The Company of **Biologists**

RESEARCH ARTICLE

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

Juliane Thoener[‡], Christian König[‡,§], Aliće Weiglein[*], Naoko Toshima, Nino Mancini, Fatima Amin and Michael Schleyer[§]

ABSTRACT

Across the animal kingdom, dopamine plays a crucial role in conferring reinforcement signals that teach animals about the causal structure of the world. In the fruit fly *Drosophila melanogaster*, dopaminergic reinforcement has largely been studied using genetics, whereas pharmacological approaches have received less attention. Here, we apply the dopamine-synthesis inhibitor 3-Iodo-L-tyrosine (3IY), which causes acute systemic inhibition of dopamine signaling, and investigate its effects on Pavlovian conditioning. We find that 3IY feeding impairs sugar-reward learning in larvae while leaving task-relevant behavioral faculties intact, and that additional feeding of a precursor of dopamine (L-3,4-dihydroxyphenylalanine, L-DOPA), rescues this impairment. Concerning a different developmental stage and for the aversive valence domain. Moreover, we demonstrate that punishment learning by activating the dopaminergic neuron PPL1-γ1pedc in adult flies is also impaired by 3IY feeding, and can likewise be rescued by L-DOPA. Our findings exemplify the advantages of using a pharmacological approach in combination with the genetic techniques available in *D. melanogaster* to manipulate neuronal and behavioral function.

KEY WORDS: Pharmacology, Optogenetics, Reward, Punishment, PPL DAN, L-DOPA

INTRODUCTION

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

Leibniz Institute for Neurobiology, Department of Genetics, 39118 Magdeburg, Germany.
*Present address: Otto von Guericke University Magdeburg, Institute of Anatomy, 39118 Magdeburg, Germany.
[‡]These authors contributed equally to this work

[§]Authors for correspondence (mschleye@lin-magdeburg.de; ckoenig@lin-magdeburg.de)

J.T., 0000-0002-8597-751X; A.W., 0000-0003-4626-328X; M.S., 0000-0001-8020-1483

of the dopaminergic system. Here, we employ a complementary approach using pharmacological intervention.

Since the 1970s, both adult and larval *D. melanogaster* have been established as powerful model organisms to investigate Pavlovian conditioning, using odors as the conditioned stimulus (CS) and various types of rewarding and punishing unconditioned stimuli (US) (adults: Busto et al., 2010; McGuire et al., 2005; Perisse et al., 2013; Quinn et al., 1974; larvae: Diegelmann et al., 2013; Gerber and Stocker, 2007; Scherer et al., 2003; Thum and Gerber, 2019; Widmann et al., 2018). The genetic tools available for *D. melanogaster* have allowed the neurogenetic mechanisms of learning and memory to be investigated, and revealed many striking similarities between the dopaminergic systems of flies and mammals, including humans (reviewed in Yamamoto and Seto, 2014). To mention but a few, flies and mammals share the majority of genes involved in dopamine synthesis, secretion and signaling (Clark et al., 1978; Karam et al., 2020; Riemensperger et al., 2011; Yamamoto and Seto, 2014), as well as the crucial role of dopaminergic neurons in reinforcement signaling (Burke et al., 2012; Liu et al., 2012; Schroll et al., 2006; Schwaerzel et al., 2003; Selcho et al., 2009; reviewed in Scaplen and Kaun, 2016). Of note, in *D. melanogaster* different sets of dopaminergic neurons signal appetitive or aversive reinforcement, respectively, to distinct compartments of the insects' memory center, the mushroom body, which harbors a sparse and specific representation of the olfactory environment (Diegelmann et al., 2013; Guven-Ozkan and Davis, 2014; Heisenberg, 2003; Owald and Waddell, 2015; Thum and Gerber, 2019). A similar dichotomy of appetitive and aversive reinforcement signals carried by different sets of dopaminergic neurons may also be emerging in vertebrates (Groessl et al., 2018; Lammel et al., 2012; Menegas et al., 2018). Due to the seductive power, ease and elegance of the available genetic tools in *D. melanogaster*, however, other useful techniques are used less often in the field. For example, feeding or injecting drugs, although lacking the neuronal specificity of many transgenic tools, is a convenient way of exerting acute systemic effects. Furthermore, these approaches can be combined with genetic methods like cell-specific optogenetic manipulations, allowing greater flexibility in manipulating the animals' nervous system.

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

1

(L-DOPA), a precursor of dopamine. As a result, 3IY reduces dopamine levels (Bainton et al., 2000; Fernandez et al., 2017; Neckameyer, 1996) (Fig. S1A). Feeding 3IY to flies decreases activity/locomotion and increases sleep (Andretic et al., 2005; Cichewicz et al., 2017; Tomita et al., 2015; Ueno and Kume, 2014), increases ethanol preference (Ojelade et al., 2019 preprint), and alters courtship behavior (Monier et al., 2019; Neckameyer, 1998; Wicker-Thomas and Hamann, 2008). Regarding learning and memory, 3IY feeding impairs visual and olfactory learning, as well as long-term appetitive ethanol memory in adult flies (Kaun et al., 2011; Seugnet et al., 2008; Zhang et al., 2008). Importantly, these effects of 3IY-induced dopamine deficiency can be substantially rescued by additionally feeding L-DOPA to the flies (Cichewicz et al., 2017; Monier et al., 2019; Riemensperger et al., 2011; Zhang et al., 2008).

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

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

RESULTS
Feeding 3IY for 24 h induces broad behavioral impairments in larvae

We first investigated the effects of 3IY feeding on *D. melanogaster* larvae. In an approach modified from Neckameyer (1996), cohorts of 4-day-old larvae were placed on a PET mesh soaked with a yeast solution mixed with 3IY at the indicated concentrations, or without 3IY. After 24 h, the larvae underwent a single-trial Pavlovian training with an odor and a fructose reward, following established protocols (Michels et al., 2017; Saumweber et al., 2011; Scherer et al., 2003; Weiglein et al., 2019): one cohort of larvae was trained by a paired presentation of odor and reward, and a second cohort was trained reciprocally, i.e. with separated, unpaired presentations of odor and reward. In control larvae that were kept on a yeast solution without 3IY, an appetitive associative memory was revealed by higher odor preferences after paired than after unpaired training in a subsequent test (Fig. S1B), indicated by positive performance index (PI) scores (Fig. 1B, left-most box plot). When we performed the same learning experiment with larvae fed with various concentrations of 3IY, we observed decreased memory scores with increased 3IY concentrations. Significantly reduced scores were found for a concentration of 5 mg/ml (Fig. 1B; Fig. S1B), a result we replicated in an independent experiment (Fig. 1C; Fig. S1C). However, we noticed that many larvae had died due to the treatment, and the cuticle of many of the surviving animals was darkened (not shown). We therefore wondered whether the treatment may generally impair behavioral faculties. Indeed, innate odor preference was found to be impaired in 3IY-fed larvae (Fig. 1D). This prompted us to test their basic locomotion on an empty, tasteless Petri dish without odor or sugar, and to analyze their behavior using custom-made analysis software (Paisios et al.,

2017). Typically, larvae move by relatively straight runs, interrupted by turning maneuvers indicated by lateral head movements called head casts (HC) (Fig. S1D) (Gershow et al., 2012; Gomez-Marin and Louis, 2014; Gomez-Marin et al., 2011; Paisios et al., 2017; Thane et al., 2019). Analysis of these parameters of locomotion revealed that the animals' run speed was unchanged by 3IY feeding (Fig. 1E). However, the larvae fed with 3IY systematically performed fewer and larger HCs than control animals (Fig. 1F,G; Fig. S1E-H).

