva (Ohyama et al 2015; Eichler et al 2017). More details about the neuron reconstructions can be found in Eichler et al (2017). Volume annotations were made manually using the web-based software CATMAID (Saalfeld et al 2009; Schneider-Mizell et al 2016) which was extended with a tool to allow for rapid graphical annotations of radii of contiguous cable segments with similar radius. Radial annotations were used to create a conical frustum compartment volumetric representation of the cells' morphology. Radii were placed so as to preserve approximate volume of the irregularly shaped processes including accounting for the anisotropic imaging resolution of 3.8 nm × 3.8 nm × 50 nm. The axon and dendrite of both APL neurons were defined as the two synapse rich areas along the arbour separated from the primary axon by the high strahler branch-point nearest to the soma. Reconstructed neurons and their synapses were analysed using the natverse package (http://natverse.org/) (Bates et al 2020a) in R version 3.6.2 and plotted using Blender version 2.79 with the CATMAID-to-Blender plugin (https://github.com/schlegelp/CATMAID-to-Blender) (Schlegel et al 2016).

Dendrogram representations of APL synapses and branching

The neuron dendrograms are simplified, but topologically correct, two-dimensional representations of neurons with sophisticated morphologies (Strauch et al 2018). As relative branch lengths and synapse location are preserved, the dendrograms can be employed to visualize the mapping of synapses in an easily readable way. The APL dendrograms are derived from the recent electron microscopy connectome and were created following established computational methods (Eichler et al 2017; Strauch et al 2018). Additionally, the boundaries of the mushroom body compartments as defined in Saumweber et al (2018) were superimposed onto the dendrograms.

To analyse the relative distribution of APL-to-KC and KC-to-APL connections within the mushroom body calyx, the procedure followed Schleyer et al (2020). In brief, the "cable length" distances between synapses were computed on the neuronal branches. Based on these distances, a clustering algorithm served to partition all synapses into local synapse clusters (domains). For each domain, the distances between the APL-to-KC (or KC-to-APL) synapses were then evaluated to the cluster's centroid point, which served as a measure for the spatial distribution of APL-to-KC (or KC-to-APL) within the domain.

Behavioural assays

Experimental setup

Behavioural assays were carried out in a light-shielded custom-built box, as described in Schleyer et al (2020). In brief, the box contained a 24 x 12 LED array light table (Solarox) with a 6 mm thick diffusion Plexiglas panel placed above it, providing constant light conditions and intensity for the activation of light-gated ion channels expressed in neurons of interest (see section "*Genotypes and methods for optophysiology"*). Containing-larvae Petri dishes were placed onto the diffusion panel and were surrounded by a translucent polyethylene ring. The ring featured 30 infrared LEDs mounted behind to deliver light conditions (imperceptible to the animals) allowing behavioural recording for offline tracking analysis (see section "*Video recording and tracking of locomotion*"). As the odours, we used n-amyl acetate (AM; CAS: 628-63-7, Merck) diluted 1:20 in paraffin oil (CAS: 042-47-5, AppliChem) and 1-octanol (OCT, undiluted; CAS: 111-87-5, Sigma Aldrich).

Odour-fructose reward association

A two-group, reciprocal conditioning paradigm was used following standard procedures described in Chapter I (see also Michels et al 2017). In brief, one group of larvae received the

odour presented together with the fructose reward (paired training), whereas a second group received separate presentations of the odour alone and the fructose reward alone (unpaired training). After paired or unpaired training, the larvae were tested for their odour preference and the olfactory preference score (PREF) was calculated as described in Chapter I, that is:

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

Associative memory is indicated by a difference in preference for AM after paired training compared to the reciprocal, unpaired training. These differences in AM preference were quantified by the associative memory score:

$$(2)\ Memory\ score = \frac{PREF\ (Paired) - PREF\ (Unpaired)}{2}$$

Thus, memory scores may range from 1 to -1, with positive values indicating appetitive associative memory, whereas negative values would indicate aversive associative memory. These experiments were combined with optogenetic APL activation (see section "*Genotypes and methods for optophysiology*") as mentioned along the Results section.

Odour-APL association

Based on early results in this study (see Results section), I suspected that optogenetic activation of the APL neuron might have a rewarding effect. Therefore, the associative learning paradigm described above was modified by using optogenetic APL activation (+) instead of a fructose reward (i.e. no real reward was presented). In the paired group, AM was presented along with continuous 2.5-min light illumination to activate APL, whereas empty containers were subsequently presented in darkness, also for 2.5 min (AM+/EM). In the unpaired group, odour and light were presented separately to the animals (AM/EM+). This training cycle was performed one time only, with the training sequence alternated across repetitions as described in Chapter I. After training, the larvae were tested on a fresh, pure Petri dish and their odour preference as well as the memory score were calculated as detailed above (equation 1, equation 2).

In addition, a differential two-odour version of the paradigm using APL activation as the reinforcer was used. In brief, this was performed as explained above except that instead of using empty containers, those containers were filled up with 1-octanol (OCT, undiluted). As described in Chapter I, differential conditioning followed the logical structure of training as

either AM+/OCT or in the reciprocal case AM/OCT+ (again, training sequence was alternated across repetitions of the experiments). Then, the larvae were tested for their choice between AM and OCT on a fresh, pure agarose Petri dish and data were analysed, with due adjustment, as detailed above (equation 1, equation 2).

Whenever variations in the above paradigms were used, these are mentioned along with the Results section.

Innate olfactory behaviour

The odour preference of experimentally naïve larvae was performed following standard procedures (Saumweber et al 2011). After being collected and briefly rinsed, ~ 30 animals were immediately transferred onto a pure-agarose plate in presence of one odour-filled container and another empty container disposed on opposite sides. Naïve odour preference was calculated after 3 min following equation (1). To probe for an effect of APL activation on innate olfactory behaviour, the test was carried out either without light stimulation, or with light stimulation.

Genotypes and methods for optophysiology

For APL activation experiments, transgenic larvae expressing either ChR2XXL or Chrimson in APL were used. To do so, SS01617-GAL4 (APL-GAL4) was crossed to UAS-ChR2XXL or to UAS-CsChrimson::mVenus as the effector. Double heterozygous progeny (abbreviated as APL>ChR2XXL or APL>Chrimson) was used for activation of the APL neuron; larvae heterozygous for either the GAL4 element (APL>+) or the UAS element (+>ChR2XXL or +>Chrimson) were used as the driver- and the effector-genetic control, respectively. To obtain the driver controls, SS01671-GAL4 was crossed to w^{1118} (Bloomington Stock Center no. 3605, 5905, 6326). As for effector controls, a strain lacking a GAL4 domain but containing the two split-GAL4 landing sites (attP40/attP2) was crossed to UAS-ChR2XXL or UAS-CsChrimson::mVenus. For experiments using Chrimson, the flies were raised on food supplemented with all-*trans* retinal (100 mM final concentration; cat: R2500; CAS: 116-31-4, Sigma Aldrich), unless mentioned otherwise.

For MBON activation experiments, UAS-ChR2XXL was crossed to three different drivers: either R36G04-GAL4 covering the two calyx MBONs in each hemisphere (Saumweber et al 2018; Bloomington Stock Centre no. 49940; abbreviated as MBONa1,a2-GAL4), the split-GAL4 line SS02006 covering only one calyx MBON in each hemisphere (Eschbach et al 2020b; kindly provided by M. Zlatic, University of Cambridge; abbreviated as MBONa1-GAL4), or SS01417 covering one, or in some cases both calyx MBONs in each hemisphere (**Figure S5**;

Eschbach et al 2020b; kindly provided by M. Zlatic, University of Cambridge; abbreviated as MBONa2-GAL4). Again, double heterozygous progeny (abbreviated as MBONa1,a2>ChR2XXL, MBONa1>ChR2XXL or MBONa2>ChR2XXL) was used for activating the calyx MBONs; larvae heterozygous for either the R36G04-GAL4 driver (MBONa1,a2>+) or the effector (+>ChR2XX) were obtained. The mentioned custom-built box (see section "*Experimental setup*") was equipped for illumination from a blue LED light table when using ChR2XXL (wavelength: 470 nm; intensity: 107 µW / cm^2; Solarox), or from a red LED light table when using Chrimson (wavelenght: 630 nm; intensity: 350 µW / cm^2; Solarox).

For MBON inactivation experiments, the GtACR1 light-gated chloride channel was expressed in the calyx MBONs. To do so, either R36G04-GAL4, SS02006-GAL4 or SS01417-GAL4 were crossed to UAS-GtACR1::YFP (König et al 2019; Bloomington Stock Centre no. 9736; kindly provided by R. Kittel, University of Leipzig). Double heterozygous progeny (MBONa1,a2>GtACR1, MBONa1>GtACR1 or MBONa2>GtACR1) was used for silencing the calyx MBONs; larvae heterozygous for either the R36G04-GAL4 driver (MBONa1,a2>+) or the effector (+>GtACR1) were obtained as described in the preceding paragraph and were used as the genetic controls. A green LED light table (wavelength: 520 nm; intensity: 160 µW/cm^2; Solarox) was used for illumination.

For all cases, the timing of illumination is mentioned for each respective experiment along with the Results section. As all effectors are sensitive to daylight, breeding of all transgenic animals was performed in darkness ensured by black covers wrapped around the food vials. All behavioural experiments were carried out in parallel for the respective experimental group and genetic controls; investigators were blind with respect to genotypes.

Video recording and tracking of locomotion

Larval chemotaxis was recorded throughout the test and analysed as described by Paisios et al (2017). In brief, four behavioural features of larvae from the genotype APL>ChR2XXL were analysed both for learned and innate olfactory behaviour combined with APL activation:

First, the olfactory preference (PREF time, in s) was calculated as:

$$(3)\ PREF\ time = \frac{Time\ spent\ on\ AM\ side - Time\ spent\ on\ EM\ side}{Total\ duration}$$

Thus, preference scores may range from +1 to -1, with positive scores showing that larvae spent more time on the odour side, whereas negative values would indicate more time spent on the non-odour side.

Second, the head cast (HC) rate modulation was calculated as:

$$(4)\ HC\ rate\ modulation = \frac{\#HC/\mathrm{s}\ (away\ from\ AM) - \#HC/\mathrm{s}\ (toward\ AM)}{\#HC/\mathrm{s}\ (away\ from\ AM) + \#HC/\mathrm{s}\ (toward\ AM)}$$

Thus, positive scores indicate odour approach i.e. larvae make more HCs when crawling away from the odour than when crawling towards it. Oppositely, negative scores indicate odour avoidance.

Third, the HC reorientation (°) was calculated as:

$$(5)\ HC\ reorientation = abs\ (before\ HC) - abs\ (after\ HC)$$

The absolute heading angle (abs) indicates how oriented the larva's head is as regards the odour. For instance, at abs 180° or 0° the odour is located behind or in front of the animal, respectively. Thus, positive values indicate odour approach i.e. the head cast directs the larva towards the odour instead of far from it. Conversely, negative values suggest odour avoidance.

Fourth, the run speed modulation was calculated as:

$$(6)\ Run\ speed\ modulation = \frac{Run\ speed\ toward\ AM - Run\ speed\ away\ AM}{Run\ speed\ toward\ AM + Run\ speed\ away\ AM}$$

Thus, positive run speed modulation indicates that animals would slow down whenever they head away from the odour, and speed up when they move towards it.

Pharmacological manipulation of dopamine synthesis

To test for an implication of the dopaminergic system in odour-APL associative learning, a systemic pharmacological approach was used to disrupt dopamine synthesis (Neckameyer 1996; Kaun et al 2011; Thoener, König et al 2020). This approach was combined with behavioural experiments using optogenetic APL activation as the reinforcer (see section Odour-

APL association experiments) and followed procedures described in Thoener, König et al (2020). In brief, a 0.5 mg/ml yeast solution was produced and kept for up to one week at 4 °C. The dopamine-synthesis inhibitor 3-Iodo-L-tyrosine (3IY; CAS: 70-78-0, Sigma Aldrich; concentration: 5 mg/ml) was added to samples of 2 ml yeast solution. In the instances mentioned in the Results section, the dopamine precursor 3,4-dihydroxyphenylalanine (L-DOPA; CAS: 59-92-7, Sigma Aldrich; concentration: 10 mg/ml) was added to a yeast solution with or without 3IY. After mixing on a shaker for 1 hour, solutions were transferred into vials containing two pieces of PET mesh. Third-instar progeny of the APL-GAL4 driver crossed to UAS-ChR2XXL (APL>ChR2XXL) were transferred from their food vials to the respective yeast solutions. After a feeding period of 4 hours at 25 °C and 60-70 % relative humidity, larvae were briefly washed in water and immediately used in behavioural experiments.

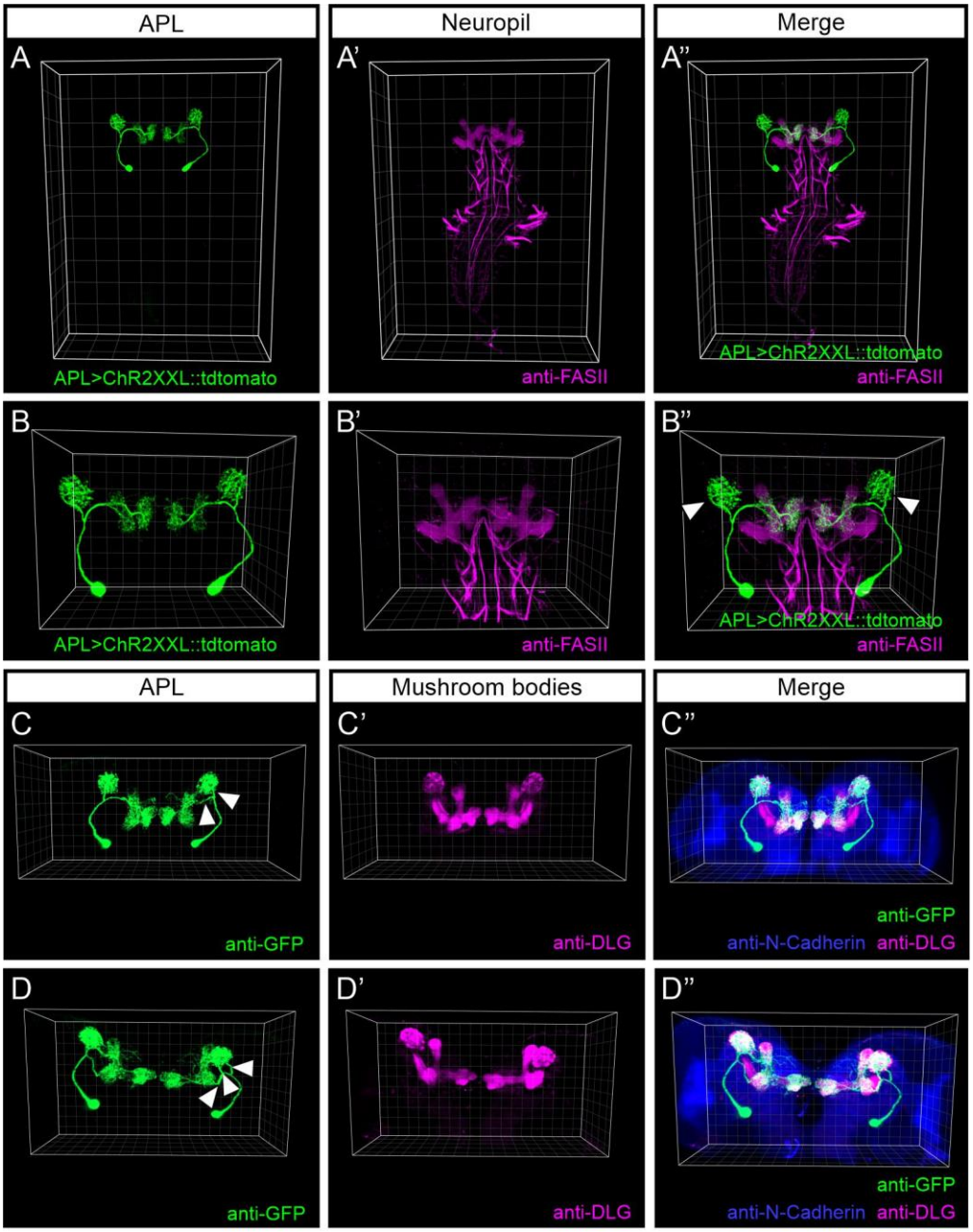
Statistics

For the behavioural data shown in **Figures 19-27**, statistical analysis was performed as mentioned in Chapter I/ Mancini et al (2019), except mentioned otherwise. All behavioural experiments were conducted in parallel for the respective experimental group and genetic controls.

Results

Organization of the APL neuron

I first investigated the expression pattern of the APL-GAL4 driver in third-instar larvae. Combining it with the UAS-ChR2XXL::tdtomato effector and using the resulting fluorescence signal confirms that APL-GAL4 specifically covers APL (**Figure 14A-B''**) (Saumweber et al 2018). As expected, APL sends its projections separately into the calyx and the lobes of the mushroom bodies (**Figure 14B''**, **C''**) (Masuda-Nakagawa et al 2014; Mayseless et al 2018; Saumweber et al 2018). Of note, in 10 of 11 preparations of third-instar larval brains with the APL-driver and UAS-mCD8::GFP as the effector, the primary neurite splits in two already after a relatively short distance (**Figure 14C-C''**). In one preparation three branches were observed from the primary axon, in both hemispheres (**Figure 14D-D''**). This made wonder how invariant the compartmental coverage of APL in the lobes is, in particular in third-instar larvae that were intended to be used in further behavioural analyses. Across five specimen of third-instar larval brains with the APL-driver and UAS-mCD8::GFP as the effector, coverage of the calyx and of the compartments was similar between the APL neurons of both hemispheres


APL
Neuropil
Merge
A
A'
A''
APL>ChR2XXL::tdtomato
anti-FASII
APL>ChR2XXL::tdtomato
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B
B'
B''
APL>ChR2XXL::tdtomato
anti-FASII
APL>ChR2XXL::tdtomato
anti-FASII
APL
Mushroom bodies
Merge
C
C'
C''
anti-GFP
anti-DLG
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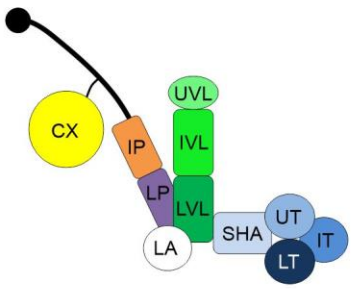
E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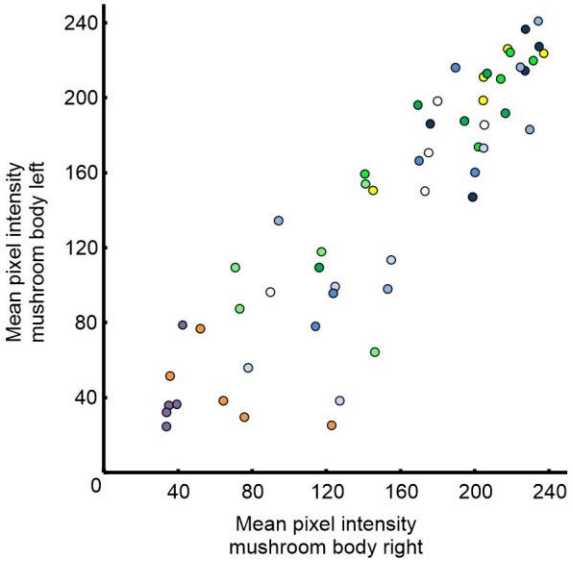
240
200
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0
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240
Mean pixel intensity mushroom body left
Mean pixel intensity mushroom body right

Figure 14. Expression of the APL-GAL4 driver strain is restricted to the APL neuron. **(A-A'')** 3D view of the expression pattern from the APL-GAL4 driver in a third-instar larval brain visualized using the fluorescence signal from the UAS-ChR2XXL::tdtomato effector (APL>ChR2XXL::tdtomato; green). Axon-rich regions of the mushroom body peduncle and lobes can be discerned as reference after labelling with a primary monoclonal mouse anti-FASII antibody and a secondary polyclonal goat anti-mouse Alexa Fluor 488 antibody (anti-FASII; magenta). Transgene expression is specific to the hemispherically unique APL neuron. Data were acquired with a 20x glycerol objective; grid edge lengths: 50 µm. **(B-B'')** As in (A-A''), providing a close-up view of the mushroom bodies, revealing that APL sends projections into the calyx and a subset of the compartments of the medial and vertical lobes. White arrowheads in (B'') point to the calyx which is innervated by APL but is largely devoid of the axonal FASII marker. Data were acquired with a 63x glycerol objective; grid edge lengths: 20 µm. **(C-D'')** As in (B-B''), but the APL-GAL4 driver was crossed to UAS-mCD8::GFP as the effector in third-instar larvae. APL membranes can be visualised after labelling with a primary polyclonal rabbit anti-GFP antibody and a secondary polyclonal goat anti-rabbit Alexa Fluor 488 antibody (anti-GFP; green). The mushroom bodies are labelled by a primary monoclonal mouse anti-DLG antibody and a secondary polyclonal goat anti-mouse Alexa Fluor 568 antibody (anti-DLG; magenta); neuropils can be discerned as reference by a primary monoclonal rat anti-N-Cadherin antibody and a secondary polyclonal goat anti-rat Alexa Fluor 647 antibody (anti-N-Cadherin; blue). Close-up analysis of APL's morphology revealed two, or in one case three branches (white arrowheads in C, D, respectively) dividing from the primary neurite; of note, these numbers of branches do not differ between both hemispheres (N = 11 brains). Data were acquired with a 16x glycerol objective; grid edge lengths: 20 µm. **(E)** For each mushroom body compartment, the mean pixel intensities of APL labelling in the right hemisphere versus the left hemisphere are plotted (compartmental colour code according to the mushroom body schematic). The observed correlation indicates no inter-hemispheric difference in APL morphology (Pearson correlation (r)= 0.9747; $p < 0.05$). The sample size (number of brains) is given within the figure.