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

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

Given the reported role of dopamine and the TH enzyme in development and cuticle formation (Friggi-Grelin et al., 2003; Hsouna et al., 2007; Neckameyer, 1996; Neckameyer and White, 1993; reviewed in Verlinden, 2018), the timing of 3IY feeding is likely to have an impact. In order to minimize developmental effects, it seems desirable to apply 3IY as late as possible in the larval life cycle (and yet early enough to be able to finish the experiment before the larvae start to pupate). We therefore reduced the duration of 3IY feeding to 4 h, which allowed for the feeding of 3IY to 5-day-old animals. After this shortened feeding protocol too, memory scores were reduced compared to controls (Fig. 2A; Fig. S2A). Critically, the animals' basic behavioral faculties turned out to be intact: no impairment in innate odor preference (Fig. 2B) or sugar preference (Fig. 2C) was detectable. Thus, the shortened feeding of 3IY specifically impaired associative memory without impairing task-relevant behavioral faculties (nor did we observe any dead or darkened larvae; not shown). This conclusion was also supported by a more detailed analysis of locomotion that revealed only very mild differences to controls (Fig. 2D–F, for more details, see Fig. S2B–E). However, we cannot rule out the possibility of impairments in locomotion or other basic behavioral faculties after the animals underwent the training procedure, caused e.g. by fatigue or adaptation to the stimuli used. Given that we used a very short one-trial training paradigm (about 6 min in total), such effects seem not too likely. Notably, we detected a small increase in the HC rate after 4 h of 3IY feeding (Fig. 2E). This effect seems to be contradictory to the decrease in the HC rate after 24 h of 3IY feeding (Fig. 1F). A closer look revealed that after 4 h feeding the HC rate is increased only for large HC (Fig. S2B,C). After 24 h feeding, the same effect is observed, but additionally the rate of small HC is reduced (Fig. S1E,F), resulting in a total decrease of the HC rate. How these effects of 3IY feeding exactly come about remains unclear.

We next tried to rescue the effect of 3IY on the TH enzyme by additionally feeding the animals with L-DOPA (Fig. S1A). To this end, we fed animals either with plain yeast solution (control), or 5 mg/ml 3IY, or with both 5 mg/ml 3IY and 10 mg/ml L-DOPA. The memory scores were impaired in larvae fed with 3IY alone (Fig. 3A; Fig. S3A), replicating the results from Fig. 2A. These reduced memory scores were restored to control levels by additionally feeding L-DOPA to the larvae (Fig. 3A; Fig. S3A). Innate odor and sugar preferences were not affected by either 3IY or combined 3IY and L-DOPA feeding, confirming that both effects were specific for associative learning (Fig. 3B,C). Importantly, while a repetition of the experiment from Fig. 3A replicated the finding that L-DOPA feeding can restore memory
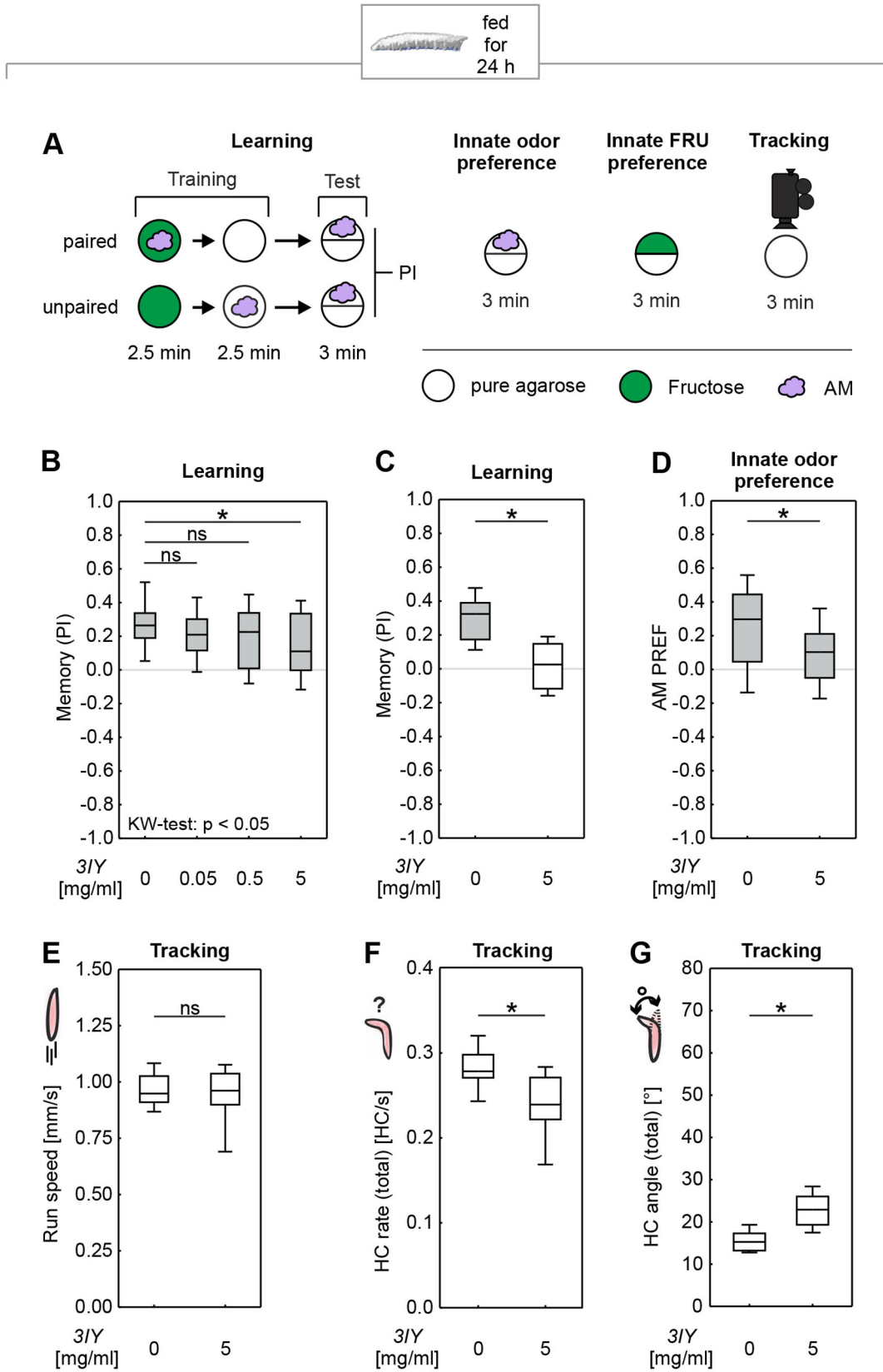
Fig. 1. See next page for legend.

Fig. 1. Feeding 3IY to *D. melanogaster* larvae for 24 h broadly impairs behavior. (A) Larvae were either trained in a learning paradigm, tested for their innate odor or fructose (FRU) preference, or behavior was analyzed offline using video recording. In the one-odor learning paradigm, cohorts of larvae were trained by either paired or unpaired presentations of an odor (purple cloud) and sugar (green circle), and subsequently tested for odor preference. Note that in every other experiment the training sequence was reversed to what is depicted. To test the innate odor preference larvae had the choice between odor on one side of the Petri dish and no odor on the other. Likewise, innate FRU preference was tested by presenting FRU (green semicircle) on one half of the Petri dish and pure agarose (white semicircle) on the other half of the Petri dish. To track the locomotion, larvae were video recorded on a dish filled with agarose, without any particular stimuli. (B) Feeding different concentrations of 3IY for 24 h led to memory impairment (KW: $H=8.44$, d.f.=3, $P=0.378$; OSSs from left to right: $P<0.0001$; $P<0.0001$; $P=0.0019$; $P=0.0039$; $N=36$ each), with a significant reduction compared to the control only in the group with the highest tested concentration of 5 mg/ml 3IY (MWU: $U=405.00$, $P=0.0063$). All other tested concentrations did not affect memory scores compared to the control group (MWU: 0 versus 0.05 mg/ml 3IY: $U=533.50$, $P=0.1992$; 0 versus 0.5 mg/ml 3IY: $U=518.00$, $P=0.1447$). (C) As seen in B, larvae fed with 5 mg/ml 3IY showed impaired memory (MWU: $U=19.00$, $P=0.0024$; OSSs from left to right: $P=0.0005$; $P=0.7744$; $N=12$ each) in an independent repetition. (D) An innate preference test revealed lower preference for the tested odor in the group fed with 5 mg/ml 3IY compared to the control group (MWU: $U=852.00$, $P=0.0061$; OSSs from left to right: $P<0.0001$; $P=0.0066$; $N=50$ each). (E) Offline analysis of larval behavior revealed no difference in run speed between control larvae and larvae fed with 5 mg/ml 3IY (MWU: $U=192.00$, $P=0.8392$, $N=20$ each). Regarding head casts, larvae fed with 5 mg/ml 3IY compared to control larvae showed (F) fewer head casts (MWU: $U=76.00$, $P=0.0008$, $N=20$ each) but (G) made larger head casts (MWU: $U=28.00$, $P<0.0001$, $N=20$ each). Grey boxes reflect behavioral scores relative to chance levels (zero) significant at $P<0.05$ in OSS tests with Bonferroni–Holm correction. KW tests are indicated within the figure. Asterisks and numbers above horizontal lines reflect significance or lack thereof in MWU tests. Box plots represent the median as the midline, 25 and 75% as the box boundaries, and 10 and 90% as the whiskers. See Fig. S1 for preference scores underlying the PIs and detailed head cast analysis.

scores upon 3IY treatment, we also showed that the feeding of L-DOPA alone did not increase memory scores (Fig. 3D; Fig. S3B).

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

After demonstrating the effect of 3IY feeding on associative learning about natural sugar rewards in larvae, we sought to combine 3IY feeding with genetic manipulations of the dopaminergic system, and at the same time to study how broadly applicable the 3IY approach might be. Therefore, we applied it to a different learning paradigm, by using (i) adult flies instead of larvae; (ii) a two-odor differential paradigm instead of a one-odor, 'absolute' paradigm; and (iii) an optogenetic punishment instead of a natural taste reward (Fig. 4). Specifically, we expressed the blue-light-gated cation channel channelrhodopsin-2-XXL as the optogenetic effector (*ChR2-XXL*; Dawydow et al., 2014) in a single dopaminergic neuron per brain hemisphere, called PPL1-γ1pedc (alternative nomenclatures PPL1-01 and MB-MP1), as covered by the Split-GAL4 driver strain *MB320C* (Aso et al., 2014). This neuron, when optogenetically activated, carries an internal punishment signal sufficient to establish an aversive associative memory when paired with an odor (Aso and Rubin, 2016; Hige et al., 2015; König et al., 2018) (Fig. 4B, left-most box plot). Upon feeding 3IY for 48 h before training, memory scores were decreased, an effect that was restored by L-DOPA feeding (Fig. 4B; Fig. S4A). The effect of 3IY in reducing memory scores increased with increasing 3IY concentrations (Fig. 4C; Fig. S4B),

and was equally observed in female and male flies (Fig. 4D; Fig. S5). Critically, 3IY feeding left innate odor preference to either odor unaffected (Fig. 4E,F), which also implies that the animals' locomotor abilities were intact to an extent that allowed normal odor preferences. We therefore did not perform detailed locomotion analyses. Furthermore, feeding L-DOPA alone did not increase memory scores (Fig. 4G; Fig. S4C). Thus, feeding 3IY specifically impaired associative learning via PPL1-γ1pedc activation in adult flies, but kept their task-relevant behavioral capacities intact.

DISCUSSION

The present study demonstrates that both in larval and adult *D. melanogaster*, and in two very different kinds of tasks, feeding 3IY can specifically impair associative learning while innate task-relevant behavior remains intact. In either case, the observed memory impairment was rescued by feeding L-DOPA, suggesting that the 3IY-impairment was indeed caused by an inhibition of the TH enzyme that catalyzes the synthesis of L-DOPA. Regarding adult flies, these results are in line with previous studies that showed that 3IY feeding impairs associative learning about ethanol, quinine or electric shock (Kaun et al., 2011; Seugnet et al., 2008; Zhang et al., 2008). Here, we find a similar impairment of learning about optogenetic PPL1-γ1pedc activation. Previously, a constitutive RNA-interference knockdown of TH in PPL1-γ1pedc revealed that punishment learning by PPL1-γ1pedc activation is dependent on dopamine synthesis in this same neuron (König et al., 2018). Using the more acute, albeit systemic approach of feeding 3IY, we provided an independent confirmation of these results (Fig. 4). Regarding larvae, genetic approaches have uncovered an important role of dopamine for odor-taste associative learning (Rohwedder et al., 2016; Selcho et al., 2009). This is further supported here by an independent pharmacological approach (Figs 2 and 3). Although not unexpected, these results are interesting in themselves by demonstrating for the first time that an acute inhibition of TH impairs associative learning in larvae. This is critical to disentangle acute effects from potential developmental impairments or their compensation.