(**Figure 14E**). GFP signals were consistently strong in the calyx, close to absent in the two peduncle compartments, weak in the upper vertical lobe and in the shaft of the medial lobe, and moderate to strong in the remaining compartments (**Figure 14E**). Taking together the present and published data, I conclude that the larval APL innervates the calyx and six of the 10 compartments, namely the lateral appendix, the upper, intermediate and lateral vertical lobe, as well as the upper and lateral toe (**Figures 4A**, **Figure 14**) (Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018). I next confirmed that APL is GABAergic **(Figure 15A-B'')** and studied the regional organization of pre- and post-synaptic sites of APL using the APL-GAL4 driver together with the double effector UAS-Dsyd-1::GFP/UAS-DenMark (Masuda-Nakagawa et al 2014; Owald et al 2015a). According to earlier reports, APL is pre-synaptic in the calyx, whereas it is post-synaptic in both the calyx and the lobes (**Figure 15C-D''**) (Masuda-Nakagawa et al 2014).

The above results match the situation in first-instar larva, as shown here for a volume reconstruction of APL generated from the electron microscopy reconstruction of the mushroom body in Eichler et al (2017) (**Figure 16A, B**). Specifically, that volume reconstruction shows that APL's relatively slender axonal and dendritic branches separately arise from a thicker primary neurite (**Figure 16C, D**), similar to the locust homologue of APL called GGN (Papadopoulou et al 2011; Ray et al 2020). The electron microscope dataset of Eichler et al (2017) further allowed mapping the site of the synapses for the different classes of synaptic

partners of APL onto its volume reconstruction (**Figure 16E-F**). Furthermore, the connectomics results allowed deriving dendrograms of APL, that is, two-dimensional representations of APL

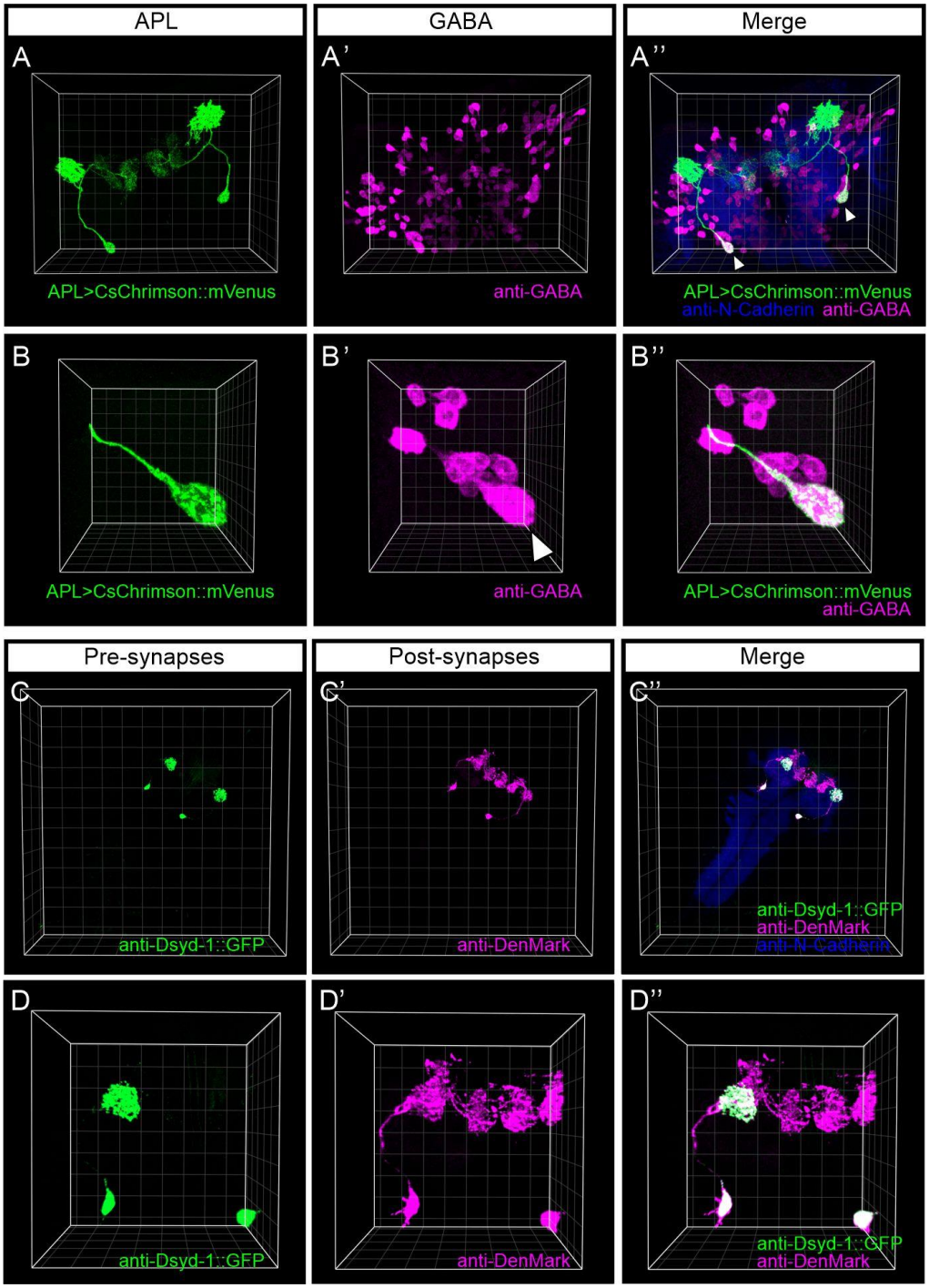

Figure 15. The larval APL neuron is GABAergic and is pre-synaptic in the calyx and post-synaptic in both the calyx and the lobes. (A-A'') 3D view of the expression pattern from the APL-GAL4 driver in the third-instar larval brain visualized using the fluorescence signal from the Chrimson effector (APL>CsChrimson::mVenus; green). GABAergic signals can be visualised after labelling with a polyclonal rabbit anti-GABA antibody and a polyclonal Cy5-conjugated goat anti-rabbit antibody (anti-GABA; magenta). The white arrowheads in (A'') point to an overlap of the GABA signal and the fluorescence signal in the APL soma. Neuropil regions are visualized as reference by using a primary monoclonal rat anti-N-Cadherin antibody and a secondary polyclonal goat anti-rat Cy3 antibody (anti-N-Cadherin; blue); grid edge lengths: 20 µm. **(B-B'')** As in (A-A''), providing a close-up view of the APL soma. The white arrowhead in (B') points to the APL

soma surrounded by additional GABAergic cells; grid edge lengths: 5 µm. Data were acquired with a 63x glycerol objective. **(C-C'')** The APL-GAL4 driver was crossed to a double effector with both, UAS-Dsyd-1::GFP and UAS-DenMark to label the pre- and post-synaptic sites of the APL neuron in third-instar larvae. Pre-synaptic regions of APL can be visualised after labelling with a polyclonal FITC-conjugated goat anti-GFP antibody (anti-Dsyd-1::GFP; green). Post-synaptic regions are revealed after labelling with a primary polyclonal rabbit anti-DsRed antibody and a secondary polyclonal goat anti-rabbit Cy5 antibody (anti-DenMark; magenta). Neuropil regions are visualized as reference by using a primary monoclonal rat anti-N-Cadherin antibody and a secondary polyclonal goat anti-rat Cy3 antibody (anti-N-Cadherin; blue). The pre-synaptic marker Dsyd-1 is mainly restricted to the calyx, while the post-synaptic marker DenMark localizes to both the calyx and a subset of the compartments in the lobes, confirming the regional synaptic polarities of the larval APL neuron (Masuda-Nakagawa et al 2014; Eichler et al 2017). Data were acquired with a 16x glycerol objective. Scale bars represent 50 µm. **(D-D'')** As in (C-C''), providing a close-up view of the pre- and post-synaptic regions of APL; grid edge lengths: 25 µm.

preserving branch lengths and synaptic locations in a topographically correct manner (**Figure 17**). It can be discerned within such a topology that wherever they coexist, the synapses that APL entertains with mushroom body extrinsic neurons are not segregated from but intermingled with the connections to the mushroom body intrinsic neurons, the KCs (**Figure 17B, C**). In the lobes, the almost exclusively post-synaptic sites of APL are relatively sparse (**Figure 17B, C**) and with some variation in topology between the APL neuron of the left versus the right brain hemisphere (for the right hemisphere APL neuron, see **Figure S4A-C**). In the calyx, reciprocal synapses between APL and the KCs are organized in four, synapse-rich centre-surround structures such that APL-to-KC synapses are observed towards their centre, while KC-to-APL synapses are located mainly at their surround (**Figure 17D; Figure S4D**).

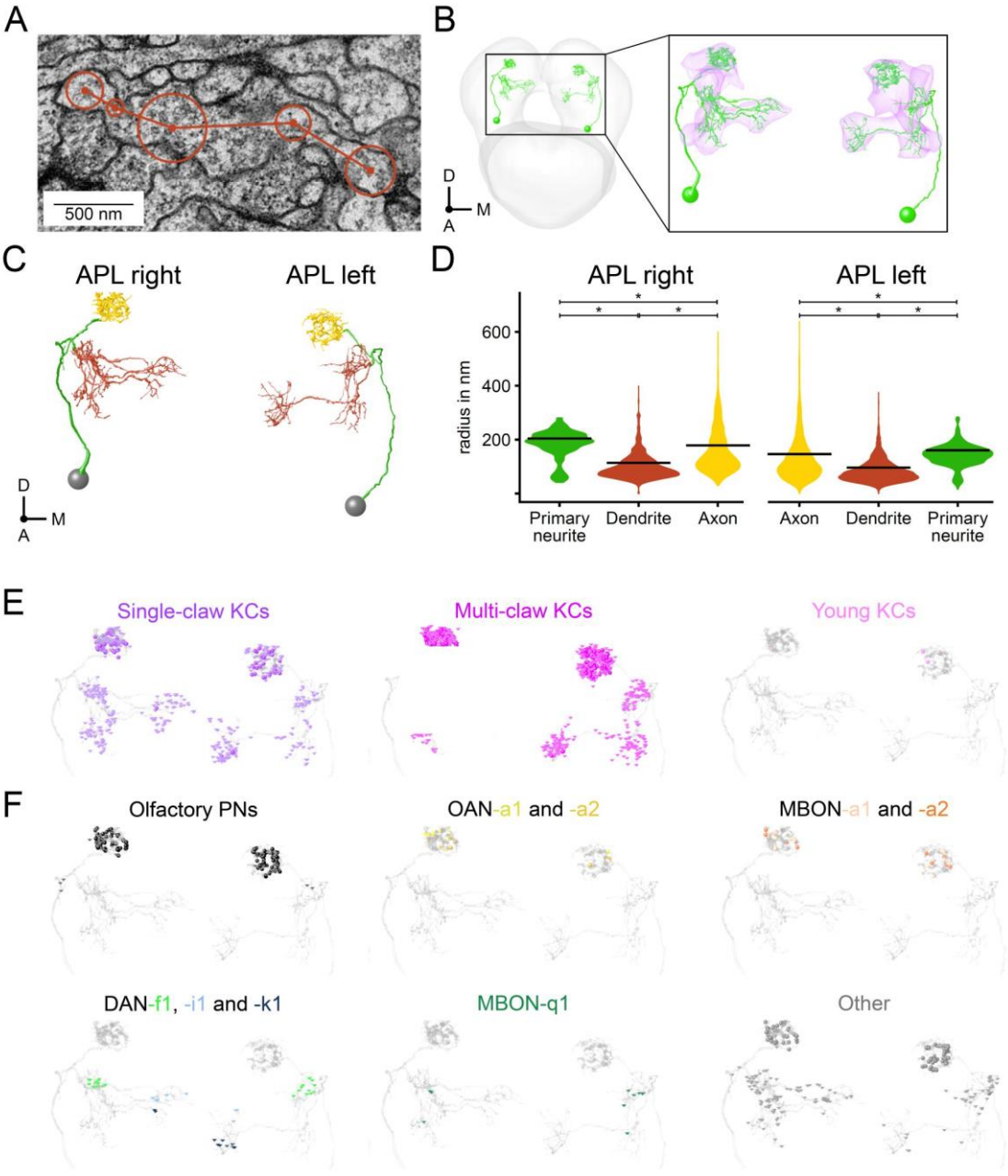

Figure 16. Volume reconstruction of the larval APL neuron. (A) Electron microscopy cross section of the APL neuron in a first instar larva. Points connected by lines represent the skeletonized reconstruction of the neuron (for details see Eichler et al 2017). Circles represent radii annotations for volume reconstruction. Scale bar represents 500 nm**. (B)** Reconstructed volume of the left- and the right-hemisphere APL neuron (green) in the context of the complete nervous system (left; grey mesh), and in a close-up of the mushroom body region (right; magenta). **(C)** Reconstructed volume of both APL neurons separated into axonal (yellow) and dendritic (red) regions, and the primary neurite and its branches (green). **(D)** Quantification of the radii of the APL neurons, showing that the primary neurite is thicker than the axonal regions (Cohen's d effect size 0.30 and 0.17 on the right and left, respectively), which in turn are thicker than the dendritic regions (0.95 and 0.76, respectively). Data are displayed as violin plots; bars represent mean; * refers to significant differences between the APL regions in t-test comparisons (* $p < 0.05$). **(E-F)** Pre- and post-synaptic sites annotated by dots and triangles, respectively, selectively for different types of connected neuron, namely: (E) single-claw, multi-claw and young KCs; (F) neurons with connections in the calyx (top row: olfactory PNs; OAN a1, a2; MBON a1, a2), as well as neurons that have otherwise been studied in functional experiments such as DAN-i1 (Saumweber et al 2018; Schleyer et al 2020), DAN-f1 (Eschbach et al 2020; Weiglein et al 2020; Schleyer et al in prep) and DAN-k1 (Saumweber et al 2018). Neurons with less than two synapses with APL in both hemispheres and are shown as "Other". In (B-C), A: anterior; D: dorsal; M: medial.

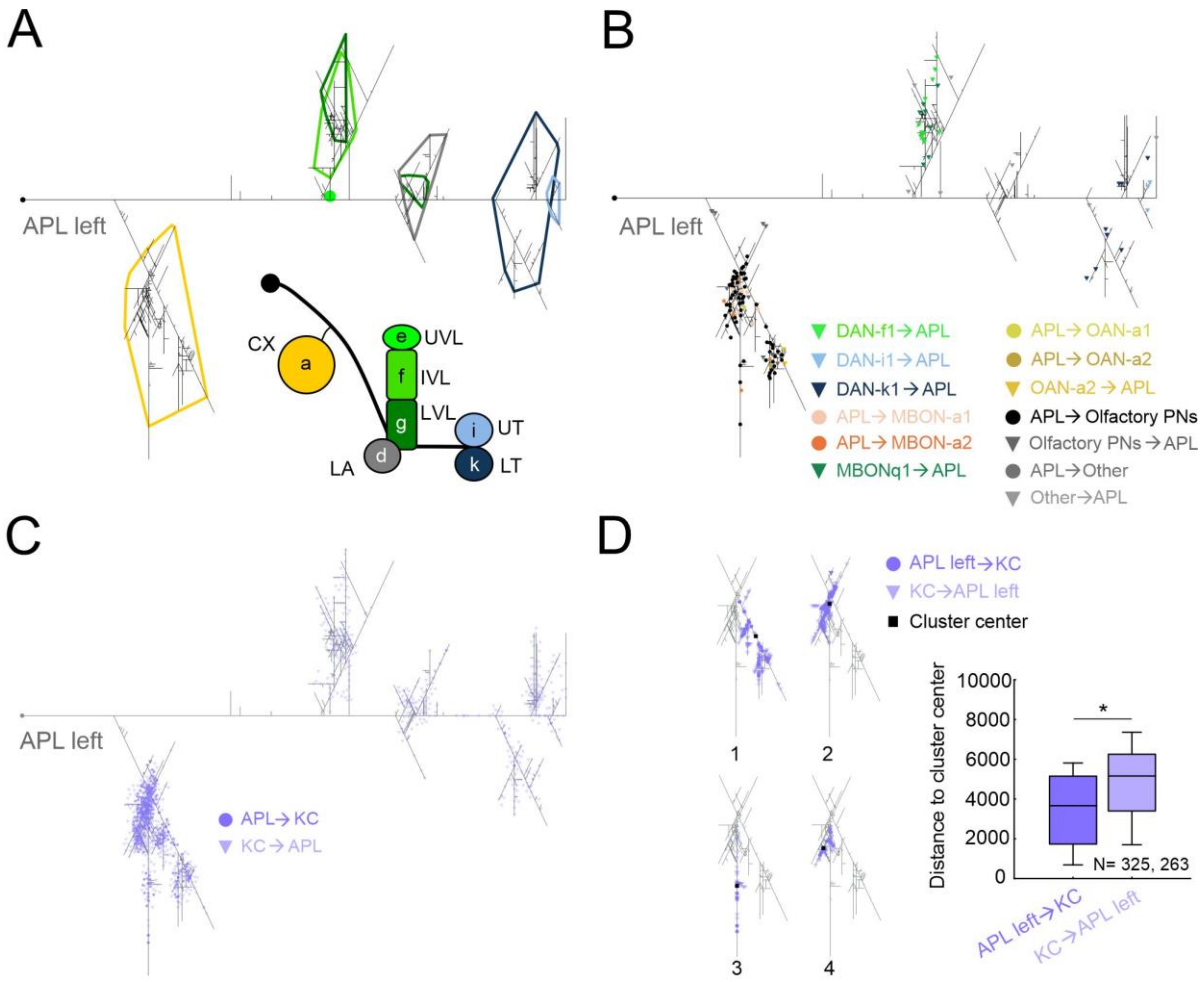

Figure 17. Dendrogram analysis of the larval APL neuron. (A) Two-dimensional dendrogram of the APL neuron from the left hemisphere, based on an electron microscope reconstruction in a first-instar larva (data from Eichler et al 2017). Branch size and synapse locations are preserved in a topographically correct manner. Coloured envelopes indicate the mushroom body calyx and compartments innervated by APL, shown schematically in the inset (see also Figure 4A, B). **(B)** Repartition of synapses on the left-hemisphere APL neuron with the indicated mushroom body extrinsic neurons. Pre- and post-synaptic sites of APL are annotated with dots and triangles, respectively (colour code according to Figure 16F). **(C)** As in (B), but showing synaptic sites of the left-hemisphere APL with the mushroom body intrinsic neurons, the Kenyon cells (KCs); dark purple dots and bright purple triangles show APL-to-KC and KC-to-APL synapses, respectively. **(D)** Cluster analysis revealed that calycal synaptic sites of the left-hemisphere APL with the KCs are organized in four clusters (1-4). As the accompanying quantification shows, most of the APL-to-KC synapses (dark purple dots) are observed towards the centre of these clusters (dark square) whereas KC-to-APL synapses (bright purple triangles) are observed mainly at their surround. Data are displayed as box plots, the middle line showing the median, the box boundaries the 25, 75 % quantiles, and the whiskers the 10, 90 % quantiles. The sample sizes (number of synapses) are given within the figure. * refers to MWU comparisons between APL-to-KC and KC-to-APL synapses (* $p < 0.05$). Corresponding analyses for the right hemisphere APL neuron can be found in Figure S4.