Indeed, our experiments demonstrate why drug feeding offers a valuable additional approach to manipulate the dopaminergic system of *D. melanogaster*. It is easy to apply, quick, comparably cheap, and it allows inducing the desired effect shortly before the experiment. The approach also does not require generating new fly strains, but can be easily combined with the use of already available genetic tools. As an example, for the experiments shown in Fig. 4 we optogenetically activated a specific dopaminergic neuron, while inhibiting the TH enzyme in a both systemic and inducible manner. In order to perform the same type of manipulation by genetic means alone, one would have to combine at least five genetic constructs for driving expression of channelrhodopsin-2-XXL in the neuron of interest, as well as of an RNAi against TH in the whole body, plus e.g. a Gal80[ts] construct to make the expression of the RNAi inducible. Although that is certainly possible, feeding 3IY is the quicker and easier option. Also, the effects of the drugs can be titrated relatively conveniently by adjusting the concentration and the duration of feeding (Figs 1 and 2). This makes it possible to find a trade-off between maximizing the intended effect on learning and memory and minimizing developmental side effects, or effects on locomotion or sensory function. Furthermore, drugs with comparable effects in different organisms allow for elegant translational research across different species.
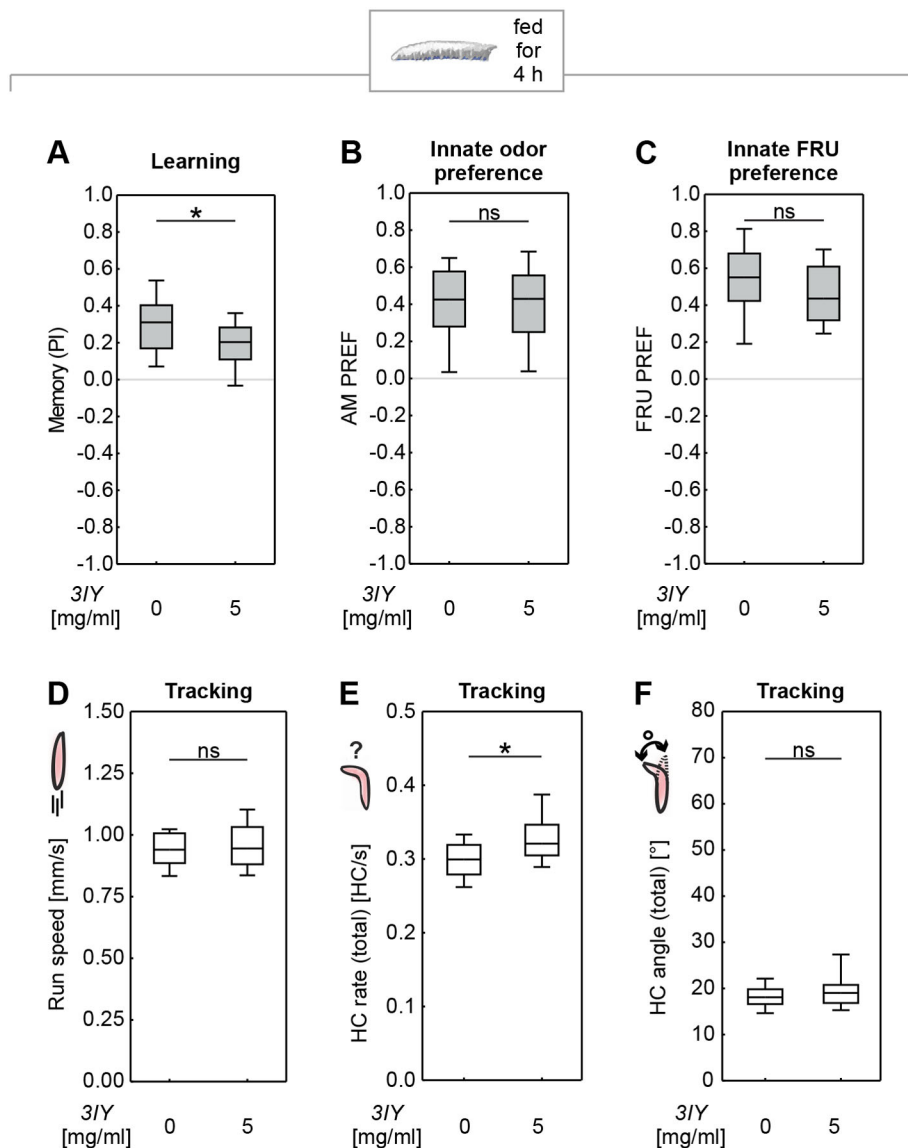
Fig. 2. Feeding 3IY to *D. melanogaster* larvae for 4 h impairs memory but leaves innate behavior intact. (A) Larvae fed with 5 mg/ml 3IY for 4 h showed impaired memory compared to the control group (MWU: *U*=125.00, *P*=0.0439; OSSs from left to right: *P*<0.0001; *P*=0.0004; *N*=20 each). Innate preference for (B) the odor (MWU: *U*=230.50, *P*=0.7963; OSS: *P*<0.0001 each; *N*=22 each) or (C) FRU (MWU: *U*=344.00, *P*=0.1188; OSS: *P*<0.000 each; *N*=30 each) was not affected. Video tracking of the larvae revealed (D) no difference in run speed (MWU: *U*=254.00, *P*=0.4897, *N*=24 each), (E) a slight increase in HC rate for larvae fed with 5 mg/ml 3IY (MWU: *U*=174.00, *P*=0.0193, *N*=24 each), and (F) no difference in HC angles (MWU: *U*=247.00, *P*=0.4037; *N*=24 each). See Fig. S2 for preference scores underlying the PIs and detailed analysis of head casts. For further details, see Fig. 1.

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

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

MATERIALS AND METHODS
General

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

For larval behavior experiments, we used third instar, feeding-stage wild-type Canton Special larvae of either sex, aged 4 or 5 days after egg laying, as mentioned along with the results. For adult behavior experiments, the split-GAL4 driver strain *MB320C* (detailed information can be found in the relevant database http://splitgal4. janelia.org/cgi-bin/splitgal4.cgi as well as in Aso et al., 2014), covering the PPL1-γ1pedc neurons (alternative nomenclatures: PPL1-01 and MB-MP1), was crossed to *UAS-ChR2-XXL* (Bloomington, stock number: 58374, Dawydow et al., 2014) as the effector and kept in darkness throughout to avoid optogenetic activation by room light. Flies of either sex, aged 1 to 4 days after hatching, were used.

Prior to behavioral experiments, animals were fed with solutions of 3-Iodo-L-tyrosine (3IY; stored at −20°C; CAS: 70-78-0, Sigma-Aldrich, Steinheim, Germany) and/or 3,4-dihydroxyphenylalanine (L-DOPA; CAS: 59-92-7, Sigma-Aldrich) at concentrations of 5 mg/ml and 10 mg/ml, respectively, as explained in more detail below. To facilitate reproducibility, we measured the absorption of
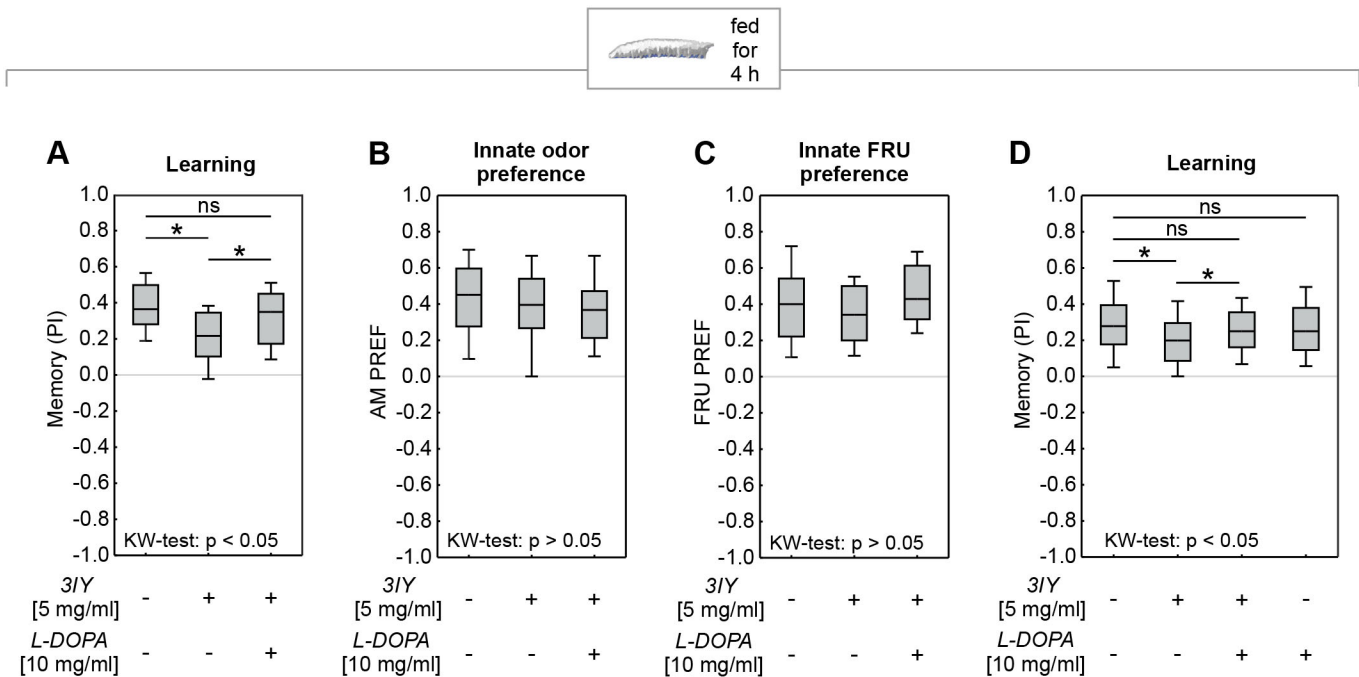
Fig. 3. Memory impairment in *Drosophila* larvae due to 3IY can be rescued by additionally feeding L-DOPA. (A) Feeding L-DOPA in addition to 3IY rescued the memory impairment (KW: *H*=10.69, d.f.=2, *P*=0.0048; OSSs from left to right: *P*<0.0001; *P*=0.0005; *P*<0.0001; *N*=26 each). Feeding 5 mg/ml 3IY alone for 4 h impaired memory (MWU: *U*=165.50, *P*=0.0016), whereas additionally feeding 10 mg/ml L-DOPA rescued memory impairment (MWU: *U*=215.00, *P*=0.0250) and led to memory scores comparable to the control group (MWU: *U*=286.00, *P*=0.3459). Feeding either drug did not affect innate approach to (B) odor (KW: *H*=2.02, d.f.=2, *P*=0.3650; OSSs from left to right: *P*<0.0001; *P*<0.0001; *P*<0.0001; *N*=28 each) or (C) FRU (KW: *H*=2.42, d.f.=2, *P*=0.2977; OSSs from left to right: *P*<0.0001; *P*<0.0001; *P*<0.0001; *N*=24 each). (D) As shown in A, feeding 3IY impaired memory scores, and this impairment was rescued by additional L-DOPA feeding (KW: *H*=14.06, d.f.=3, *P*=0.0028; OSSs: *P*<0.0001 each; *N*=96 each; MWU: no drug versus 3IY alone: *U*=3262.50, *P*=0.0005; no drug versus 3IY+L-DOPA: *U*=4084.50, *P*=0.1743; 3IY alone versus 3IY+L-DOPA: *U*=3673.00, *P*=0.0152). Feeding L-DOPA alone had no effect on memory scores (MWU: no drug versus L-DOPA alone: *U*=4246.50, *P*=0.3484). Given this lack of effect of feeding L-DOPA alone, we did not perform an additional control for innate odor and sugar preference for this experimental condition. See Fig. S3 for preference scores underlying the PIs. For further details, see Fig. 1.

the solutions in the UV-visible spectrum, using a NanoDrop 2000c spectrometer (ThermoFisher Scientific, Dreiich, Germany). For 5 mg/ml 3IY in distilled water, we found the wavelength of maximal absorption to be 280 nm, and the average absorption at this wavelength to be 4.46. For 10 mg/ml L-DOPA in distilled water, we determined a wavelength of maximal absorption of 280 nm, and an absorption at this wavelength of 7.66.

Feeding of 3IY to larval *D. melanogaster*
A 0.5 mg/ml yeast solution was prepared from fresh baker's yeast (common supermarket brands) diluted in tap water and stored for up to 5 days at 4°C in a closed bottle. Samples of 2 ml yeast solution were filled into a 15 ml Falcon tube and kept for a few minutes in a warm water bath. 3-Iodo-L-tyrosine (3IY; stored at −20°C; CAS: 70-78-0, Sigma-Aldrich) was added at a concentration of 5 mg/ml to the respective sample, if not mentioned otherwise. Notably, in contrast to earlier studies using 10 mg/ml or more (Neckameyer, 1996; Wang et al., 2013), we were not able to dissolve concentrations higher than 5 mg/ml. In some experiments, 3,4-dihydroxyphenylalanine (L-DOPA; CAS: 59-92-7, Sigma-Aldrich) was added at a concentration of 10 mg/ml, either to pure yeast solution, or to yeast solution with 5 mg/ml 3IY.

The solutions were thoroughly mixed by attaching the Falcon tubes to a shaker at high speed for approximately 60 min. Empty vials of 5 cm diameter were equipped with two layers of mesh (PET, 500 μm mesh size). Samples of the mixed yeast solution with or without additional substances were distributed onto the mesh of one vial. Larvae of the third instar feeding stage were collected from the

fly food by adding 15% sucrose solution (*D*-Sucrose; CAS: 57-50-1, Roth, Karlsruhe, Germany; in dH₂O) so that the larvae floated up and could be transferred to a Petri dish filled with tap water using a tip-cut plastic pipette. After being rinsed in water, the larvae were loaded onto a filter (pluriStrainer 70 μm, pluriSelect Life Science, Leipzig, Germany) to separate them from water and small food particles, and transferred with a brush to one of the prepared vials. For yeast solutions containing different drugs and/or concentrations, different brushes were used. The larvae were left to feed on the respective yeast solution for 24 or 4 h at 25°C and 60–70% relative humidity. The desired number of larvae were collected with a brush, briefly rinsed in water, and afterwards used in the respective experiment.