Regional synaptic polarity of APL across metamorphosis

Given the conserved regional synaptic polarity of APL across larval stages (see preceding section) and given that APL persists into adulthood yet in adults is not regionally polarized, we examined how APL develops across metamorphosis (Wu et al 2013; Lin et al 2014; Mayseless et al 2018; Saumweber et al 2018). Towards this end, genetically encoded protein 'tags' coupled

with chemical fluorophore ligands were used (Kohl et al 2014; Sutcliffe et al 2017; Meissner et al 2018). The synaptic reporter synaptotagmin fused to the tag SNAPm (Syt1-SNAPm) allowed to label pre-synapses, and the reporter telencephalin fused to the tag CLIPm (TLN-CLIPm) allowed to label post-synapses (Kohl et al 2014). These constructs were expressed in APL throughout development using the intersectional driver APLi-GAL4 that is specifically expressed in APL in both larvae and adults (Lin et al 2014; Mayseless et al 2018). In addition, UAS-mCD8::GFP was expressed to visualize APL membranes. In third-instar larvae of the genotype APLi/Syt1:SNAP > mCD8::GFP/TLN:CLIP, pre-synaptic staining was mostly found in the calyx (**Figure 18A'**) while post-synaptic staining was distributed in the calyx and the lobes (**Figure 18A''**), consistent with previous observations (**Figure 15**) (Masuda-Nakagawa et al 2014). Already at 6 h after puparium formation, pre-synaptic structures that were more punctuated were detected (**Figure 18B'**, **C'**) and observed overall fewer post-synaptic structures (**Figure 18B''**, **C''**), consistent with the previously reported pruning of APL secondary neurites during pupal stages (Mayseless et al 2018). Interestingly, at 12 h after puparium formation — a stage where APL pruning is almost at its peak (Mayseless et al 2018; Puñal et al 2021) — both pre- and post-synaptic structures were still detectable (**Figure 18D-D'''**), although some post-synaptic structures were observed detached from the primary neurite (**Figure 18D'', D'''**; yellow arrowhead). Nonetheless, the polarized organization of APL in third-instar larvae was no longer observed at the adult stage, as both pre- and post-synaptic markers were detected across both the calyx and the lobes of the mushroom bodies (**Figure 18E-F'''**) (Wu et al 2013; Lin et al 2014).

Taken together, these results indicate that while APL is regionally polarized throughout larval stages, it undergoes rearrangement during metamorphosis to give rise to a regionally more diffuse organization. In addition, the DPM neuron, one of the main APL synaptic partner involved in memory consolidation in adults, does not exist in larvae (Pitman et al 2011; Wu et al 2011; Eichler et al 2017; Saumweber et al 2018). Based on these differences, and despite the rich recent insights gained into the function of APL in adults, a detailed look into the function of the larval APL neuron is warranted (Inada et al 2017; Zhao et al 2019; Zhou et al 2019; Amin et al 2020; Apostolopoulou and Lin 2020; Kanellopoulos et al 2020; Yamagata et al 2021).

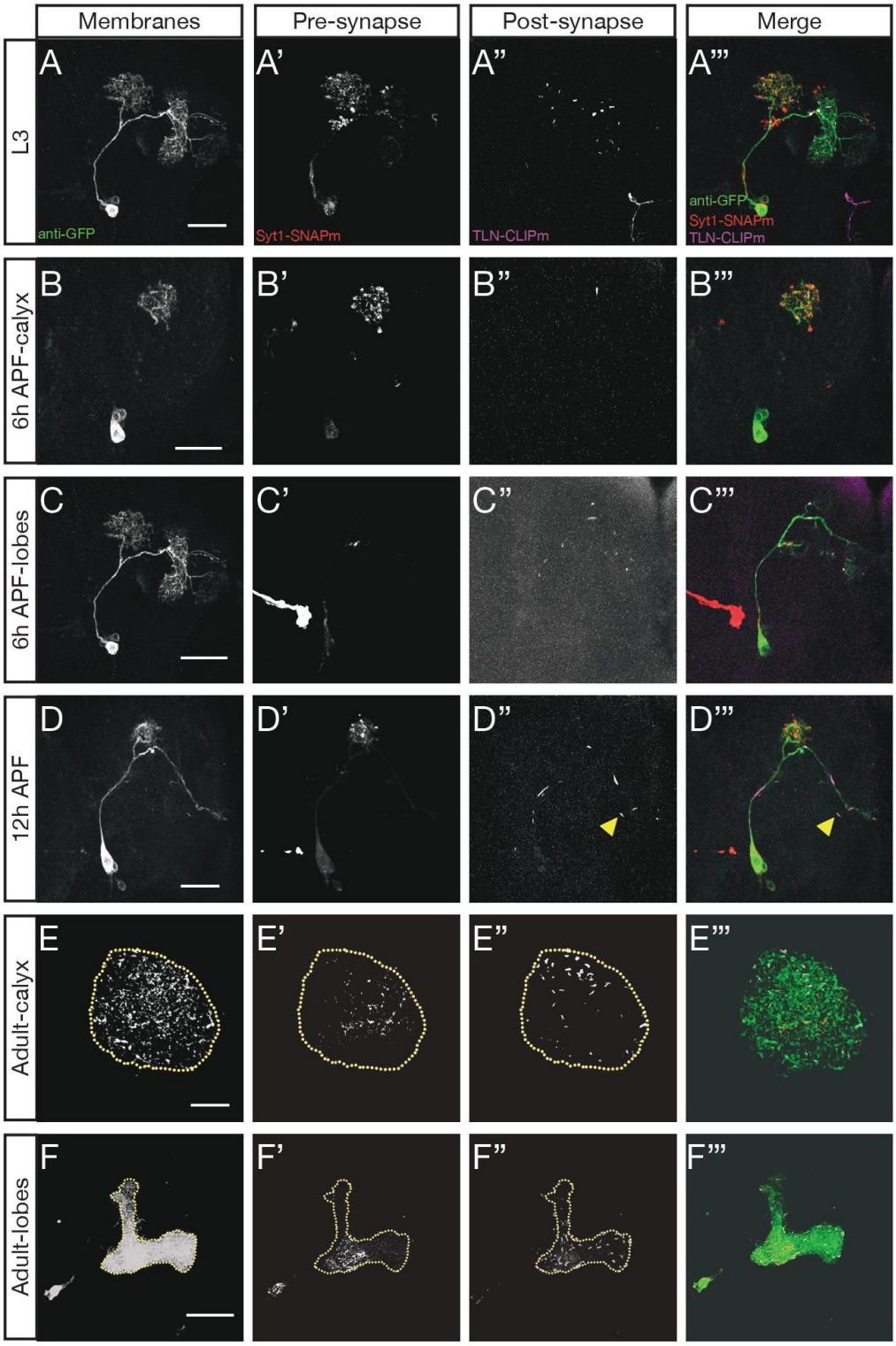

Figure 18. Regional synaptic polarity of APL across metamorphosis. Confocal maximal projection images of stainings for mCD8::GFP, Syt1::SNAP, and TLN::CLIP (Kohl et al 2014) driven by the APL-specific intersectional driver APLi (Lin et al 2014; Mayseless et al 2018) at the developmental times: **(A-A''')** third-instar larva (L3); **(B-C''')** 6h after puparium formation (6h APF: calyx: B-B'''; lobes: C-C'''); **(D-D''')** 12h APF; **(E-F''')** adult (calyx: E-E'''; lobes: F-F'''). Brains were stained with a polyclonal chicken anti-GFP antibody to label the APL neuron (A-F). To label pre-synapses (A'-F') and post-synapses (A''-F''), the pre-synaptic reporter synaptotagmin was fused to the chemical tag SNAPm (Syt1-SNAPm), and the post-synaptic reporter telencephalin was fused to CLIPm (TLN-CLIPm), respectively (Kohl et al 2014). Merged images are shown in (A'''-F'''). In third-instar larva, pre-synaptic staining was largely restricted to the calyx (A') while post-synaptic staining was distributed in both the calyx and the lobes (A''). At 6h APF, both pre- and post-

synaptic staining are similarly distributed as in the larvae (B'-C'''); of note, pre-synaptic structures seemed to be more punctated (B', C') and fewer post-synaptic structures were detectable (B'', C''). Indeed as late as 12h APF, both pre- and post-synaptic structures were still detectable (D', D''); post-synaptic structures appeared to be detached from the primary neurite (D'', D'''; yellow arrowhead). In adults, both pre- and post-synaptic markers were detectable in both the calyx and the lobes (E-F'''). Data were acquired with a 40x oil objective; scale bars represent 30 μm.

Memory scores are abolished upon activating APL throughout odour-fructose training

I first asked whether optogenetically activating APL affects associative memory. Third-instar larvae were trained in a standard Pavlovian conditioning paradigm, using an odour (*n*-amylacetate) as the conditioned stimulus, and a fructose reward as the unconditioned stimulus (Scherer et al 2003; Neuser et al 2005; Saumweber et al 2011; Michels et al 2017). One group of larvae received the odour presented together with the fructose reward (paired training), whereas a second group received separate presentations of the odour and the fructose reward (unpaired training). After training, these two groups were tested for their odour preference. A difference in odour preference between paired and unpaired training thus reflects associative memory, and is quantified by the memory score. According to the convention, positive memory scores reflect appetitive associative memory, whereas negative scores would reveal aversive memory (equation 2; Materials and Methods section). As mentioned in Chapter I, paired and unpaired training both establish associative memory, yet of opposite "sign": after paired training the odour predicts the occurrence of the reward, leading to an associative increase in odour preference. In contrast, unpaired training establishes the odour as a predictor of the non-occurrence of the reward and supports an associative decrease in odour preference (for a detailed discussion, see Schleyer et al 2018).

By repeating an experiment from Saumweber et al (2018), APL was optogenetically activated throughout odour-fructose training (**Figure 19**). Confirming their report, odour-fructose memory scores in the experimental genotype (APL>ChR2XXL) were reduced to chance levels upon such treatment, and reduced relative to genetic controls (**Figure 19A**). The same abolishment of memory scores was observed in a shortened, one-trial version of this experiment (**Figure 19B**). For practical reasons, this shortened experimental design was used throughout the rest of the study. In addition, the expression of ChR2XXL in APL was directly confirmed by immunohistochemistry (**Figure 19C**). Critically, the behaviour of experimentally naïve larvae toward the odour was unaffected by APL activation (i.e. innate odour preference: **Figure 19D**, Saumweber et al 2018). Also, as shown here from offline analysis of video tracking data, APL activation did not affect the modulation of the microbehavioural patterns of locomotion by which naïve odour preferences come about (**Figure 19E-I**).

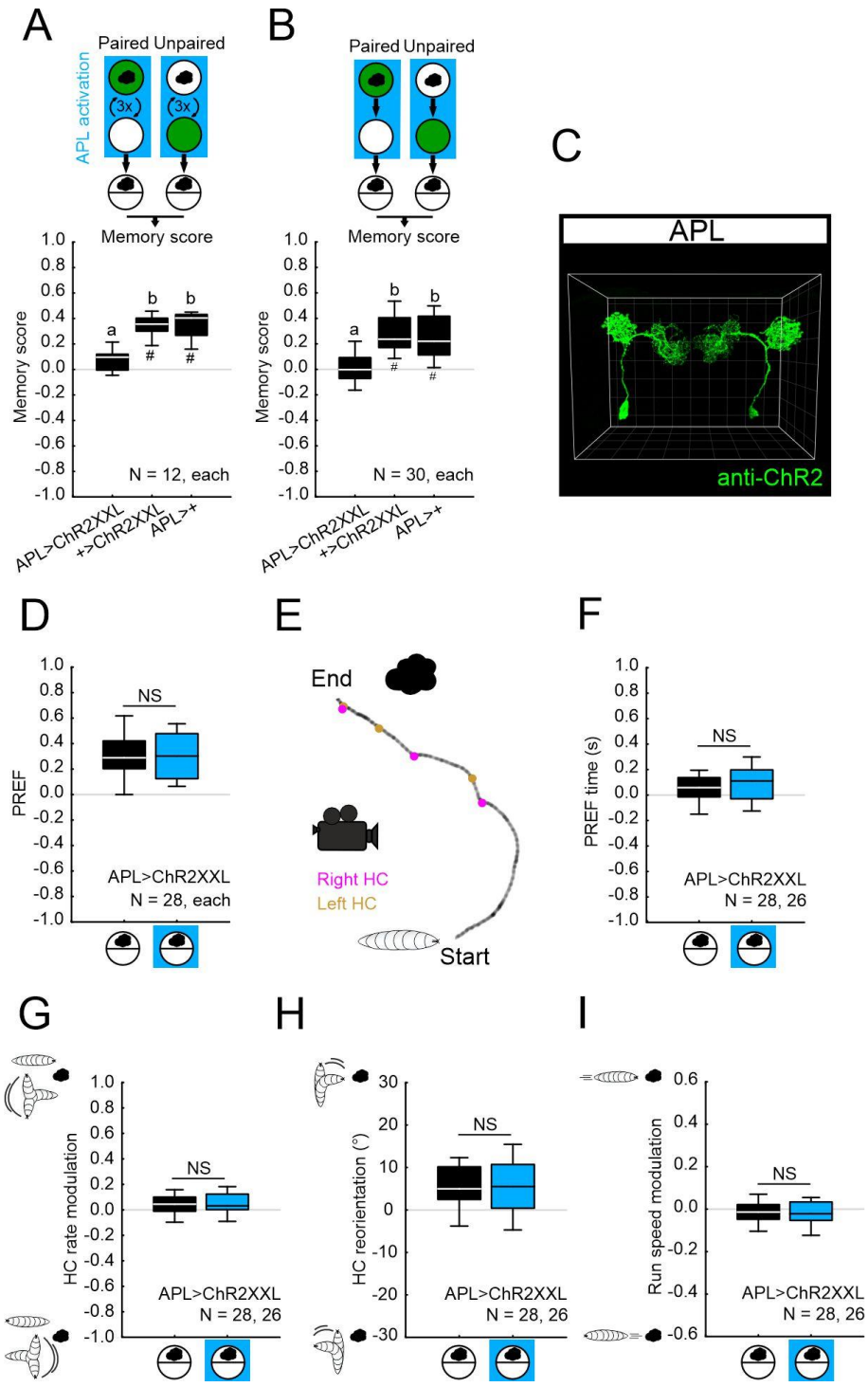

Figure 19. Memory scores are abolished upon activating APL throughout training. (A) Larvae were trained such that in one set of animals, the odour *n*-amyl acetate (dark cloud) was paired with the fructose reward (green-filled circle indicating a Petri dish) alternated with blank trials (open circle) whereas in a reciprocal set, the odour was presented unpaired from the fructose reward; please note that here and throughout this study, the sequence of training events was as depicted in half of the cases, and in reverse order in the other half of the cases. The APL neuron was optogenetically activated with blue light illumination (blue rectangle) during the complete training phase. Larvae from both group were then tested for their odour preference, and associative memory was quantified by the memory score as the difference in preference between these reciprocally trained groups of animals. Double heterozygous larvae of the genotype APL>ChR2XXL were used for APL activation; larvae heterozygous for the GAL4 (APL>+) or the effector construct (+>ChR2XXL) were used as the genetic controls. Optogenetic activation of the APL neuron during the complete training phase abolished associative memory scores. **(B)** The same effects were observed in a shortened, one-training cycle version of this

experiment. **(C)** Full projection of the expression pattern from the APL-GAL4 driver crossed to UAS-ChR2XXL in the third-instar larval brain. ChR2XXL is visualized by a primary monoclonal mouse anti-ChR2 antibody and a secondary polyclonal donkey anti-mouse Cy3 antibody. Confirming the results from Figures 14-15, this reveals strong and specific transgene expression in the APL neuron of both hemispheres (anti-ChR2XXL; green). Data were acquired with a 63x glycerol objective; grid edge lengths: 20 µm. **(D)** The behaviour of experimentally naïve larvae from the experimental genotype (APL>ChR2XXL) toward *n*-amyl acetate (black cloud) was tested, with or without APL activated during testing (blue square). Naïve odour preference was unaffected by APL activation. **(E-I)** The behaviour of larvae in (D) was videorecorded and analysed offline as described in Paisios et al (2017). (E) shows a short video-recorded sample of larval behaviour with successive runs and head casts (HCs). Displayed is the track of the midpoint with a successive regular darker - lighter pattern indicating peristaltic movements. Specifically, three microbehavioural features were analysed in addition to the olfactory preference i.e. the time spent by the larvae on the odour and the non-odour side (F), the HC rate modulation (G), the HC reorientation (H), and the run speed modulation (I). In all cases APL activation had no influence on larval behaviour. In (A-B) different letters refer to significant differences between groups in MWU comparisons with a Bonferroni-Holm correction ($p< 0.05$); In (D, F-I) NS refers to an absence of significance between groups in MWU comparisons (NS $p> 0.05$); in (A-B) $^{\#}$ refers to OSS comparisons to chance levels (i.e. to zero), also with a Bonferroni-Holm correction ($^{\#} p< 0.05$). Other details as in Figures 5-13.

Activating APL either in the presence or in the absence of the odour reduces memory scores

As argued in Saumweber et al (2018), the abolishment of memory scores upon activating APL during the complete training phase (**Figure 19**) may arise because APL provides an inhibitory GABAergic signal onto the KCs, based on ealier reports in adults (Lin et al 2014). Therefore, a strong activation of APL would silence the KCs, hence preventing a proper odour representation in the mushroom body and thereby also preventing odour-fructose memory formation. If so, memory formation should be disrupted when activating APL while the odour is presented, rather than when activating APL while the odour is not presented. To our surprise, however, *in both cases* odour-fructose memory scores were partially reduced compared to a control condition in which APL was not activated at all (**Figure 20, left**). Concerning these residual memory scores, the interpretation of odour-fructose memory was considered as a learned search for the fructose reward (Saumweber et al 2011; Schleyer et al 2011). This interpretation implies that memory is behaviourally expressed if the sought-for fructose reward is indeed absent during the test, but is not expressed if the testing is carried out in presence of the sought-for fructose reward. This was indeed the case in all three cases: (i) when not activating APL during training at all, (ii) when activating APL during odour presentation or (iii) when activating APL in the absence of odour (**Figure 20, right**) – please note that innate olfactory behaviour is not changed in the presence of fructose or other tastants (Schleyer et al 2011). In other words, also the remaining memory scores after APL activation during either period of the training reflect a search for the fructose reward.

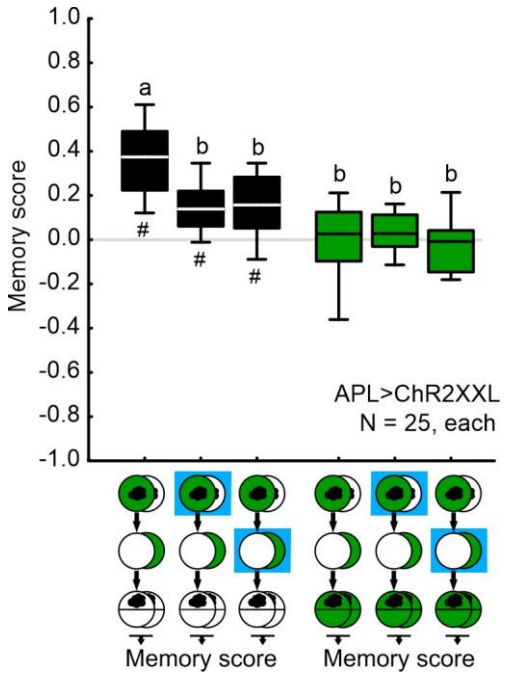

Figure 20. Activating APL in only the presence or in only the absence of odour reduces memory scores. Activating APL optogenetically (blue square) either only when the odour was presented during training, or only when the odour was not presented during training reduced memory scores to about half of control animals which did not receive any APL activation (black filled box plots). Testing the animals in the presence of the training reward (i.e. fructose) abolished the behavioural expression of memory in all cases (green filled box plots). The sample sizes and the genotype are given within the figure. # refers to OSS comparisons to chance levels (i.e. to zero) with Bonferroni-Holm correction (# $p < 0.05$); different letters refer to significant differences between groups in MWU comparisons also with a Bonferroni-Holm correction ($p < 0.05$). Other details as in Figure 19.

Differential effects of activating APL only in the presence or only in the absence of the odour

As mentioned, the observation that odour-fructose memory was impaired while activating APL in the absence of the odour was unexpected. In order to understand these results, I separately analysed the odour preference scores underlying the memory scores from **Figure 20**. In all three cases — (i) when APL was not activated at all (**Figure 21A**), (ii) when it was activated while the odour was presented (**Figure 21B**), and (iii) when it was activated while the odour was not presented (**Figure 21C**) — odour preference scores after paired versus unpaired training were indistinguishable from each other when the fructose reward was present during testing (open boxes in **Figure 21A-C**). In other words, in all cases learned search ceased once the sought-for reward was found. As discussed in detail in Schleyer et al (2018), this allows pooling the odour preferences after paired and unpaired training to determine baseline levels of odour preference, cleared of associative memory (stippled lines in **Figure 21A-C**). In all three cases, these baseline preference scores were intermediate between the paired-trained and the unpaired-trained animals that were tested in the absence of fructose, consistent with earlier reports (Schleyer et al 2018). This is adaptive because after paired training the larvae search for fructose where the odour is, whereas after unpaired training they search for fructose where the odour is not, and fittingly in either case their search is suppressed in the presence of the sought-for fructose. Important for the current context, however, is that these baseline levels were strikingly different depending on the contingency between APL activation and odour presentation (**Figure 21D**): as compared to the control baseline scores when not activating APL at all (stippled line

in **Figure 21A**; plotted in **Figure 21D, left**), the baseline scores were increased when APL was activated in the presence of the odour (stippled line in **Figure 21B**; plotted in **Figure 21D, middle**), and were decreased when APL was activated in the absence of the odour (stippled line in **Figure 21C**; plotted in **Figure 21D, right**). In other words, activating APL paired with odour increased odour preferences, while activating APL unpaired from odour presentation decreased odour preferences (**Figure 21D**) – as if activating APL had a rewarding effect! The next experiment directly tested this hypothesis.

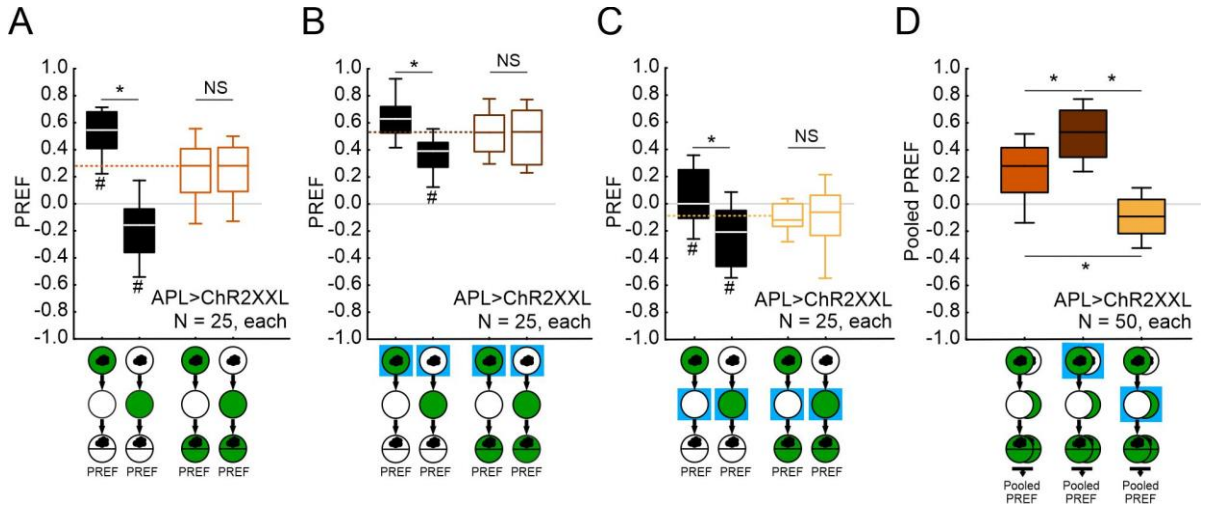

Figure 21. Differential effects of activating APL only in the presence or only in the absence of the odour. Considering the preference scores (PREF) underlying the associative memory scores from Figure 20 reveals that odour preference scores are higher after paired than after unpaired training with odour and fructose reward (black-line plots to the left), a difference that is abolished when testing is carried out in the presence of the training reward (right most-coloured plots). This is adaptive because learned search for the reward is obsolete in its presence. These preference scores can thus be pooled to serve as baseline odour preference cleared of associative memory (stippled line). This reveals that odour preference scores are higher than baseline after paired training and lower than baseline after unpaired training. **(B-C)** shows the same upon activating APL during training (blue square) only during odour presentation (B), or unpaired from odour presentation (C). Strikingly, baseline levels of odour preference differ between these three training conditions **(D)**: As compared to the control condition without APL activation, baseline odour preference scores (Pooled PREF) are increased when activating APL together with odour presentation, and decreased while activating APL unpaired from odour presentation. The sample sizes and the genotype are given within the figure. In (A-D) * and NS refer to MWU comparisons between groups with a Bonferroni-Holm correction (* $p < 0.05$; NS $p > 0.05$); in (A-C) $^{\#}$ refers to MWU comparisons to baseline levels of odour preference also with a Bonferroni-Holm correction ($^{\#}$ $p < 0.05$). Other details as in Figures 19-20.

Activating APL has a rewarding effect

To directly test whether optogenetically activating APL has a rewarding effect, animals were trained paired or unpaired odour presentations with APL activation instead of the fructose reward. This established positive memory scores in the experimental genotype, differing significantly from the genetic controls (**Figure 22A**). Thus, activating APL is rewarding and can establish an associative olfactory memory. Similar results were observed when using a

brief-stimulation protocol (**Figure 22B, C**). Such associative "odour-APL memory" was transient and lasted for less than 10 min (**Figure 22D**).

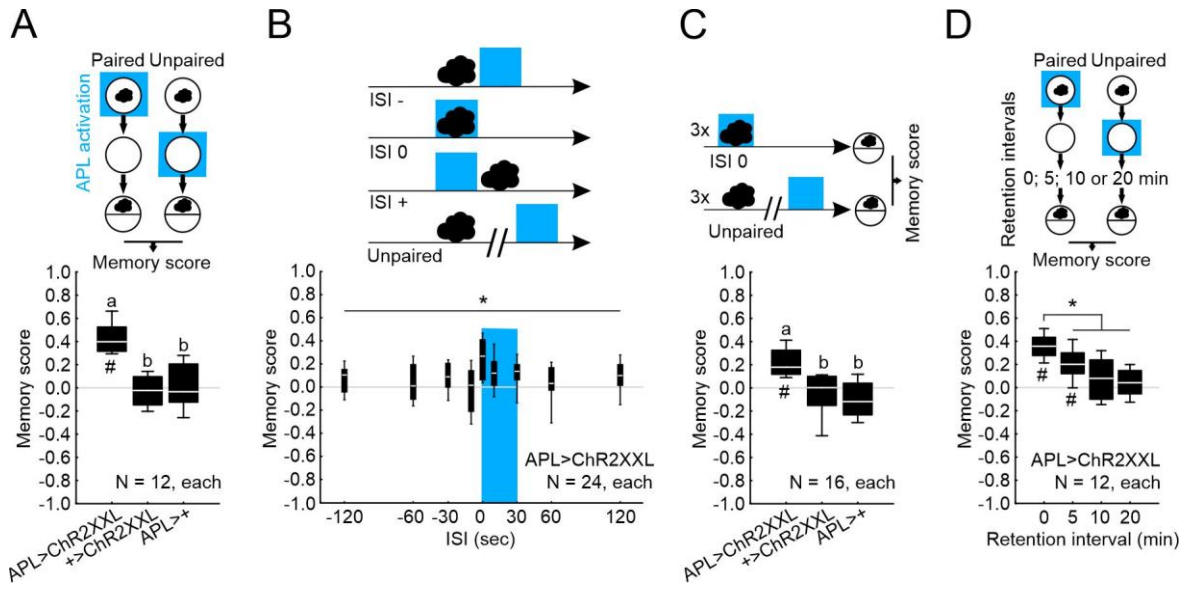

Figure 22. Activating APL has a rewarding effect. (A) Animals were trained by presenting an odour presented paired with, or unpaired from, activation of APL using ChR2XXL as the effector and blue light illumination (blue square). The effect of APL activation as a reward is quantified by positive memory scores, differing significantly from the genetic controls. **(B)** Larvae from the experimental genotype (APL>ChR2XXL) were trained as in (A) (i.e. paired or unpaired) but with modifications of the paradigm according to Weiglein et al (2020). Specifically, odour presentation and APL activation lasted for 30 s each at different timings relative to their onset (inter-stimulus interval, ISI): either the odour was presented *before* the APL activation (negative ISI values), *during* the APL activation (ISI 0), or *after* the APL activation (positive ISI values); in all cases reciprocal training involved odour presentation unpaired from APL activation. Three training trials were performed, followed by the test of odour preference. Memory scores differed dependent on the ISI. **(C)** Repetition of the experiment from (B) for simultaneous presentation of odour and APL activation (ISI 0), including genetic controls. Positive memory scores for the experimental genotype (APL>ChR2XXL) indicate that a brief stimulation of APL is sufficient to be rewarding, an effect that was not observed in the genetic controls. **(D)** Larvae from the experimental genotype (APL>ChR2XXL) were trained as described in (A) and tested either immediately after training (retention interval 0 min) or 5, 10 or 20 min after training. Expression of odour-APL memory was observed immediately after training and was still detectable at a 5 min retention interval; it was significantly reduced compared to immediate testing when assessed at 5, 10 or 20 min retention intervals. The sample sizes and the genotypes are indicated within the figure. In (A, C) different letters refer to significant differences between groups in MWU comparisons with a Bonferroni-Holm correction ($p < 0.05$); in (B) ***** refers to a KW multiple-group comparison (***** $p < 0.05$); in (D) ***** refers to significant differences between groups in MWU comparisons with a Bonferroni-Holm correction (***** $p < 0.05$). $^{\#}$ refers to OSS comparisons to chance levels (i.e. to zero), also with a Bonferroni-Holm correction ($^{\#}$ $p < 0.05$). Other details as in Figures 19-21.

In addition, an offline analysis of videorecorded larval locomotion revealed that the same aspects of larval "microbehaviour" were modulated by odour-APL memory **(Figure 23)** as for odour-taste reward associative memories, namely the rate of head casts and their orientation but not run speed (Schleyer et al 2015b; Paisios et al 2017; Thane et al 2019; Saumweber et al 2018; Schleyer et al 2020).

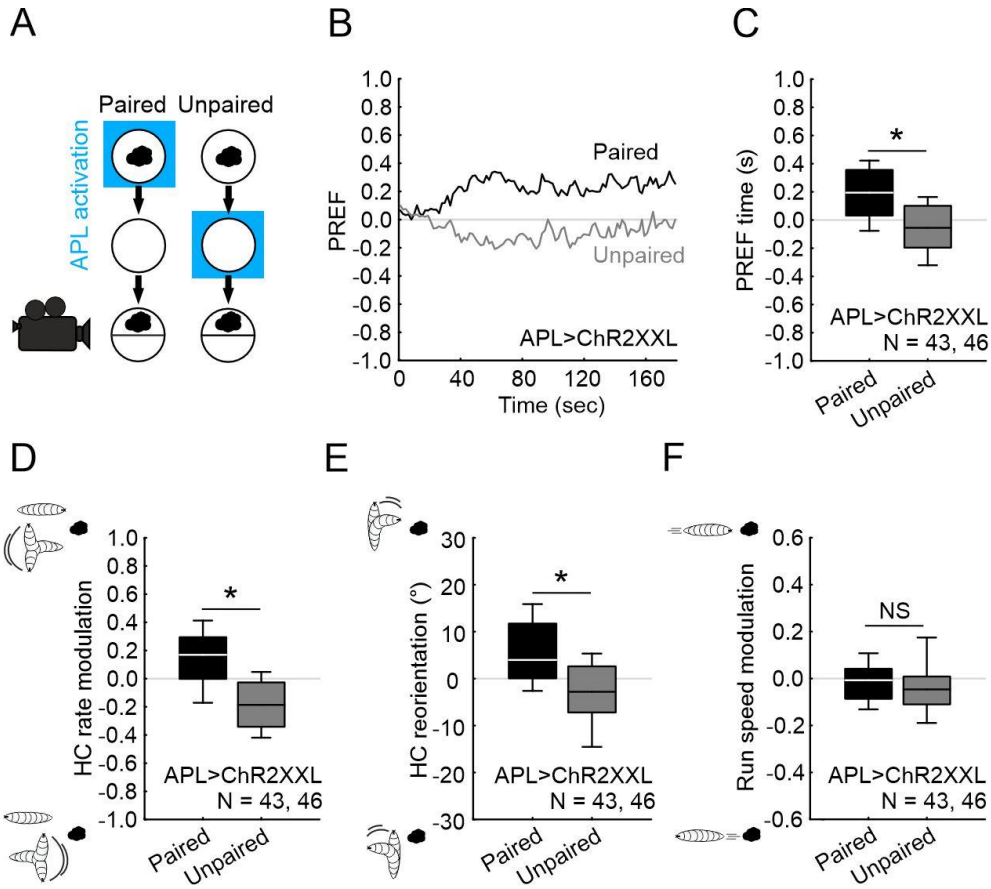

Figure 23. Activating APL modulates larval search behaviour as real taste rewards. (A) The behaviour of larvae from the genotype APL>ChR2XXL was recorded after paired or unpaired training with odour and APL activation; based on data available from Figure 22A, D and Figure 24A. **(B)** Larvae showed a higher preference for the odour after paired training than after unpaired training; dataset splitted into 100 bins (1.8 sec, each), showing the median of odour preferences across Petri dishes over time. **(C)** Larvae from the paired group spent more time on the odour side than to the non-odour side during testing, while the contrary was observed for the unpaired group. **(D)** Larvae trained paired exhibited more HCs when crawling away from the odour than when moving towards it; the contrary was true for the unpaired group. **(E)** Larvae from the paired goup oriented their HCs more in the direction of the odour as compared to larvae from the unpaired group. **(F)** The run speed when heading towards versus when headed away from the odour did not differ between paired- and unpaired-trained animals. Similar results were observed when using Chrimson as the optogenetic effector (not shown). The sample sizes (number of Petri dishes) and the genotype are given within the figure. In (C-E) * refers to significant differences between groups in MWU comparisons (* $p < 0.05$). In (F) NS refers to an absence of significance between groups in MWU comparisons (NS $p > 0.05$). Other details as in Figures 19-22.

Following what has been reported about fructose as a taste reward, if odour-APL memory scores reflect a learned search for the training reward (i.e. here APL activation), they should be abolished if the test is carried out in presence of that sought-for reward (Schleyer et al 2015a; see also **Figure 20**, **Figure 21**). The experiment from **Figure 22A** was therefore repeated with an additional experimental condition for which APL was also activated throughout the test. This prevented the behavioural expression of odour-APL memory (**Figure 24A**). The same was observed for a two-odour, differential conditioning version of the paradigm, using 1-octanol as the second odour (**Figure 24B)**, implying that a mnemonically eligible odour-specific

representation can be established in the mushroom body under these conditions. In line with our earlier results from **Figure 19D**, naïve odour preferences were unaffected by APL activation **(Figure 24C-E)**. Thus, APL activation has two kinds of effect previously reported for taste rewards: it both, induces associative memory when paired with odour during training (**Figure 22, Figure 24**) with the same microbehavioural 'footprint' as for taste rewards (**Figure 23**), and it terminates the search behaviour that is based on this memory during the test (**Figure 24**). These two effects of reward are adaptive because they aid the animals to search for the reward on the one hand, and prevent the animals from drifting away once the reward is found on the other hand. Both these two reward-like effects of APL activation, and the lack of effect of APL activation on naïve odour preference, were confirmed using Chrimson as the effector (**Figure 25A-D**). Of note, the termination of search upon APL activation during testing was only partial for Chrimson (**Figure 25C**).

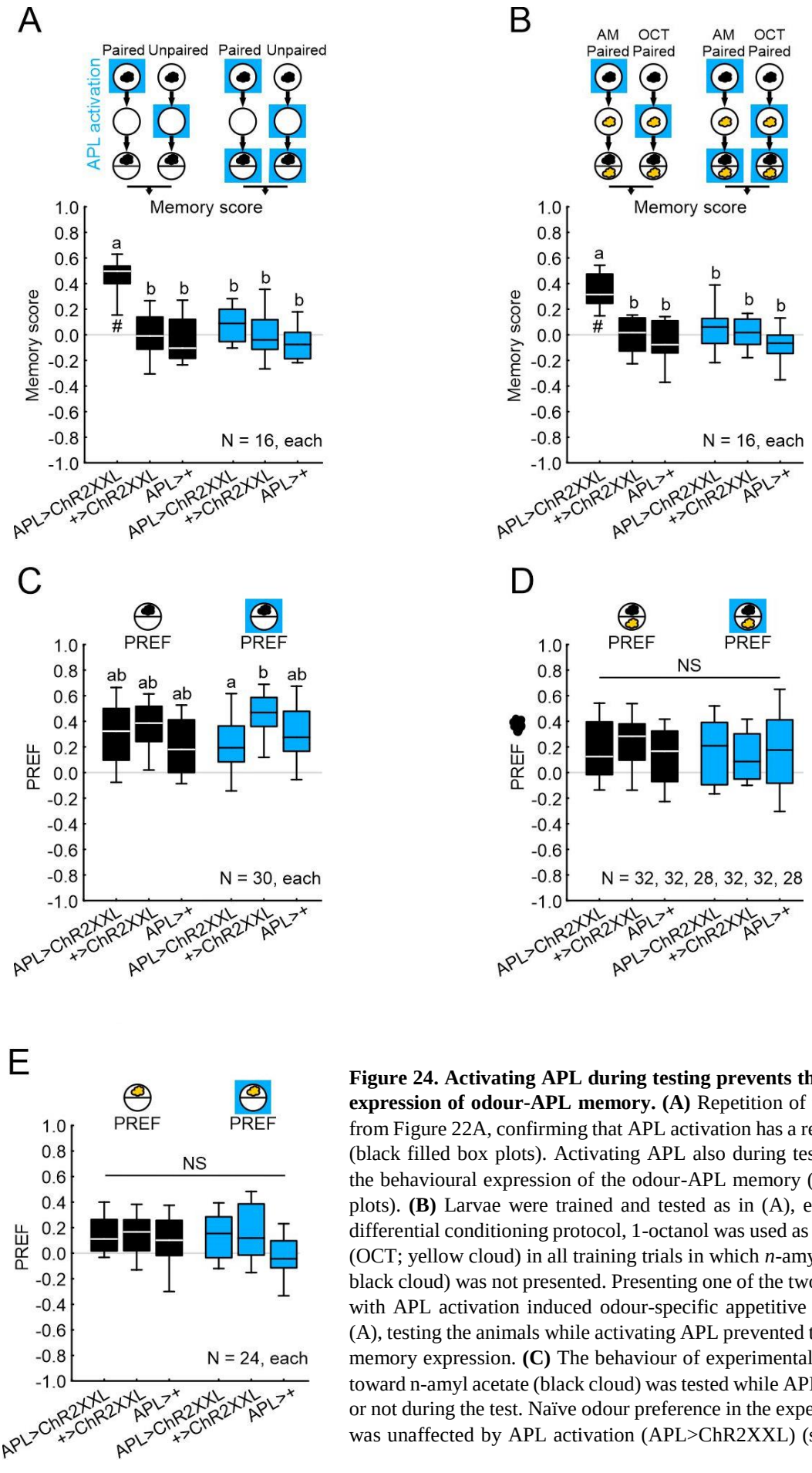

Figure 24. Activating APL during testing prevents the behavioural expression of odour-APL memory. (A) Repetition of the experiment from Figure 22A, confirming that APL activation has a rewarding effect (black filled box plots). Activating APL also during testing prevented the behavioural expression of the odour-APL memory (blue filled box plots). **(B)** Larvae were trained and tested as in (A), except that in a differential conditioning protocol, 1-octanol was used as a second odour (OCT; yellow cloud) in all training trials in which *n*-amyl acetate (AM; black cloud) was not presented. Presenting one of the two odours paired with APL activation induced odour-specific appetitive memory; as in (A), testing the animals while activating APL prevented the behavioural memory expression. **(C)** The behaviour of experimentally naïve larvae toward n-amyl acetate (black cloud) was tested while APL was activated or not during the test. Naïve odour preference in the experimental group was unaffected by APL activation (APL>ChR2XXL) (see also Figure

19D), with the caveat that it did differ from the effector (+>ChR2XXL), but not from the driver control (APL>+). **(D)** As in (C), except that OCT was used as a second odour (yellow cloud). Naïve odour preference in the experimental group was unaffected by APL activation (APL>ChR2XXL), and did not differ from the genetic controls. **(E)** As in (C-D), except that OCT was used as a single odour (yellow cloud). Again, naïve odour preference in the experimental group was unaffected by APL activation (APL>ChR2XXL), and did not differ from the genetic controls. The sample sizes and the genotypes are given within the figure. In (A-C) $^{\#}$ refers to OSS comparisons to chance levels (i.e. to zero) with a Bonferroni-Holm correction ($^{\#}$ $p< 0.05$); different letters refer to significant differences between groups in MWU comparisons, also with a Bonferroni-Holm correction ($p< 0.05$). In (D-E) NS refers to an absence of significance between groups in KW comparisons (NS $p> 0.05$). Other details as in Figures 19-23.