Larval behavior
Odor-fructose associative learning
Experiments for appetitive odor-fructose associative memory (Saumweber et al., 2011; Scherer et al., 2003) were performed using a one-odor, single-training-trial protocol described in Weiglein et al. (2019) (Fig. 1A, left). For example, two custom-made Teflon containers of 5 mm diameter were filled with 10 μl of odor substance (*n*-amylacetate, AM; CAS: 628-63-7, Merck, Darmstadt, Germany; diluted 1:20 in paraffin oil; CAS: 8042-47-5, AppliChem, Darmstadt, Germany) and closed with lids perforated with 5–10 holes, each of approximately 0.5 mm diameter. These odor containers were located on opposite sides of a Petri dish (9 cm inner diameter; Nr. 82.1472 Sarstedt, Nümbrecht, Germany) filled with 1% agarose solution (electrophoresis grade; CAS: 9012-36-6,
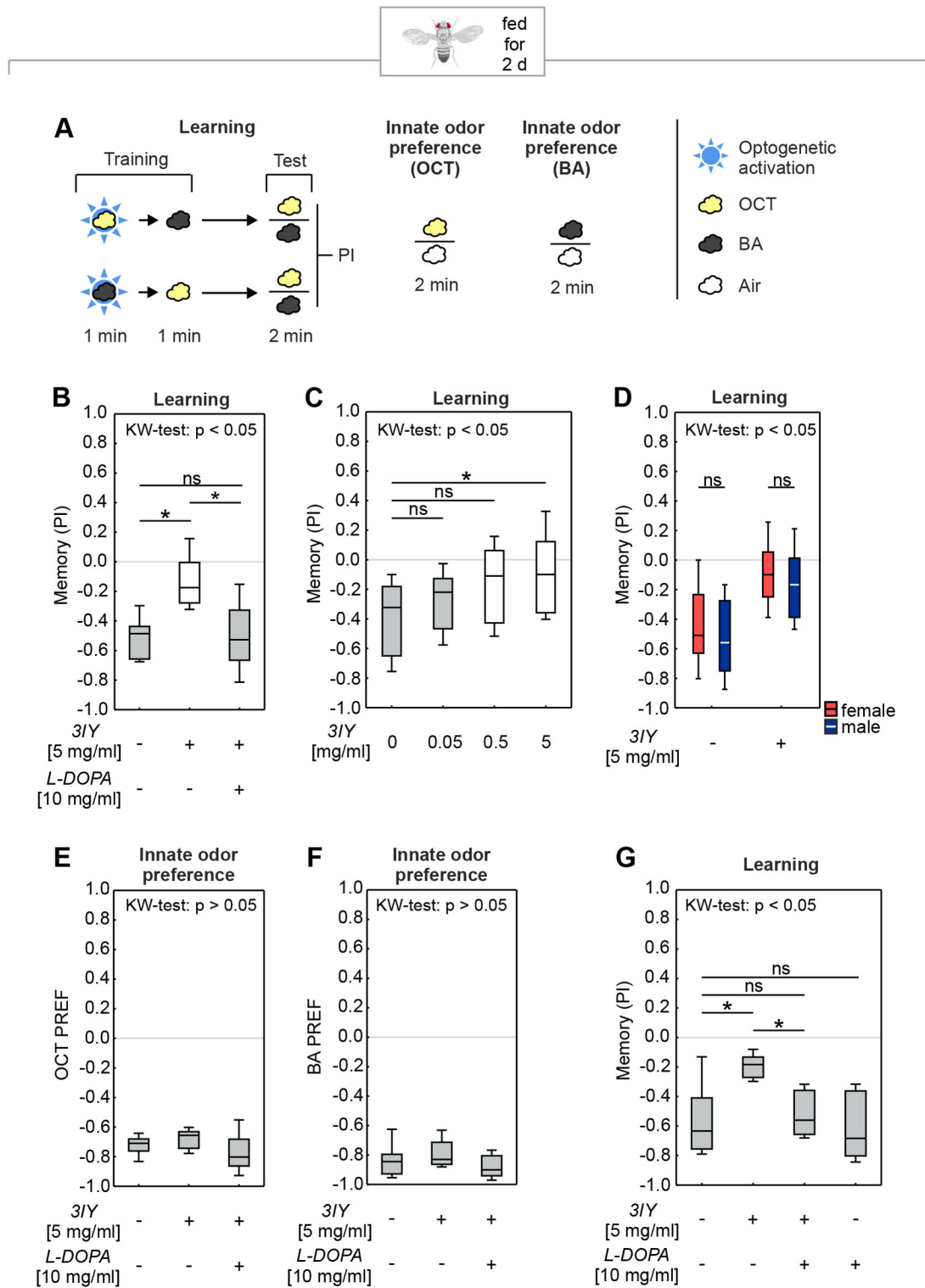
Fig. 4. See next page for legend.

Roth, Karlsruhe, Germany) and additionally containing fructose (FRU; 2 M; purity 99%; CAS: 57-48-7 Roth, Karlsruhe, Germany) as a taste reward (+). Cohorts of approximately 30 larvae were placed at the center of the Petri dish and allowed to move about the Petri dish for 2.5 min. Subsequently, they were transferred with a brush to a fresh Petri dish that was filled with plain, tasteless agarose and equipped with two empty Teflon containers (EM). For each cohort trained in such a paired way (paired training; AM+/EM), a

second cohort of larvae received the odor unpaired from the fructose reward (unpaired training; EM+/AM). In half of the cases the sequence of events was reversed (EM/AM+, AM/EM+, respectively).

After one training trial, the larvae were transferred to a fresh, tasteless test Petri dish with AM on one side and an EM container on the opposite side. The larvae were left to distribute for 3 min and then counted to evaluate their preference for AM. The number of

Fig. 4. Feeding 3IY to adult *D. melanogaster* impairs optogenetically induced memory but leaves innate behavior intact. (A) Flies were either trained in a learning paradigm and tested afterwards or were tested for their innate odor preference. In the learning paradigm, cohorts of flies were trained by pairing one of two odors (yellow/black cloud) with optogenetic activation of PPL1-γ1pedc (blue star), and subsequently tested for their choice between the two odors. Note that the sequences of the odors and the optogenetic activation was shuffled across experiments as explained in the methods section. In the innate odor preference test, flies were given the choice between an odor (yellow/black cloud) and air (white cloud). (B) 3IY feeding led to an impaired performance index compared to the control group (MWU: $U=2.00$, $P=0.0019$). Additional L-DOPA feeding (KW: $H=11.89$, d.f.=2, $P=0.0026$; OSS from left to right: $P=0.0078$; $P=0.2891$; $P=0.0078$; $N=8$ each) rescued this impairment of memory scores (MWU: 3IY alone versus 3IY+L-DOPA: $U=6.00$, $P=0.0074$) to the control level (MWU: no drug versus 3IY+L-DOPA: $U=29.00$, $P=0.7929$). (C) 3IY concentrations significantly influenced PI values (KW: $H=11.08$, d.f.=3, $P=0.0113$; OSSs from left to right: $P=0.0005$; $P=0.0018$; $P=1.0$; $N=16$, 14, 16, 15). The highest concentration of 5 mg/ml 3IY significantly reduced memory compared to the control group (MWU: $U=47.00$, $P=0.0042$). All other tested concentrations of 3IY had no significant effect with the given sample sizes (MWU: 0 versus 0.05 mg/ml 3IY: $U=83.00$, $P=0.2361$; 0 versus 0.5 mg/ml 3IY: $U=72.00$, $P=0.0365$). (D) Analysis of gender differences of pooled data from B,C,G revealed no gender specific effects of 3IY (KW: $H=43.40$, d.f.=3, $P=0.0001$; MWU: 0 mg/ml 3IY: $U=577.50$, $P=0.2496$; 5 mg/ml 3IY: $U=526.00$, $P=0.3124$; OSSs from left to right: $P<0.001$; $P<0.001$; $P=0.0895$; $P=0.0090$; $N=37$, 37, 35, 35). Red and blue boxes represent females and males, respectively. (E,F) Innate odor avoidance of (E) OCT and (F) BA was not affected by 3IY and/or L-DOPA feeding (KW: OCT: $H=4.42$, d.f.=2, $P=0.1097$; OSSs from left to right: $P<0.0001$; $P<0.0001$; $P<0.0001$; $N=12$ each) (KW: BA: $H=2.71$, d.f.=2, $P=0.2575$; OSSs from left to right: $P<0.0001$; $P<0.0001$; $P<0.0001$; $N=12$ each). (G) In a repetition of the experiment shown in B, feeding L-DOPA in addition to 3IY rescued the 3IY-induced memory impairment (KW: $H=14.68$, d.f.=3, $P=0.0021$; OSSs: $P<0.0001$ each; $N=13$, 12, 11, 12; MWU: no drug versus 3IY alone: $U=29.00$, $P=0.0083$; no drug versus 3IY+L-DOPA: $U=59.00$, $P=0.4869$; 3IY alone versus 3IY+L-DOPA: $U=12.00$, $P=0.0009$). Importantly, L-DOPA alone had no effect on the memory scores (MWU: no drug versus L-DOPA alone: $U=68.00$, $P=0.6053$). Given this lack of effect of feeding L-DOPA alone, we did not perform an additional control for innate odor preference for this experimental condition. See Fig. S4 for preference scores underlying the PIs, and Fig. S5 for a full display of all adult fly behavioral results separated by gender. For further details, see Fig. 1.