Manipulating activity in the calyx MBONs has no reinforcing effect

Considering the circuit mechanisms by which APL activation exerts a rewarding effect, I focused on the calyx MBONs to which APL is presynaptic (MBONa1 and MBONa2; also known as "Odd" neurons: **Figure 16F, Figure 17B**; Slater et al 2015; Eichler et al 2017; Saumweber et al 2018). I reasoned that if activating the GABAergic APL neuron exerts its rewarding effect by inhibiting the calyx MBONs, silencing them paired or unpaired from an odour should also trigger an appetitive memory. Using the chloride channel GtACR1 as the effector, this was not the case, however (**Figure 26A**). Considering the possibility that instead of being inhibited by the GABAergic signal from APL, the calyx MBONs get activated (e.g. through post-inhibitory rebound activation: Apostolopoulou & Lin 2020), the same experiment was repeated except that the MBONs were optogenetically activated using ChR2XXL as the effector. Again, no rewarding effect was observed upon such manipulation (**Figure 26B**). Before ruling out the two calyx MBONs as involved in the rewarding effect of APL, it seemed important to separately test for the effects of manipulating each of them separately. Indeed, activating MBONa1 and MBONa2 induces approach and avoidance, respectively (Eschbach et al 2020b). Thus, these MBONs might exert a rewarding and punishing effect, respectively, which would sum to zero when manipulating them together. However, neither silencing nor activating either one of the calyx MBONs yielded evidence for such oppositely-reinforcing effects (**Figure 26C, D**; these experiments include groups tested in the presence of the light for the respective optogenetic effectors as this can promote aversive memory expression: Weiglein et al 2020; Schleyer et al in prep). These results make it unlikely that the rewarding effect of APL activation involves the APL-to-MBONa1/a2 connections.

Given the resemblance between taste reward memories and the ones established by APL activation, and given the functional role of dopamine in conveying reward signals (Rohwedder et al 2016; Thoener, König et al 2020), I next asked for the dopamine-dependency of APL's rewarding effect.

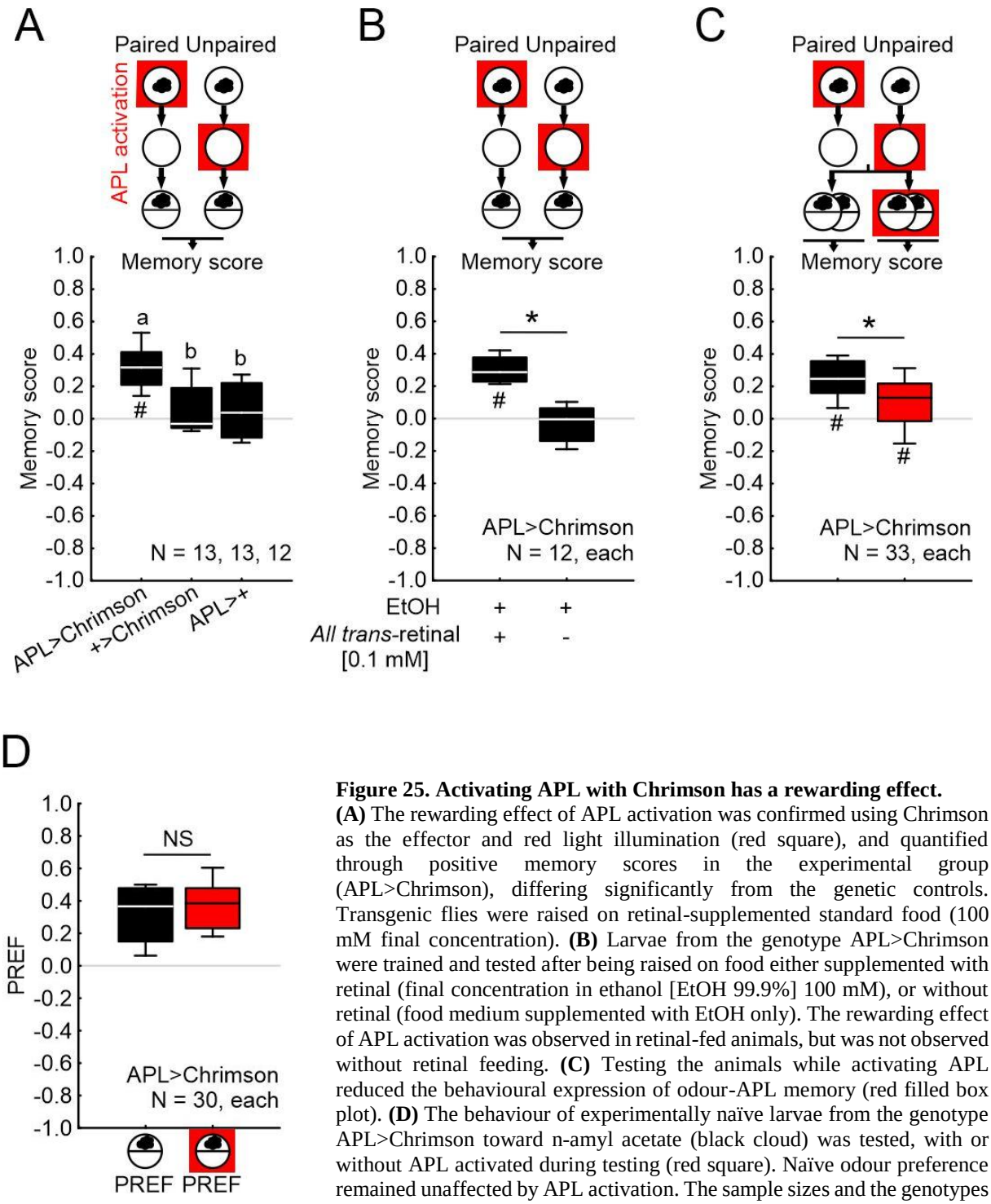

Figure 25. Activating APL with Chrimson has a rewarding effect.
(A) The rewarding effect of APL activation was confirmed using Chrimson as the effector and red light illumination (red square), and quantified through positive memory scores in the experimental group (APL>Chrimson), differing significantly from the genetic controls. Transgenic flies were raised on retinal-supplemented standard food (100 mM final concentration). **(B)** Larvae from the genotype APL>Chrimson were trained and tested after being raised on food either supplemented with retinal (final concentration in ethanol [EtOH 99.9%] 100 mM), or without retinal (food medium supplemented with EtOH only). The rewarding effect of APL activation was observed in retinal-fed animals, but was not observed without retinal feeding. **(C)** Testing the animals while activating APL reduced the behavioural expression of odour-APL memory (red filled box plot). **(D)** The behaviour of experimentally naïve larvae from the genotype APL>Chrimson toward n-amyl acetate (black cloud) was tested, with or without APL activated during testing (red square). Naïve odour preference remained unaffected by APL activation. The sample sizes and the genotypes are given within the figure. In (A) different letters refer to significant differences between groups in MWU comparisons with a Bonferroni-Holm correction ($p< 0.05$). In (B-C) * refers to significant differences between groups in MWU comparisons also with a Bonferroni-Holm correction (* $p< 0.05$). In (D) NS refers to an absence of significance between groups in MWU comparisons (NS $p> 0.05$). In (A-C) $^{\#}$ refers to OSS comparisons to chance levels (i.e. to zero) with a Bonferroni-Holm correction ($^{\#} p< 0.05$). Other details as in Figures 19-24.

Inhibition of dopamine signalling impairs odour-APL memory

An acute and systemic pharmacological approach was used to disrupt the dopamine-synthesis pathway (Neckameyer 1996; Bainton et al 2000; Fernandez et al 2017; Thoener, König et al 2020; **Figure 27A, B**). The dopamine-synthesis inhibitor 3IY was applied at a dose which leaves task-relevant behavioural faculties, i.e. innate odour preference and locomotion, intact (Thoener, König et al 2020). Larvae fed with 3IY for 4 hours prior conditioning exhibited reduced memory scores (**Figure 27C**). These memory defects were rescued in larvae which were additionally fed with the dopamine precursor L-DOPA (**Figure 27D**; of note, L-DOPA alone did not increase memory scores: **Figure 27D**).

These results suggest that the rewarding effect of APL activation involves a downstream dopaminergic mechanism.

Discussion

The present study confirms and extends our knowledge of the morphology of the GABAergic larval APL neuron, its regional synaptic polarity, the symmetrical coverage of the calyx and a subset of mushroom body compartments, the topology of all of its chemical synapses, its development through metamorphosis, and of the exquisite specificity of the transgenic driver strain that is available for studying it (**Figures 14, 15**). Together with previous studies, this establishes APL as both, the most complex and the most comprehensively described neuron of the larval mushroom body, including its larval-specific features (Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018; preprint by Jürgensen et al 2021; see also Introduction section). All these findings are consistent with a function of the reciprocal connection between APL and the KCs in the calyx for a sparsening of sensory representations across the mushroom body, serving their stimulus-specific association with reinforcement (**Figure 4C**). Surprisingly, however, and possibly in addition to such a role in the sparsening of the KC representation, the present study reveals a rewarding effect of optogenetically activating APL (**Figure 22**). Our behavioural experiments were then designed to ascertain key features of this unexpected rewarding effect and to take first steps to understand how it comes about (**Figures 22-27**).

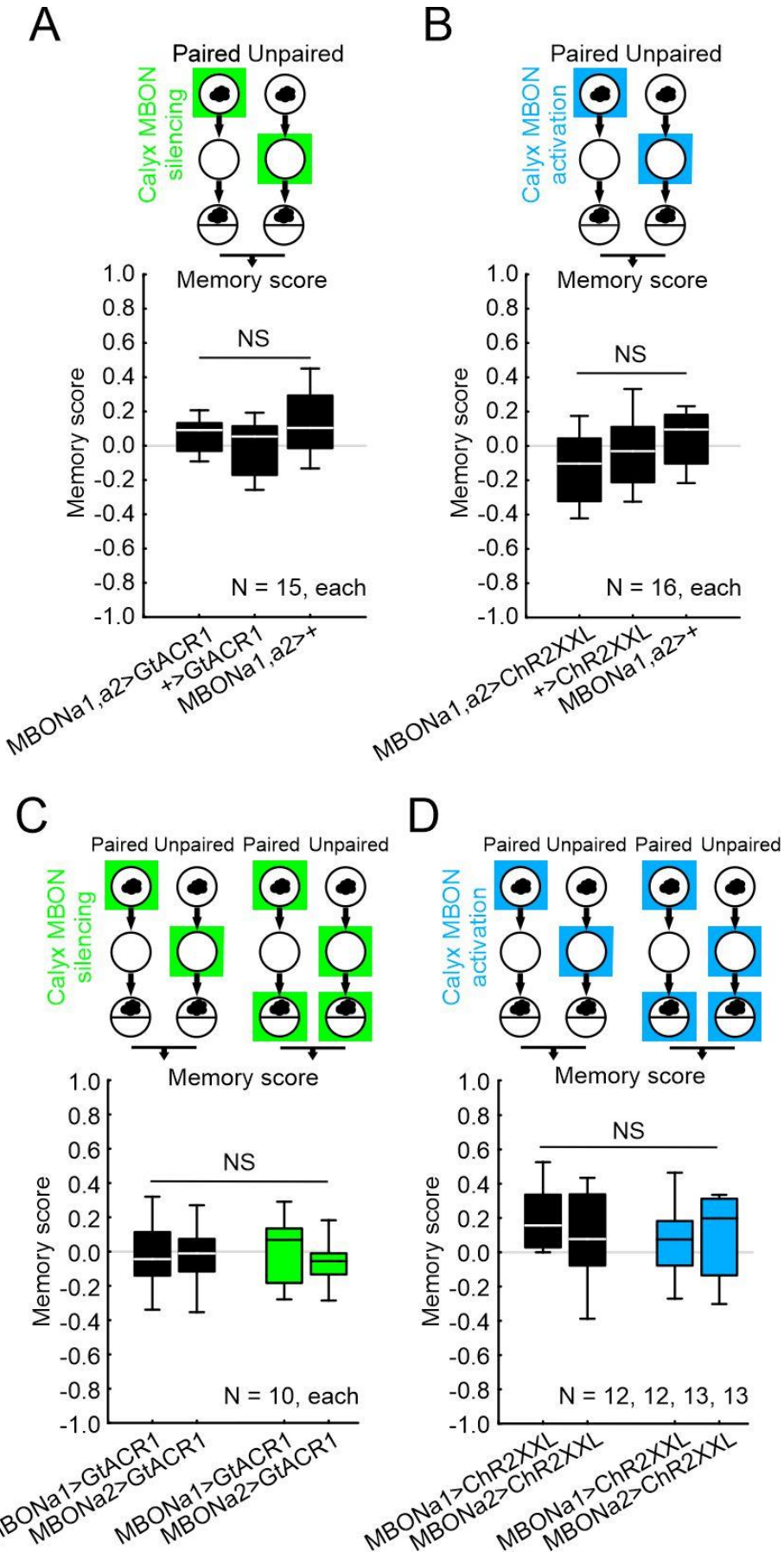
A
Paired Unpaired
Calyx MBON silencing
Memory score
Memory score
NS
N = 15, each
MBONa1,a2>GtACR1
+>GtACR1
MBONa1,a2>+
B
Paired Unpaired
Calyx MBON activation
Memory score
Memory score
NS
N = 16, each
MBONa1,a2>ChR2XXL
+>ChR2XXL
MBONa1,a2>+
C
Paired Unpaired Paired Unpaired
Calyx MBON silencing
Memory score
Memory score
NS
N = 10, each
MBONa1>GtACR1
MBONa2>GtACR1
MBONa1>GtACR1
MBONa2>GtACR1
D
Paired Unpaired Paired Unpaired
Calyx MBON activation
Memory score
Memory score
NS
N = 12, 12, 13, 13
MBONa1>ChR2XXL
MBONa2>ChR2XXL
MBONa1>ChR2XXL
MBONa2>ChR2XXL

Figure 26. Manipulating activity in the calyx MBONs has no reinforcing effect. (A) Larvae were trained paired or unpaired with odour presentations and silencing of the two calyx MBONs using GtACR1 as the effector and green light illumination (green square). Silencing the two calyx MBONs had no rewarding effect as larvae from the experimental genotype (MBONa1,a2>GtACR1) did not behave differently from the genetic controls. **(B)** Activating the two calyx MBONs likewise had no rewarding effect. **(C-D)** Silencing (C) or activating (D) the two calyx MBONs separately had no opposite reinforcing effect, either. The sample sizes and the genotypes are given within the figure. NS refers to an absence of significance between groups in MWU comparisons (NS p> 0.05). Other details as in Figures 19-25.

Features of the rewarding effect of APL activation

I discovered that activating APL optogenetically can induce an appetitive associative olfactory memory. As for odour-sugar association and for the rewarding effect of activating the dopaminergic DAN-i1 neuron, this is the case after already one training trial (Weiglein et al 2019; **Figure 22A**). The association process requires a fairly strict temporal coincidence of odour and APL activation (**Figure 22B, C**), which is reminiscent of the punishing effect of the dopaminergic DAN-d1 neuron in the aversive domain (Weiglein et al 2021). Such relatively

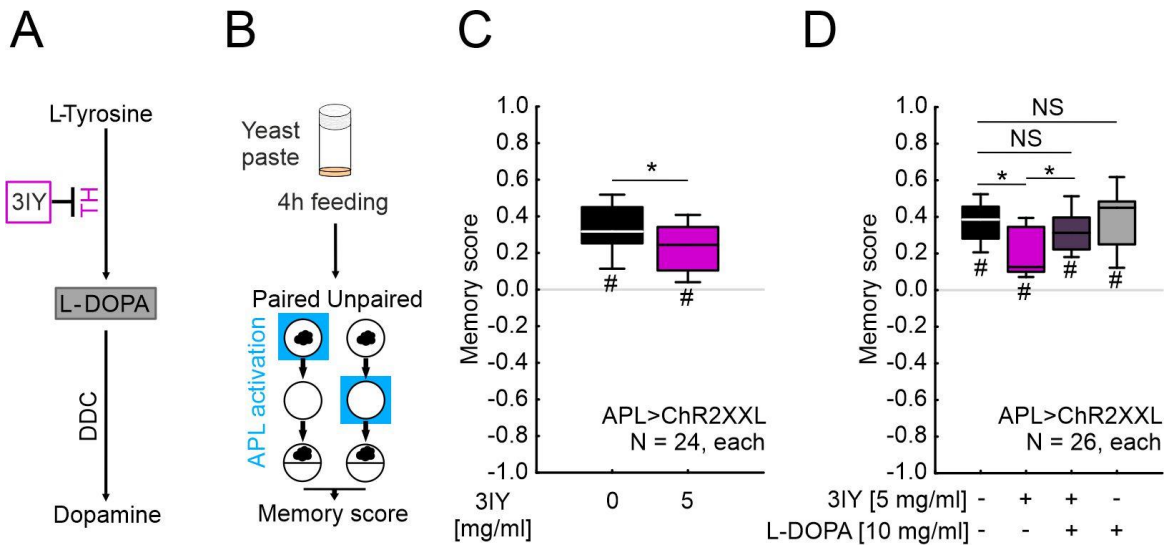

Figure 27. Inhibition of dopamine signalling impairs odour-APL memory. A systemic pharmacological approach was used to disrupt dopamine signalling (Thoener, König et al 2020). **(A)** Sketch of dopamine biosynthesis. The enzyme tyrosine hydroxylase (TH) converts the amino acid L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA). In a next step the enzyme dopa-decarboxylase (DDC) converts L-DOPA to dopamine. Application of 3-Iodo-L-tyrosine (3IY) inhibits the TH enzyme. **(B)** Third-instar APL>ChR2XXL larvae were transferred from their food vials to a yeast solution either supplemented with or without 3IY. After 4 hours of such feeding, animals were trained and tested as in Figure 22A. **(C)** 3IY-fed larvae exhibited impaired odour-APL memory scores as compared to 3IY-unfed control larvae. **(D)** As in (C) except that the yeast solution was prepared either without additional substances, or added with 3IY only, or with 3IY plus L-DOPA (a dopamine precursor), or with L-DOPA only, at the indicated concentrations. Again, reduced memory scores were observed in 3IY-fed larvae; feeding them additionally with L-DOPA rescued that memory impairment, leading to scores similar to that of control animals. L-DOPA alone had no impact on odour-APL memory. The sample sizes and the genotypes are given within the figure. In (C, D) $^{\#}$ refers to OSS comparisons to chance levels (i.e. to zero) with a Bonferroni-Holm correction ($^{\#}$ p< 0.05); ***** refers to significant differences between groups in MWU comparisons with a Bonferroni-Holm correction (***** p< 0.05). Other details as in Figures 19-26.

narrow windows for association formation match classic reports for odour-sugar association in honeybees and the association between odour and electric shock in adult *Drosophila* (Menzel & Bittermann 1983; Tully & Quinn 1985; Menzel 1990). At the technical level, this suggests that the present procedures and in particular the kinetics of the opening and the closing of the ChR2XXL channels are fast enough for performing meaningful behavioural experiments. Memories for single-trial odour-APL association decay within a few minutes (**Figure 22D**), as do one-trial memories for odour-sugar association (Weiglein et al 2019). Matching odour-tastant, as well as odour-DAN memories, odour-APL memories express as modulations of the frequency and direction of turning manoeuvres, but not via modulations of run speed (Paisios et al 2017; Schleyer et al 2020; **Figure 23**).