larvae (#) on the AM side, on the EM side, and in a 10 mm-wide middle zone was counted. Larvae crawling up the sidewalls of the Petri dish were counted for the respective side, whereas larvae on the lid were excluded from the analysis (<5%). A preference index (AM PREF) was calculated:

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

AM PREF values range from +1 to −1, with positive values indicating AM preference and negative values indicating avoidance of AM.

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

$$PI = \frac{(AM\ PREF\ Paired\ -\ AM\ PREF\ Unpaired)}{2}. \qquad (2)$$

Performance indices range from +1 to −1. Positive PIs indicate appetitive associative memory; negative values indicate aversive associative memory.

Innate odor preference tests
Cohorts of approximately 20–30 experimentally naïve larvae were collected, briefly washed in tap water, and placed onto a Petri dish

with an AM container on one side and an EM container on the other side (Fig. 1A, second from left). After 3 min, the odor preference was determined as detailed in Eqn 1.

Innate fructose preference tests
Split Petri dishes were prepared freshly approximately 4 h before the experiment, following the procedures described in König et al. (2014) such that one half of the Petri dish (9 cm diameter) was filled with agarose with 2 M FRU, and the other half with plain agarose (Fig. 1A, second from right). Approximately 20–30 larvae were collected, rinsed in tap water, and placed onto the center of a split Petri dish. After 3 min, the number of larvae (#) on the fructose side, on the pure agarose side, and in a 10 mm-wide middle zone was counted. Fructose preference was calculated as follows:

$$FRU\ PREF = \frac{(\#Fructose\ -\ \#Agarose)}{\#Total}. \qquad (3)$$

FRU PREF scores range from +1 to −1, with positive values indicating fructose preference and negative values indicating avoidance.

Analyses of locomotion
Cohorts of approximately 20 larvae were placed on an empty, plain-agarose-filled Petri dish without odor or reward (Fig. 1A, right). For 3 min, they were video-recorded while they freely moved in the dish. The videos were analyzed offline using custom-made tracking software described in Paisios et al. (2017). In brief, larvae alternately perform relatively straight forward-locomotion, called runs, and lateral head movements, called head casts (HC) that are often followed by changes in direction. This leads to a typical zig-zagging pattern of locomotion (Gershow et al., 2012; Gomez-Marin and Louis, 2014; Gomez-Marin et al., 2011). As described in detail by Paisios et al. (2017), an HC was detected whenever the angular velocity of a vector through the animal's head exceeded a threshold of 35°/s and ended as soon as that angular velocity dropped below the threshold again. The time during which an animal was not head-casting was regarded as a run, deducting 1.5 s before and after an HC to exclude the decelerating and accelerating phases that usually happen before and after an HC, respectively. Three aspects of behavior were analyzed: the run speed was determined as the average speed (mm/s) of the larval midpoint during runs; the rate of HCs was determined as the number of HCs per second (HC/s); and the size of HCs was determined by the HC angle. Accordingly, the animal's bending angle as the angle between vectors through the head and tail was determined before and after an HC. Then, the HC angle was calculated as the difference between the animal's bending angle after an HC and the bending angle before an HC. For a detailed description, see Paisios et al. (2017).

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

Feeding of 3IY to adult *D. melanogaster*
For 3IY feeding in adult flies, a 5% sucrose solution (CAS: 57-50-1, Hartenstein, Würzburg, Germany) was prepared. This solution was either used pure, or mixed with 5 mg/ml 3IY, or with 10 mg/ml L-DOPA, or with both, in an analogous manner to that described above for the larval case. Hatched adults of the genotype *MB320C; ChR2-XXL* were collected in fresh food vials and kept under the

normal culture conditions mentioned above, at least overnight and at most until 4 days after hatching. Flies were transferred to new vials containing a tissue (Fripa, Düren, Germany) soaked with 1.8 ml of sucrose solution that either did or did not contain 3IY and/or L-DOPA, as mentioned in the results section. After 40–48 h under otherwise normal culture conditions, the flies were trained and/or tested *en masse*.

Adult behavior

Odor-PPL1-γ1pedc associative learning

For the memory assays, we followed the procedures described in König et al. (2018), unless mentioned otherwise (Fig. 4A, left). Approximately 100 flies were loaded into a small transparent tube in a custom-made setup (CON-ELEKTRONIK, Greussenheim, Germany), and were trained and tested at 23–25°C and 60–80% relative humidity. Training was performed in dimmed red light, which is largely invisible to flies and does not stimulate the ChR2-XXL effector; testing was performed in darkness. For the application of blue light, a 2.5 cm-diameter and 4.5 cm-length hollow tube with 24 LEDs mounted on the inner surface was placed around the transparent training tubes harboring the flies. As odorants, 50 µl benzaldehyde (BA) and 250 µl 3-octanol (OCT) (CAS 100-52-7, 589-98-0; both from Fluka, Steinheim, Germany) were applied to 1 cm-deep Teflon containers of 5 and 14 mm diameter, respectively. From these containers, odor-loaded air was shunted into the permanent air stream flowing through the apparatus. During training, the flies were presented with both odors for 1 min with a 3 min resting interval in between, but only one of the odors was paired with 1 min of blue light (465 nm) for optogenetic activation of PPL1-γ1pedc, whereas the other odor was presented alone (either BA-paired or OCT-paired training, respectively). In half of the cases training started with the odor paired with light (CS+); in the other half training started with the odor without light activation (CS-; for details see electronic supplement Fig. S1B of König et al., 2019). For the subsequent test, the flies were given a 3 min accommodation period, after which they were transferred to the T-maze-like choice point. The test configuration between the two odors used during training was prepared and balanced so that either BA or OCT were present at front versus rear position over the course of all experiments. After 2 min testing time, the arms of the maze were closed and the flies on each side were counted to calculate a benzaldehyde preference index (BA PREF):

$$BA\ PREF = \frac{(\#BA\ -\ \#OCT)}{\#Total}. \tag{4}$$

Thus, positive scores indicate preference for BA and negative scores preference for OCT. From the BA PREF scores of two independently trained fly groups after BA-paired and OCT-paired training, a performance index (PI) was calculated as follows:

$$PI = \frac{(BA\ PREF\ BA\text{-}Paired\ -\ BA\ PREF\ OCT\text{-}Paired)}{2}. \tag{5}$$

Positive performance indices thus reflect appetitive associative memory, negative values aversive associative memory.