These findings suggest that driving APL does not only establish an appetitive memory during conditioning, but it can also prevent the expression of this behavioural memory when also activated during testing (**Figure 24A, B**). This is consistent with earlier reports using natural taste reward (e.g. fructose) or optogenetic activation of DAN-i1 as a "virtual" reward (Schleyer et al 2015a; Schleyer et al 2020). In contrast, manipulating APL activity is without measurable effect on innate olfactory behaviour, that is behaviour in gradients of odours for which the larvae are experimentally naive (**Figure 24C-E**). This is consistent with the little, if any, impact on naïve behaviour upon manipulating the function of the *entire* mushroom body (Heimbeck et al 2001; Parnas et al 2013).

From these results, it seems unlikely that activating APL completely silences the KCs. I do not exclude that manipulating APL activity by optogenetically activating it may affect KC activity and therefore odour representation within the mushroom body. Still, the odour-APL memory can be odour-specific (**Figure 24B**), suggesting that odour representations might not be affected by APL actvation. It could be that the odorants used in our study are distinct enough such that sparseness is not affected by manipulating APL; this would be in line with previous results showing that interfering with APL activity in adult flies impairs learning for similar, but not dissimilar odours (Lin et al 2014).

Thus, the rewarding effect of APL activation was observed across various repetitions and variations of the experiment, by using two independent optogenetic effectors, proper genetic and (for Chrimson) pharmacological controls. Unexpected as it is, the APL rewarding effect and the resulting memories do not appear to be in any way odd in comparison to more canonical internal reinforcement via DANs, or in relation to 'real world' reinforcements. Also, the present results appear to be compatible with previous observations reported in Saumweber et al (2018).

Effect of APL activation on odour-fructose memories: revisiting the hypothesis

By replicating an experiment from Saumweber et al (2018), I confirmed that activating APL optogenetically during odour-fructose training abolishes associative olfactory memory scores (**Figure 19A, B**). From this result it was proposed that activating APL would silence the KCs, thus preventing odour-fructose memory formation (Saumweber et al 2018). By revealing that driving APL exerts a rewarding effect, I provide an alternative explanation: in addition to a "real-world" fructose reward presented paired or unpaired from the odour, presenting a "virtual" reward (i.e. APL activation) throughout odour-fructose training prevents larvae from learning the predictive relationship between the odour and the fructose reward (*"regardless of the presence or absence of the odour, I will always get rewarded"*). Yet, additional effects of APL activation on odour respresentation within the mushroom body should not be excluded.

From APL to DANs?

By using a systemic pharmacological approach, I found that odour-APL learning is 3IY-sensitive and can be rescued by L-DOPA (**Figure 27**), suggesting a role of dopamine signalling in odour-APL memory formation. Here I discuss the involvement of potential synaptic pathways that may modulate DAN activity.

From APL via MBONs to DANs? APL makes pre-synaptic contacts with the two calyx MBONs (MBONa1 and MBONa2, also called Odd neurons) that project back indirectly onto DANs, mediate chemotactic behaviour, and are respectively approach- and avoidance-promoting when optogenetically activated (Slater et al 2015; Eichler et al 2017; Saumweber et al 2018; Eschbach et al 2020a, 2020b). Thus, activating APL might modulate MBON activity, further leading to indirect physiological effect on DANs; presenting an odour simultaneously would then allow the formation of an appetitive memory for that odour. However, presenting an odour with manipulating calyx MBON activity had no reinforcing effect (**Figure 26**), making the above scenario rather unlikely.

From APL via KCs to DANs synapses? APL establishes reciprocal connections with almost all KCs (Eichler et al 2017; Saumweber et al 2018). Interestingly, and similar to the present results, activating all KCs optogenetically while presenting odour is sufficient to induce an appetitive olfactory memory, which depends on the newly discovered KC-to-DANs synapses (Lyutova et al 2019). Given that APL is GABAergic and therefore presumably inhibitory (**Figure 15A-B''**; Lin et al 2014), activating it likely reduces KC activity. Thus, opposite manipulations of the KCs might lead to the same effect by modulating DAN activity via KC-to-DAN synapses.

Open questions

The rewarding effect of APL activation reported here raises several questions concerning its physiology and function.

Can signals in APL travel from the lobes to the calyx? Here I confirm previous reports that in larvae APL is both pre- and post-synaptic in the calyx, but only post-synaptic in the lobes (Masuda-Nakagawa et al 2014; Eichler et al 2017; **Figure 15C-D''**). In adults APL is pre- and postsynaptic throughout the mushroom body and provides local inhibition within distinct mushroom body compartments (Tanaka et al 2008; Aso et al 2014a; Inada et al 2017; Amin et al 2020). The clear synaptic polarity of the larval APL may suggest a local circuitry in the calyx only. Still the different morphology of the larval APL neuron (e.g. smaller size) may allow passive signal propagation throughout the mushroom body (for discussion, see Amin et al 2020). Alternatively, the larval APL undergoing extensive rearrangement during development (**Figure 18**) may have different electrical properties than its adult counterpart, which is presumably non-spiking (Papadopoulou et al 2011; Mayseless et al 2018; Amin et al 2020).

Does APL release co-transmitter(s)? Here I confirm that APL is GABAergic in larvae (**Figure 15A-B''**; Masuda-Nakagawa et al 2014). It is tempting to speculate that in larvae APL conveys a reward signal via co-transmitter release, as suggested by the presence of dense-core vesicles (A.S. Thum, personal communication). In adults APL has been suggested to co-express octopamine and glutamate in addition to GABA, and to modulate associative learning via octopamine release (Wu et al 2013; Sabandal et al 2020). However, a recent transcriptome analysis suggests that in adults APL is only GABAergic, matching the situation for the larval APL in which no octopamine, acetylcholine or glutamate has been detected (Masuda-Nakagawa et al 2014; Eichler et al 2017; adult flies: Aso et al 2019). These discrepancies, likely due to different sensitivity levels of the used methods, make of the existence of a potential co-transmission in APL rather unclear and raise the question of how APL activation affects the activity of its post-synaptic partners – including the KCs.

Does driving APL have a direct physiological effect on KCs? In adults APL gets activated by the KCs, and in turn inhibits them (Lin et al 2014). If the same is expected to occur in larvae, it is still unknown whether the larval APL indeed *inhibits* the KCs (Masuda-Nakagawa et al 2014). If so, and as argued in Saumweber et al (2018), a strong APL activation would silence almost all KCs, but would hardly explain how an associative memory could be formed under these conditions. Arguably, the clear APL synaptic polarization in larvae (i.e. the fact that APL pre-synapses are almost absent in the lobes) may imply an overall reduced inhibition towards the KCs, such that the "sparsening" effect of APL could be relatively weaker in larvae as

compared to adults; at the same time, the rewarding effect potentially brought about by connections to downstream synaptic partners (see section below) could become "unmasked" in the larva. Alternatively, optogenetic APL activation could lead to a depolarization of the KCs through, for example, post-inhibitory rebound activation as reported by Apostolopoulou & Lin (2020) in adult flies; in this study, however, APL was continuously activated during four days using dTrpA1 as the effector. In contrast, the relatively short time windows of APL activation used here (**Figure 22**), together with the long-lasting opening state of ChR2-XXL, makes it challenging to directly relate the present findings to the ones in adults (Dawydow et al 2014; Apostolopoulou & Lin 2020). Finally, it could be that instead of being inhibitory, GABA is excitatory in third-instar larvae and further becomes inhibitory in adults. This developmental switch from excitatory-to-inhibitory effect of GABA has been reported in mammals, as well as in *Drosophila* motoneurons during pupal life (Ben-Ari 2002; Ryglewski et al 2017); whether it also occurs in mushroom body neurons remains unknown. In any event, how KC activity is affected by APL activation in larvae should be addressed through functional imaging approaches now available in this model (Lyutova et al 2019; Escbach et al 2020a,b). As suggested by the present results, this may involve downstream synaptic partners, such as DANs.

Does driving APL have an indirect physiological effect on DANs? As mentioned above, Lyutova et al (2019) have shown that driving the KCs establishes an appetitive olfactory memory. Specifically, this manipulation leads to an increased activity in DANs from the pPAM cluster, allowing the transmission of a reward signal during odour stimulation (Lyutova et al 2019). Similarly, driving APL optogenetically could increase the activity of the pPAM neurons indirectly (via post-inhibitory rebound activation of the KCs: Apostolopoulou & Lin 2020), and/or supress spontaneous activity of punishing DANs.

Final remarks and conclusion

Overall, it seems likely that manipulating APL activity broadly affects the whole mushroom body, such that more than one pathway could be involved in odour-APL memory formation. In other words, all faint trends or partial effects observed in some cases (**Figures 26, 27**) might "sum up" to trigger the observed APL rewarding effect. The present findings thus provide a study case of unexpected, complex circuit features within the larval mushroom body, and pave the way for future functional and physiological studies.

General discussion

How do brains organize behaviour? Clearly, a comprehensive picture is still far from being achieved. Nonetheless, considerable progress has been made using organisms that are simple enough to be experimentally tractable but complex enough to be worth studied. The larva of *Drosophila* provides this fortunate balance. In this thesis, I have probed cognitive flexibility in larvae by developing a reversal learning paradigm both in absolute and differential conditioning, and in both valence domains. Further, I have discovered that the larval APL neuron displays unexpected rewarding properties upon optogenetic activation, and investigated this neuron and its rewarding properties in detail. These findings are discussed and put into perspective.

Larval *Drosophila* as a simple, tractable study case of cognitive flexibility

The present work reveals that larvae can reverse previously learned odour-taste contingencies. To the best of my knowledge, these results constitute the first demonstration of non-elemental learning abilities in larval *Drosophila*. Reversal learning is a non-elementary form of learning in the sense that the switch of contingencies from the first to the second training phase induces a transient ambiguity that needs to be overcome by the animals (Hadar & Menzel 2010). This differs from elementary learning tasks, in which cues are unambiguously associated with each other (Giurfa 2003). The availability of a reversal learning paradigm in larvae does not only enrich the behavioural toolbox for this model but will hopefully also prompt investigation of the underlying mechanisms and potentially conserved principles. Analogous to the mammalian orbitofrontal cortex, the mushroom bodies are necessary for reversal learning in insects (adult flies: Ren et al 2012; honey bees: Devaud et al 2007; Boitard et al 2015; vertebrates: Iversen & Mishkin 1970; Fellows & Farah 2003; Schoenbaum et al 2003; Delamater 2007; Stalnaker et al 2009; Ghahremani et al 2010). In addition, the involvement of GABAergic signalling pathways in solving ambiguous tasks such as reversal learning has been characterized across species (flies: Wu et al 2012; Ren et al 2012; honey bees: Boitard et al 2015; vertebrates: Bissonette et al 2010; Morellini et al 2010). In flies, downregulating ionotropic GABA receptors in the mushroom body or reducing GABA levels in APL impairs reversal performance (Wu et al 2012; Ren et al 2012). Similarly in honey bees a cluster of feedback GABAergic neurons from the protocerebro-calycal tract, reminiscent of APL, is likely required for achieving reversal through ionotropic, but not metabotropic, GABA receptors in the mushroom

body calyces (Rybak & Menzel 1993; Boitard et al 2015; Cabirol et al 2018). Thus, in the light of the abovementioned findings it is tempting to speculate that in larvae, too, achieving reversal learning requires the mushroom body-APL network. However, this clearly remains speculative not least because of the different connectivity of the larval APL as compared to adults and its surprising rewarding effect upon optogenetic activation (see also Chapter II).

The availability of fully annotated connectomes in *Drosophila* inspires the analysis of the functionality of neural circuits and, in the present case, to understand how reversal learning takes place. A recent study in adult flies has revealed that after initial odour-shock training, omitting the unconditioned punishment during the subsequent reversal phase leads to increased activity in rewarding DANs, thus allowing for proper odour-shock contingency reversal (McCurdy et al 2021). Critically, these parallel opposing mnemonic processes as well as those uncovered for memory extinction involve recurrent MBON-to-DAN connections, which also exist in larvae (Eichler et al 2017; Felsenberg et al 2018; Eschbach et al 2020a; Otto et al 2020; McCurdy et al 2021). Thus, the conservation of circuit motifs hints at shared processes across life stages in *Drosophila*, and possibly other insect species, and may give food for thought for exploring potential mechanistic principles in mammals (Schiller et al 2010; Zhang et al 2015; Luo et al 2018). Of note is that in their study, McCurdy et al (2021) used a differential, two-odour paradigm and mainly focused on the reversal of paired odour-shock memories. Whether the reversal of unpaired memories requires the same or distinct neural pathways could be addressed in larvae by using the one-odour, absolute version of the present paradigm, in either of the valence domains (**Figures 9-13**).

From a biomedical perspective, given that neuropsychiatric disorders such as obsessive-compulsive disorder and addiction are characterized by deficits in cognitive flexibility, the present reversal paradigm may provide a simple and genetically tractable study case of biomedical relevance (Izquierdo & Jentsch 2012; Gruner & Pittenger 2017).

Towards an extension of the behavioural toolbox?

As discussed in the previous section, the present reversal learning paradigm enriches the behavioural toolbox for the larva. This is important because all the newly discovered connections in the larval mushroom bodies suggest much richer functionality in these structures than previously thought (Thum & Gerber 2019). Therefore, the development of paradigms to probe the system beyond the standard tasks is now required. Recent modelling approaches suggest that the newly discovered circuit motifs support memory extinction or even more complex learning tasks, such as sensory pre-conditioning, second-order conditioning, or

context-dependent conditioning (Wessnitzer et al 2012; Eschbach et al 2020a; Springer & Nawrot 2021; for behavioural data in other insects: Tabone & de Belle 2011; Müller et al 2000; Matsumoto & Mizunami 2004; Menzel 2012; mammals: Brogden 1939). Although these types of learning have been observed across many species, empirical evidence is lacking in *Drosophila* larvae, calling for the development of corresponding paradigms. Given that the process of extinction arguably is part of reversal learning – yet both learning processes likely require distinct neural pathways in adult flies – the present reversal paradigm may suggest extinction in larvae (Felsenberg et al 2018; Otto et al 2020; McCurdy et al 2021). Nonetheless, a proper extinction paradigm is not yet available for the larva (for a recent modelling study, see Springer & Nawrot 2021). More recently, and after earlier attempts, larval operant conditioning has been demonstrated and is shown to involve serotonergic processes (Eschbach 2011; preprint by Klein et al 2021; for investigations in *Aplysia*: Brembs et al 2002; adult flies: Brembs & Plendl 2008; Brembs 2011; Mendoza et al 2014; Wiggin et al 2021; for a modelling study, see Wei & Webb 2018).

Newly established paradigms therefore open perspectives for teasing apart the neural coding of more advanced learning tasks. However, the abilities to perform complex learning tasks might not always be conserved across species. Although the larval brain is similarly organized to adult flies and other insects (see General introduction), its massively reduced number of neurons may suggest a poorer signal-to-noise ratio, making the larva potentially less efficient in performing complex learning tasks. This may also reflect different cognitive strategies in the larva, which is mnemonically less performing and arguably more prone to extinguish previously established memories (Weiglein et al 2019; Mancini et al 2019/Chapter I). Finally, the selection of optimal experimental parameters is crucial to demonstrate that animals can achieve – or not – a given behavioural task. In any case, probing the system beyond its capacities can be considered informative.

Bearing this in mind, the development of new protocols through careful parametric examination will be highly valuable in relating performance in complex learning tasks (or indeed the inability to do so) to identified circuitry, with a view to probing the function of these circuit motifs and implementing them in computational algorithms.

The APL neuron as a study case of unexpected complexity

Surprisingly, activating APL optogenetically together with odour presentation induces an olfactory appetitive memory in third-instar larvae. This associative memory can be odour-specific and relies on dopamine-synthesis, suggesting the involvement of downstream

dopaminergic processes. These findings are unexpected given that APL (i) is GABAergic and thus presumably inhibitory, (ii) broadly innervates the mushroom body, and (iii) is considered key in preserving the sparse coding of olfactory cues within the mushroom body. Critically, while the available evidence in larvae – including from the present thesis – is consistent with such a role in sparsening (Masuda-Nakagawa et al 2014; Eichler et al 2017; Saumweber et al 2018), the actual evidence stems from work in adult insects (adult flies: Liu et al 2007; Liu & Davis 2009; Lei et al 2013; Lin et al 2014; Inada et al 2017; Zhou et al 2019; Amin et al 2020; Apostolopoulou & Lin 2020; locust: Papadopoulou et al 2011; Ray et al 2020) (modelling studies: Ardin et al 2016; Dasgupta et al 2017; Assisi et al 2020; Rapp & Nawrot 2020; Ray et al 2020; Abdelrahman et al 2021; preprints by Jürgensen et al 2021; Zavitz et al 2021). Even so, the different connectivity of the larval APL neuron, together with its unexpected rewarding effect reported in this thesis, may suggest other or possibly additional functions than those related to sparse odour coding.

The rewarding properties of APL activation raise a central question: is this effect functional or purely "artificial"? Keeping in mind that optogenetic manipulations may not completely reflect "the real world" neuronal activity, they contribute for probing the functionality of brain circuits at single neuron level (Ehmann & Pauls 2020). For instance, activation of DANs can induce appetitive or aversive memories (Saumweber et al 2018; Eschbach et al 2020a; Schleyer et al 2020); accordingly, these modulatory neurons respond to appetitive and aversive stimuli and presumably receive inputs from gustatory centres in the SEZ to convey reinforcing signals (Riemensperger et al 2005; Claridge-Chang et al 2009; Mao & Davis 2009; Burke et al 2012; Liu et al 2012; Waddell 2013; Kirkhart & Scott 2015; Kim et al 2017; Siju et al 2020; Siju et al 2021; mammals: Matsumoto & Hikosaka 2009; Schultz 2010). In addition, presenting a real sugar reward during the recall test to larvae previously trained with odour presentation and optogenetic activation of DAN-i1 as a "virtual" reward can prevent the expression of the appetitive "odour-DAN-i1" memory (Schleyer et al 2020). In larvae appetitive memory expression is prevented only in the presence of matching rewards (i.e. between training and test), suggesting that optogenetically-induced DAN-1 activity indeed resembles a real sugar reward (Schleyer et al 2015a; Schleyer et al 2020). Whereas activating the larval APL neuron confers similar rewarding properties to those reported for real tastant rewards and DANs (**Figures 22-24**), it does not receive direct inputs from gustatory afferents or the suboesophageal zone (Eichler et al 2017). Moreover, APL receives relatively few direct projections from DANs (it does only from the left- but not the right-hemisphere DAN-i1 involved in appetitive learning, DAN-f1 involved in aversive learning, and DAN-k1 involved in odour and sugar preference),

but rather indirectly via successive synaptic steps – mainly via the KCs (**Figure 17**, **Figure S4**; Eichler et al 2017; Saumweber et al 2018). In adults APL responds to electric shocks, expresses dopamine receptors (DD2R and DopEcR), and receives inputs from DANs that suppress its activity to ensure proper learning and decision-making (Liu & Davis 2009; Aso et al 2019; Zhao et al 2019; Zhou et al 2019; Amin et al 2020). Whether the activation of APL in adults is also rewarding and whether its activity is modulated by appetitive tastants (e.g. sugars) is unknown. Of note, and despite the conservation of the neuronal circuit architecture across development, the potential "trans-fating" of neurons across metamorphosis should prompt us not to systematically assume conserved roles from one developmental stage to another (Gerhard et al 2017; J. Truman, unpublished).

Thus, given the lack of direct input from rewarding DANs, they may not be the preferred candidates to convey a reward signal to APL. What about OANs? The first-instar larval connectome revealed relatively few OAN-to-APL connections (Eichler et al 2017; **Figure 17**, **Figure S4**). Yet, third-instar larvae some OANs (OANa-1 and OANa-2; also called sVUMmd1 and sVUMmx1, respectively) may establish functional synaptic connections with APL in the calyx. Optogenetic activation of OANs throughout odour-fructose training prevents differential conditioning of similar but not dissimilar odours (Wong et al 2021). As argued by the authors, these learning impairments could be due to APL's inhibitory action through OAN activation, in accordance with previous work (Stopfer et al 1997; Lin et al 2014; for a modelling study, see Linster & Cleland 2001). This would suggest an implication of OAN-to-APL connections in regulating sparse odour coding rather than relaying a reward signal in the calyx. In honey bees, activation of the single OAN VUMmx1 innervating the calyx is rewarding (Hammer 1993). In contrast, none of the OANs identified in *Drosophila* has been shown to confer a reinforcing signal in adults or larvae (Busch et al 2009; Burke et al 2012; Saumweber et al 2018; see also General introduction). From this lack of evidence, and given that the calyx is not innervated by DANs, it is tempting to speculate that in addition to sparsening odour representation through GABA release, APL conveys a reinforcing signal via co-release of octopamine or other messengers (Wu et al 2013; Yang et al 2016; Eichler et al 2017; Eschbach et al 2020a,b; Sabandal et al 2020). In adults, but not in larvae, some reports indeed suggest octopamine as a co-transmitter of APL (Masuda Nakagawa et al 2014; Wu et al 2013). Also, some DANs (but not APL) release nitric oxide in addition to dopamine to induce oppositely valenced memories (Aso et al 2019; reviewed in Green & Lin 2020). As regards APL, however, the question of potential co-messenger(s) remains unclear and calls for future transcriptome analysis in the larva (Masuda Nakagawa et al 2014; Aso et al 2019; see also Discussion from Chapter II).

The rewarding effect of APL is dependent on dopamine synthesis. Given that both in larvae and adults APL is not shown to be dopaminergic itself, this suggests the implication of downstream dopaminergic pathways (Eichler et al 2017; Aso et al 2019). In adults, APL projects directly onto rewarding PAM-DANs to regulate reward signalling via metabotropic GABA receptors (Yamagata et al 2021). As mentioned in the preceding paragraph, the larval APL neuron rather receives few inputs only from selected DANs (**Figure 17**, **Figure S4**; Eichler et al 2017; Saumweber et al 2018). This may thus rather hint at indirect modulation of DAN activity via, for example, KC-to-DAN connections as recently observed for a broad KC activation also leading to a rewarding effect (Lyutova et al 2019; see also Discussion in Chapter II). Considering APL as inhibitory, however, its activation might be expected to induce opposite effects as compared to driving the KCs. As discussed in Chapter II, driving APL might lead to a post-inhibitory rebound KC activation, potentially eliciting increased activity in rewarding DANs. Whether the odour-APL memory relies on these circuit motifs needs to be elucidated. However, it remains puzzling that broad manipulation of the mushroom body activity (i.e. via activating KCs or by activating APL) is positively valenced; given that most DANs establish reciprocal connections with KCs, both rewarding and punishing DANs might be affected (Eichler et al 2017). Activating all KCs leads to increased activity in rewarding pPAM-DANs, but whether aversive DANs are also modulated is still unknown (Lyutova et al 2019). Speculatively, the mushroom body might be "tuned" such that, instead of being neutral, overall manipulation of its circuitry (i.e. by driving KCs directly or through rebound-activation) results in a net rewarding effect. This is potentially compatible with larval behaviour, which mainly consists in tracking down almost all kinds of resources in the environment.

Clearly, the rewarding effect of APL raises more questions than it answers. Conceivably, these reinforcing properties may result from an artefact rather than being functionally relevant: activating APL may indeed "crack the system", affecting several to many neural pathways throughout the mushroom body. Whatever the case, the present results illustrate the complexity of neural circuits, here highlighted in the mushroom body. A clear understanding of this puzzling effect will require a careful examination of APL and the physiological consequences of activating it through several approaches, such as transcriptomics, electrophysiology, or functional imaging (Avalos et al 2019; Lyutova et al 2019; Eschbach et al 2020a,b). In particular, the morphology of this neuron and the accessibility allowed by its highly specific and reliable driver appear well-suited for this endeavour, presenting exciting opportunities to gain a deeper understanding of how circuits are wired to orchestrate learned behaviour.

Towards memory-efferent processes

Chemical-synapse connectomes for *Drosophila* have revealed that more than half the synaptic classes have been overlooked in the mushroom body, suggesting a much richer and complex circuitry than expected (Eichler et al 2017; Takemura et al 2017; Li et al 2020; **Figure 4C**). A major step is now to map these circuit motifs onto memory-efferent pathways. Recent reconstructions of circuits downstream of the mushroom body suggest extensive interactions with others brain structures, including the lateral horn (larvae: Eschbach et al 2020b; adult flies: Li et al 2020; Scaplen et al 2021; Schlegel et al 2021). Lateral horn neurons (LHNs) receive direct projections from MBONs (Eschbach et al 2020b; Li et al 2020; Schlegel et al 2021). These connections have been shown to be functional in adults; in addition to mediating innate information, LHNs PD2a1/b1 are modulated by MBONs-α2sc (also called MB-V2α) to allow memory retrieval (Dolan et al 2018). Together with the requirement of the lateral horn, but probably not the mushroom body, for context-dependent long-term memory, this suggests that innate and learnt centres are functionally interacting with each other along efferent pathways (at the very latest at the level of motorneurons or muscles) rather than working as parallel, strictly separated pathways (Dolan et al 2019; Sayin et al 2019; reviewed in Chakraborty & Sachse 2021). Of note is that many manipulations of mushroom body function in its entirety – including by activating APL (**Figure 24C-E**) – have surprisingly little effect on innate behaviours (Heimbeck et al 2001; Parnas et al 2013). In any event, it seems more prudent to regard the mushroom body and the lateral horn as *mainly* involved in learnt and innate behaviour, respectively, and to keep in mind that manipulating selected output channels from the mushroom body can indeed alter innate behaviour (Aso et al 2014a; Owald et al 2015a; Chia & Scott 2020).

In larvae, recent mapping of all MBON downstream synaptic partners has revealed at least two additional features. First, some LHNs project directly onto MBONs, such as the GABAergic MBON-m1 that receives excitatory inputs from LHNs to trigger odour attraction in experimentally naïve larvae (Eschbach et al 2020b). Second, MBONs and LHNs converge onto common downstream synaptic partners (hence called "convergence neurons") which might then integrate learnt and innate valuation signals to elicit appropriate behavioural responses – as it is the case for MBON-m1 which also receives inputs from other MBONs (Eschbach et al 2020b). These convergent neurons project back onto modulatory DANs, potentially providing a circuit mechanism for prediction error, a fundamental process for learning often modelled yet until now not directly observed in *Drosophila* (Horiuchi 2019; Eschbach et al 2020a,b; Bennett et al 2021; Jiang & Litwin-Kumar 2021; Springer & Nawrot

2021; Yamagata et al 2021). Prediction error, i.e. the difference between expectation and reality, is encoded at the level of DANs across many species (vertebrates: Ljungberg et al 1992; Schultz 1997; Schultz 1998; Waelti et al 2001; Tobler et al 2003; Bayer et al 2005; Lak et al 2014; reviewed in Schultz 2016; in honey bees, at the level of the OAN VUMmx1: Hammer 1993; reviewed in Menzel 2012). The accessibility of single DANs conveying reinforcement signals (e.g. DAN-i1, DAN-f1), together with cutting-edge methods now established for the larva (e.g. functional imaging, electrophysiology) provide opportunities to look for experimental evidences in *Drosophila* (Marescotti et al 2018; Lyutova et al 2019; Schleyer et al 2020; Eschbach et al 2020a,b; Eschbach & Zlatic 2020).

In adults, and presumably in larvae, homo- and hetero-compartmental interactions between MBONs and DANs via direct or indirect synaptic steps are functionally relevant for different learning processes (Ichinose et al 2015; Felsenberg et al 2017; Felsenberg et al 2018; Pavlowsky et al 2018; König et al 2019; Eschbach et al 2020a,b; Jacob & Waddell 2020; Modi et al 2020; Ichinose et al 2021; Jacob et al 2021; McCurdy et al 2021). For instance, reduced odour-evoked activity in the glutamatergic avoidance-promoting MBON-01 (also called MBON-γ5β′2a) underlies extinction and reversal learning in adult flies (Felsenberg et al 2018; Otto et al 2020; McCurdy et al 2021). Extinction requires local feedback loops from MBON-01 to rewarding PAM-γ5 DANs, whereas reversal learning involves both local and indirect hetero-compartmental connections, suggesting distinct underlying pathways between these two learning processes (Felsenberg et al 2018; Otto et al 2020; McCurdy et al 2021). Moreover, MBON-01 receives inhibitory inputs from the GABAergic approach-promoting MBON-11 (also called MBON-γ1pedc), potentially enhancing the signal-to-noise ratio through lateral inhibition across different mushroom body compartments (larvae: Eichler et al 2017; Eschbach et al 2020b; adult flies: Owald et al 2015a; Li et al 2020). In addition, MBON-11 may suppress activity in the punishing DAN PPL1-01 (also called PPL01-γ1pedc) to prevent unrelevant olfactory cues to enter into association with a punitive electric shock (Ueoka et al 2017). Accordingly, activating or silencing MBON-11 along with odour presentations triggers appetitive or aversive olfactory memories, respectively (König et al 2019). Although some larval MBONs can induce approach or avoidance, as far as has been tested there is no evidence yet for their reinforcing effects (Eschbach et al 2020b; **Figure 26**). Notably, the calyx and the upper vertical lobe are the only mushroom body compartments featuring two MBONs with opposite valences (Eschbach et al 2020b); the significance of this observation remains to be elucidated.

The availability of large electron and light-microscopy databases, together with the cutting-edge approaches available in *Drosophila* provide great opportunities for understanding the function and physiology of neural circuits (Eichler et al 2017; Zheng et al 2018; Bates 2020a,b; Cachero et al 2020; Eckstein et al 2020; Eschbach et al 2020a,b; Huoviala et al 2020; Li et al 2020; Meissner et al 2020; Scheffer et al 2020; Zheng et al 2020; Buhmann et al 2021; for reviews, see Eschbach & Zlatic 2020; Jovanic 2020; Vogt 2020). The complete reconstruction of nerve cord in larvae and more recently in adults now makes it possible to map memory-efferent circuitry onto descending pathways, and to understand how memories are translated into specific motor programs (larvae: Lemon et al 2015; Ohyama, et al 2015; Carreira-Rosario et al 2018; Lee & Doe 2021; adult flies: Bidaye et al 2014; Feng et al 2020; Phelps et al 2021). This might provide considerable inspiration for the development of computational algorithms and intelligent robots (Webb 2002; Rapp & Nawrot 2020; Spaeth et al 2020).

Final remarks and conclusion

The past few years have revealed new features of the *Drosophila* brain circuitry, leading us to "expect the unexpected" (Thum & Gerber 2019). By contributing to the collective effort to characterize the function of neural circuits and relate them to complex behavioural tasks, the present study illustrates how expectations are subject to change. The availability of fully annotated, functional connectomes is now within reach, bringing together many forms of expertise ranging from behavioural analysis to physiological and molecular studies, and computational modelling. Such interdisciplinary consortia, together with translational studies, will hopefully contribute to a clearer picture of how behaviour is organized in a miniature brain and potentially also in more complex ones.

List of abbreviations

3IY	3-Iodo-L-tyrosine
AD	activation domain
AL	antennal lobe
AM	amyl-acetate
AN	antennal nerve
APL	anterior paired lateral neuron
BA	benzaldehyde
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
ChR2	channelrhodopsin 2
CS	conditioned stimulus
CX	calyx
DANs	dopaminergic neurons
DBD	DNA binding domain
DD2R	dopamine D2-like receptor
DO	olfactory dome
DOG	dorsal organ ganglion
DopEcR	dopamine/ecdysteroid receptor
DPM	dorsal paired median neuron
DPO	dorsal pharyngeal organ
DPS	dorsal pharyngeal sensillum
EM	electron-microscopy
GABA	gamma-aminobutyric acid
GFP	green fluorescent protein
GPCRs	G-protein-coupled receptors
GRN	gustatory receptor neuron
iACT	inner antennocerebral tract
IP	intermediate peduncle
IT	intermediate toe
KCs	Kenyon cells
KW	Kruskal-Wallis test
LA	lateral appendix
LBN	labial nerve
L-DOPA	l-3,4-dihydroxyphenylalanine
LH	lateral horn
LHN	lateral horn neuron
LN	lateral neuron
LP	lower peduncle
LT	lower toe
LVL	lateral vertical lobe
MBIN	mushroom body input neuron
MBON	mushroom body output neuron
ML	medial lobe
MN	maxillary nerve
MWU	Mann-Whitney U-test
NGS	normal goat serum

OAN	octopaminergic neuron
OCT	octanol
ORN	olfactory receptor neuron
OSS	one-sample sign test
PAM	protocerebral anterior medial
PBS	phosphate buffered saline
PD	peduncle
PN	projection neuron
pPAM	primary protocerebral anterior medial
PPL	protocerebral posterior lateral
PPS	posterior pharyngeal sensillum
SEZ	suboesophageal zone
SHA	shaft
TO	terminal organ
TOG	terminal organ ganglion
TβH	tyramine β-hydroxylase
UAS	upstream activation sequence
US	unconditioned stimulus
UT	upper toe
UVL	upper vertical lobe
VL	vertical lobe
VO	ventral organ
VOG	ventral organ ganglion
VPS	ventral pharyngeal sensillum
VUMmx1	ventral unpaired median neuron of the maxillary neuromere 1

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Supplementary material

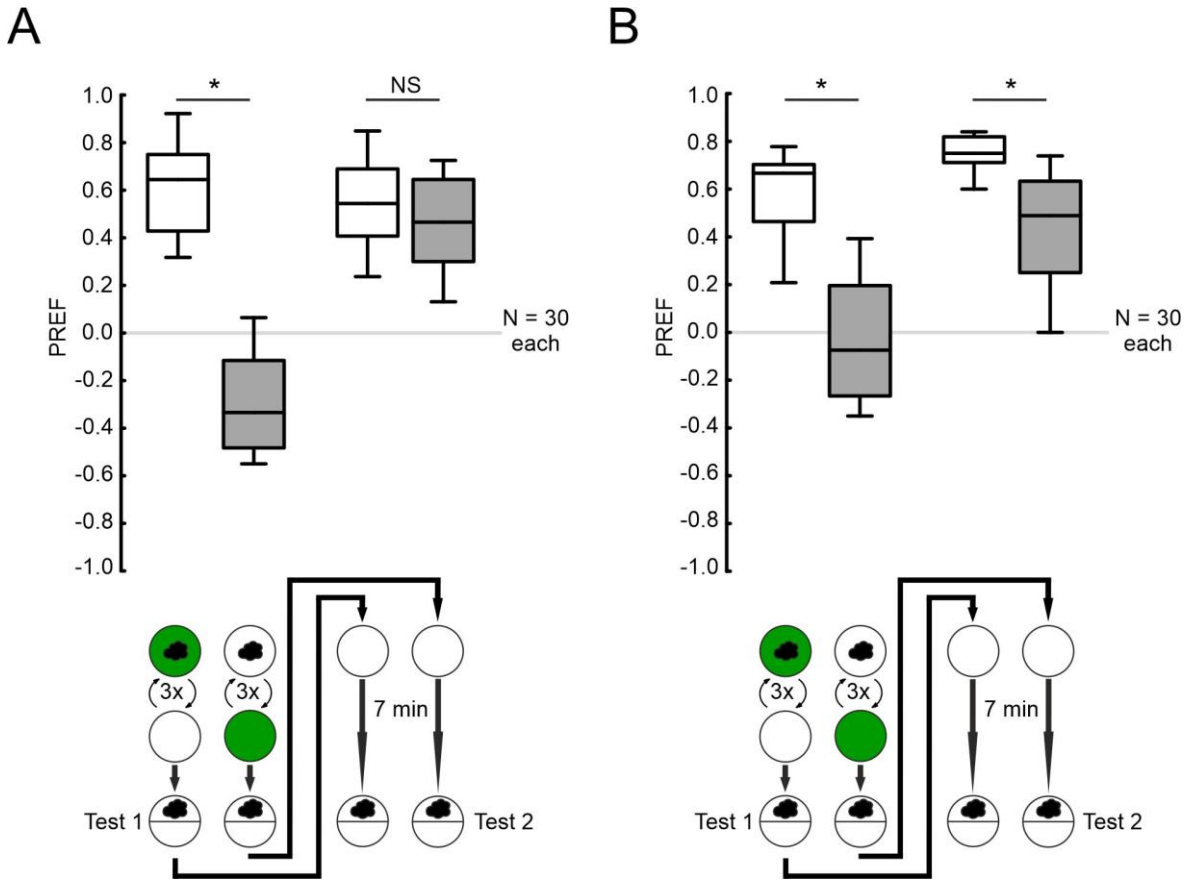

Figure S1. Temporal stability of memory, revisited. Following up on the experiment displayed in Figure 6C, this experiment sought to ascertain whether associative memory remains detectable after an initial test and a 7 min waiting period. **(A)** In a repetition of the experiment displayed in Figure 6C, the larvae were trained by either paired or unpaired presentations of odour (dark cloud) and reward (green fill of circle indicating a Petri dish with fructose and, as substrate, agarose) and tested for their odour preference (Test 1); then, the larvae were transferred to a Petri dish with only the agarose substrate (white fill of circle) for a 7 min waiting period and tested again for their odour preference (Test 2). In the first test, the larvae behaved according to the preceding training phase. In this dataset, this effect had vanished by the time of the second test. **(B)** Repetition of the experiment in (A). In this case, too, the larvae behaved according to the preceding training in the first test. In this dataset, this memory was retained until the second test 7 min later. Data are displayed as box plots, the middle line showing the median, the box boundaries the 25 and 75 % quantiles, and the whiskers the 10 and 90 % quantiles. Sample sizes are given within the figure. * and NS refer to MWU comparisons between groups (* $p < 0.05$ corrected according to Bonferroni-Holm and NS $p > 0.05$).

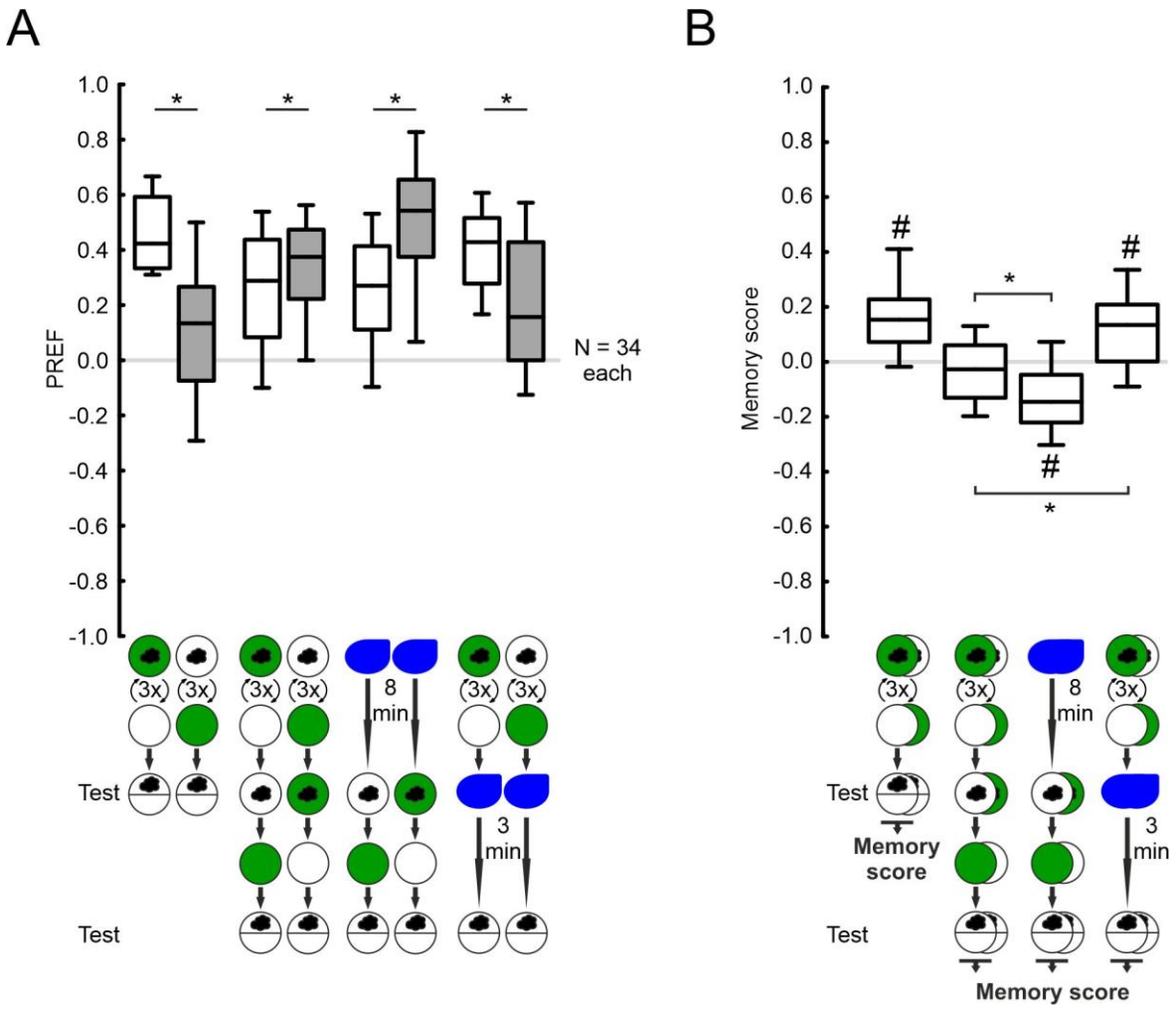

Figure S2. Shortened version of the absolute appetitive reversal learning paradigm. The larvae were trained and tested as in Figure 9, except that the duration of individual training trials was 1 min each. **(A)** The larvae were tested for their odour preference either (i) immediately after a one-phase training, (ii) after training with reversed contingencies in the first and the second training phase, (iii) after omitting the first training phase, or (iv) after omitting the second training phase. **(B)** Performance indices calculated from the preference scores in (A). Positive and negative memory scores indicate appetitive memory related to the first and the second training phase, respectively. The scores after reversed contingency training were less negative than when the first training phase was omitted, suggesting a persisting impact from the first training phase. In turn, after reversed-contingency training the scores were more negative than when the second training phase was omitted, suggesting an impact from the second training phase. Sample sizes are given within the figure. * and NS refer to MWU comparisons between groups, # refers to OSS comparisons to chance levels i.e. to zero (*, # $p < 0.05$ corrected according to Bonferroni-Holm and NS $p > 0.05$). Other details as in Figure S1.

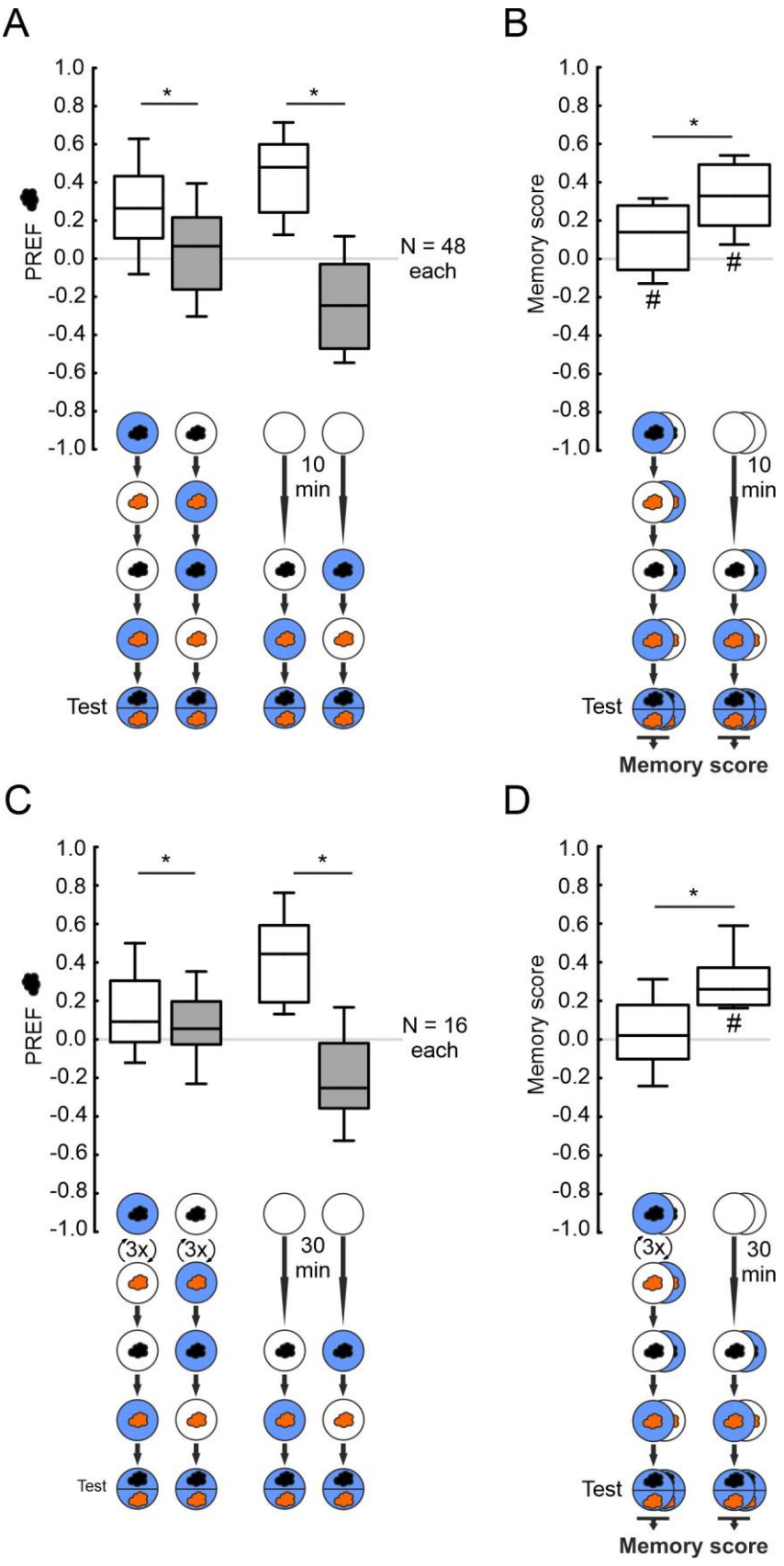

Figure S3. Increasing the amount of cycles in the first training phase makes differential aversive reversal learning more difficult. (A) Repetition of two experimental conditions from Figures 12, 13. The larvae were tested after training with reversed contingencies in the first and the second training phase, or after omitting the first training phase. Preference scores (PREF) reflect preference for n-amyl acetate (dark cloud). **(B)** Performance indices calculated from the preference scores in (A). Positive memory scores indicate aversive

memory related to the second training phase. The scores after reversed-contingency training were less positive than when the first training phase was omitted, suggesting a persisting impact from the first training phase. In addition, the scores after reversed-contingency training were significantly positive, suggesting behaviour in accordance with the second training phase. **(C)** As in (A), except that three cycles were given in the first training phase instead of one cycle. **(D)** Memory scores calculated from the preference scores in (C). As in (B), a persisting impact from the first training phase was detectable, since the memory scores after reversed-contingency training were less positive than when the first training phase was omitted. As expected, having more training cycles in the first training phase makes reversal learning more difficult. Indeed, with three training cycles in the first training phase, the impact of the second training phase was undetectable. Sample sizes are given within the figure. * and NS refer to MWU comparisons between groups, # refers to OSS comparisons to chance levels i.e. to zero (* $p < 0.05$ corrected according to Bonferroni-Holm, # $p < 0.05$ and NS $p > 0.05$). Other details as in Figure S1-2

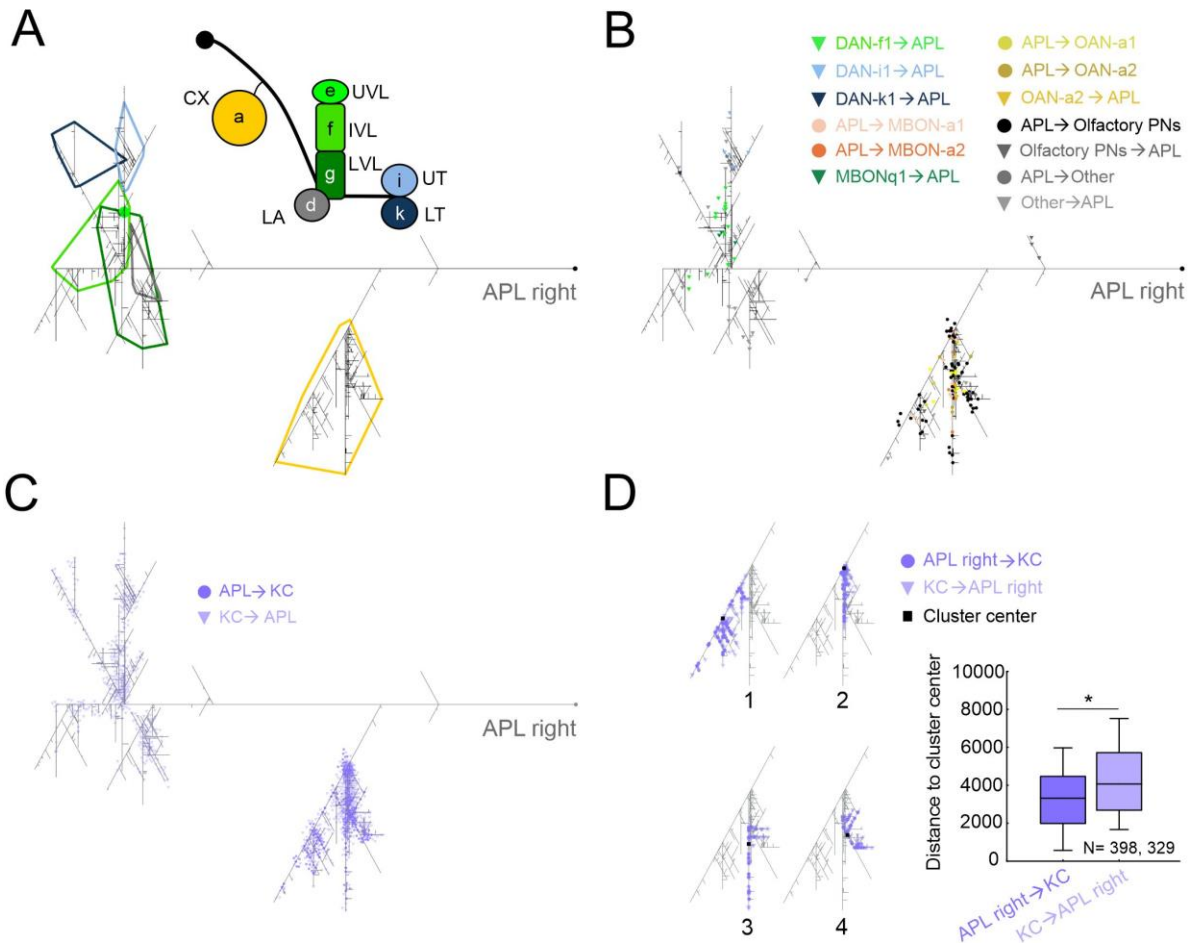

Figure S4. Dendrogram analysis of the right hemisphere larval APL neuron. As in Figure 17, but for the right hemisphere APL neuron. Despite the apparent different branching pattern of the right hemisphere APL relative to the mushroom body compartments (A) (compare with Figure 17A, but see Figure 14 for a lack of left-right asymmetry in third-instar larvae), the overall connectivity is comparable between both hemispheres (B, C). Again, cluster analysis of reciprocal APL-KC synapses within the calyx revealed four clusters (1-4), and most of the APL-to-KC synapses (dark purple dots) are found towards the centre of these clusters (dark square) whereas KC-to-APL synapses (bright purple triangles) are observed mainly at their surround. Other details as in Figure 17.

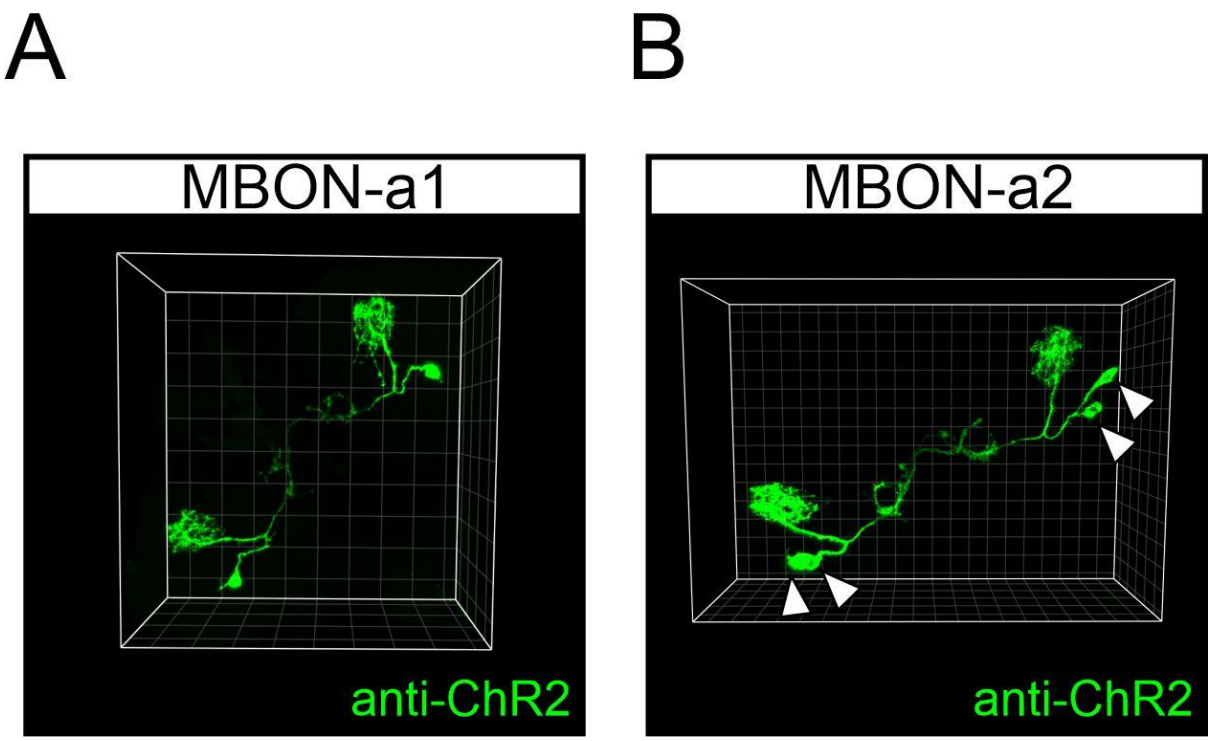

Figure S5. Expression patterns of the calyx MBON drivers used in Figure 26C-D. (A) Full projection of the expression pattern from the SS02006-GAL4 driver (MBON-a1) covering only one calyx MBON in each hemisphere (N = 7 brains tested). **(B)** As in (A), but for SS01417-GAL4 (MBON-a2) covering one, or in some cases both calyx MBONs (white arrowheads; left: cell bodies overlap one another) in each hemisphere observed in two out of N = 5 brains tested). Data were acquired with a 63x glycerol objective; grid edge lengths: 20 µm. Other details as in Figure 19C.

REAGENT or RESOURCE	SOURCE or REFERENCE	IDENTIFIERS	ADDITIONAL INFORMATION
Fly strains			
SS01671-GAL4 (split-GAL4 driver covering specifically APL in larvae) AD source strain: R21D02-GAL4 DBD source strain: R55D08-GAL4	Saumweber et al 2018	R21D02-GAL4: BDSC no. 48939 R55D08-GAL4: BDSC no. 39115	
APLi-GAL4 (intersectional driver covering specifically APL in larvae and adults)	Lin et al 2014; Mayseless et al 2018		
R36G04-GAL4 (GAL4 driver covering the calyx MBONs, plus additional neurons in the VNC in larvae)	Saumweber et al 2018	BDSC no. 49940	chrs III
SS02006-GAL4 (intersectional driver covering specifically one of the two calyx MBONs in larvae) AD source strain: R93G12-GAL4 DBD source strain: R71E06-GAL4	Eschbach et al 2020b; kindly provided by M. Zlatic, University of Cambridge		
SS01417-GAL4 (intersectional driver covering one, or in some cases both calyx MBONs in larvae) AD source strain: R52E12-GAL4 DBD source strain: R93G12-GAL4	Eschbach et al 2020b; kindly provided by M. Zlatic, University of Cambridge		
UAS-ChR2-XXL (optogenetic effector)	Dawydow et al 2014	BDSC no. 58374	chrs II
UAS-ChR2-XXL-td::tomato (reporter/optogenetic effector)	Saumweber et al 2018	FlyBase ID: FBtp0131815	chrs II
UAS-mCD8::GFP (reporter effector)	Lee and Luo 1999	BDSC no. 5137	chrs II
20xUAS-IVS-CsChrimson::mVenus (reporter/optogenetic effector)	Klapoetke et al 2014	BDSC no. 55135	chrs II
20xUAS-IVS-CsChrimson::mVenus (reporter/optogenetic effector)	Klapoetke et al 2014	BDSC no. 55136	chrs III
UAS-GtACR1::YFP (reporter/optogenetic effector)	Kindly provided by R. Kittel, Würzburg; König et al 2019	BDSC no. 9736	chrs II
UAS-Dsyd-1::GFP (pre-synaptic reporter)	Owald et al 2015a		chrs III
UAS-DenMark (post-synaptic reporter)	Nicolai et al 2010	BDSC no. 33062	chrs II
UAS-Syt1:SNAP (pre-synaptic TAG reporter)	Kohl et al 2014	BDSC no. 58379	Chrs III
UAS-TLN:CLIP (post-synaptic TAG reporter)	Kohl et al 2014	BDSC no. 58382	Chrs III
w1118		BDSC no. 3605, 5905, 6326	
attP40/attP2	Pfeiffer et al 2010		
Antibodies			
primary monoclonal mouse anti-FASII	DSHB	1D4 anti-Fasciclin II; AB_528235	1:50
primary monoclonal mouse anti-ChR2	ProGen Biotechnik	610180	1:100
primary polyclonal rabbit anti-GFP	Life Technologies	A6455	1:1000
primary polyclonal FITC-conjugated goat anti-GFP	Abcam	ab 6662	1:1000
primary monoclonal mouse 4F3 anti-DLG	Hybridoma	AB_528203	1:200
primary polyclonal rabbit anti-DsRed	Clontech	632496	1:200
primary monoclonal rat anti-N-Cadherin	Hybridoma	DN-Ex #8-s	1:50
primary polyclonal rabbit anti-GABA	Sigma Aldrich	A2052	1:500
primary polyclonal chicken anti-GFP	Aves Labs	AB_10000240	1:500
secondary polyclonal goat anti-rabbit Alexa Fluor 488	Life Technologies	A11008	1:500
secondary polyclonal goat anti-mouse Alexa Fluor 568	Life Technologies	A10037	1:500
secondary polyclonal goat anti-rat Alexa Fluor 647	Jackson IR	712-605-153	1:500
secondary polyclonal donkey anti-mouse Cy3	Jackson IR	715-165-150	1:300
secondary polyclonal goat anti-mouse Alexa Fluor 488	Invitrogen	A11001	1:200
secondary polyclonal goat anti-rabbit Cy5	Life Technologies	A10523	1:200
secondary polyclonal goat anti-rat Cy3	Life Technologies	A10522	1:200
secondary polyclonal FITC-conjugated goat anti-chicken	Invitrogen	A16055	1:300
Chemical TAG ligands (chemical substrates)			
SNAP-tag ligands (SNAP surface 549 - BG 549)	NEB	S9112S	
CLIP-tag ligands (CLIP surface 647 - BC 647)	NEB	S9234S	
Drugs			
3-Iodo-L-tyrosine (3IY)	Sigma–Aldrich	70-78-0	
3,4-dihydroxyphenylalanine (L-DOPA)	Sigma–Aldrich	59-92-7	

Softwares			
Fiji ImageJ	National Institutes of Health	SCR_002285	
Imaris 9.72	Oxford Instruments	SCR_007370	
R 3.3.2	Development Core Team 2016		
Statistika 13	StatSoft Inc	SCR_014213	
Corel Draw 2019	Corel Corporation	SCR_013674	

Supplementary Table 1

Declaration of Honour

“I hereby declare that I prepared this thesis without the impermissible help of third parties and that none other than the aids indicated have been used; all sources of information are clearly marked, including my own publications.

In particular I have not consciously:
• fabricated data or rejected undesirable results,
• misused statistical methods with the aim of drawing other conclusions than those warranted by the available data,
• plagiarized external data or publications,
• presented the results of other researchers in a distorted way.

I am aware that violations of copyright may lead to injunction and damage claims by the author and also to prosecution by the law enforcement authorities.
I hereby agree that the thesis may be electronically reviewed with the aim of identifying plagiarism.

This work has not yet been submitted as a doctoral thesis in the same or a similar form in Germany, nor in any other country. It has not yet been published as a whole.”