Innate odor preference tests

Cohorts of approximately 50 flies were loaded into the setup. After a 5 min resting interval, they were transferred to the choice point of a T-maze between an arm equipped with either BA or OCT (in the

same manner as described above), and an arm with an empty Teflon container, and allowed to distribute for 2 min (Fig. 4A, right). A preference was calculated as:

$$BA\ PREF = \frac{(\#BA\ -\ \#EM)}{\#Total}\quad \text{or}$$
$$OCT\ PREF = \frac{(\#OCT\ -\ \#EM)}{\#Total}. \tag{6}$$

Each data point in Fig. 4D and E represents the mean value of two runs tested with the odor in the front or rear T-maze position.

Statistics

Two-tailed, non-parametric statistics were used throughout to analyze the behavioral data. For comparisons of a group's scores with chance levels (zero), one-sample sign tests (OSS) were applied. To compare across multiple independent groups, Kruskal–Wallis tests (KW) with subsequent pair-wise Mann–Whitney U-tests (MWU) were used (Statistica 13, StatSoft Inc, Tulsa, USA). To ensure a within-experiment error rate below 5%, a Bonferroni–Holm (BH) correction for multiple comparisons was employed (Holm, 1979). Sample sizes (biological replications) were estimated based on previous studies with small to medium effect sizes (König et al., 2018; Weiglein et al., 2019). None of the specific experiments reported here had previously been performed in our laboratory, although the basic behavioral paradigms are regularly used. Experimenters were blind to treatment condition during the experiments with larvae, and during the fly counting for the experiments with adults. Data are presented as box plots showing the median as the middle line, the 25 and 75% quantiles as box boundaries, and the 10 and 90% quantiles as whiskers. All data from behavioral experiments are documented in Table S1.

References

Andretic, R., Van Swinderen, B. and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol.* **15**, 1165-1175. doi:10.1016/j.cub.2005.05.025

Aso, Y. and Rubin, G. M. (2016). Dopaminergic neurons write and update memories with cell-type-specific rules. *eLife* **5**, e16135. doi:10.7554/eLife.16135

Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T. T., Dionne, H., Abbott, L. F., Axel, R., Tanimoto, H. et al. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *eLife* **3**, e04577. doi:10.7554/eLife.04577

Bainton, R. J., Tsai, L. T.-Y., Singh, C. M., Moore, M. S., Neckameyer, W. S. and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol.* **10**, 187-194. doi:10.1016/S0960-9822(00)00336-5

Berke, J. D. (2018). What does dopamine mean? *Nat. Neurosci.* **21**, 787-793. doi:10.1038/s41593-018-0152-y

Burke, C. J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M. J., Das, G., Gohl, D., Silies, M., Certel, S. and Waddell, S. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* **492**, 433-437. doi:10.1038/nature11614

Busto, G. U., Cervantes-Sandoval, I. and Davis, R. L. (2010). Olfactory learning in *Drosophila. Physiology* **25**, 338-346. doi:10.1152/physiol.00026.2010

Chang, H.-Y., Grygoruk, A., Brooks, E. S., Ackerson, L. C., Maidment, N. T., Bainton, R. J. and Krantz, D. E. (2006). Overexpression of the *Drosophila* vesicular monoamine transporter increases motor activity and courtship but decreases the behavioral response to cocaine. *Mol. Psychiatry* **11**, 99-113. doi:10.1038/sj.mp.4001742

Cichewicz, K., Garren, E. J., Adiele, C., Aso, Y., Wang, Z., Wu, M., Birman, S., Rubin, G. M. and Hirsh, J. (2017). A new brain dopamine-deficient *Drosophila* and its pharmacological and genetic rescue. *Genes Brain Behav.* **16**, 394-403. doi:10.1111/gbb.12353

Clark, W. C., Pass, P. S., Venkataraman, B. and Hodgetts, R. B. (1978). Dopa decarboxylase from *Drosophila* melanogaster: purification, characterization and an analysis of mutants. *Mol. Gen. Genet. MGG* **162**, 287-297. doi:10.1007/BF00268854

Dawydow, A., Gueta, R., Ljaschenko, D., Ullrich, S., Hermann, M., Ehmann, N., Gao, S., Fiala, A., Langenhan, T., Nagel, G. et al. (2014). Channelrhodopsin-2-XXL, a powerful optogenetic tool for low-light applications. *Proc. Natl. Acad. Sci. USA* **111**, 13972-13977. doi:10.1073/pnas.1408269111

Diegelmann, S., Klagges, B., Michels, B., Schleyer, M. and Gerber, B. (2013). Maggot learning and synapsin function. *J. Exp. Biol.* **216**, 939-951. doi:10.1242/jeb.076208

Fernandez, R. W., Akinleye, A. A., Nurilov, M., Feliciano, O., Lollar, M., Aijuri, R. R., O'Donnell, J. M. and Simon, A. F. (2017). Modulation of social space by dopamine in *Drosophila* melanogaster, but no effect on the avoidance of the *Drosophila* stress odorant. *Biol. Lett.* **13**, 20170369. doi:10.1098/rsbl.2017.0369

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J. and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* **54**, 618-627. doi:10.1002/neu.10185

Gerber, B. and Stocker, R. F. (2007). The drosophila larva as a model for studying chemosensation and chemosensory learning: a review. *Chem. Senses* **32**, 65-89. doi:10.1093/chemse/bjl030

Gershow, M., Berck, M., Mathew, D., Luo, L., Kane, E. A., Carlson, J. R. and Samuel, A. D. T. (2012). Controlling airborne cues to study small animal navigation. *Nat. Methods* **9**, 290-296. doi:10.1038/nmeth.1853

Gomez-Marin, A. and Louis, M. (2014). Multilevel control of run orientation in *Drosophila* larval chemotaxis. *Front. Behav. Neurosci.* **8**, 38. doi:10.3389/fnbeh.2014.00038

Gomez-Marin, A., Stephens, G. J. and Louis, M. (2011). Active sampling and decision making in *Drosophila* chemotaxis. *Nat. Commun.* **2**, 441. doi:10.1038/ncomms1455

Groessl, F., Munsch, T., Meis, S., Griessner, J., Kaczanowska, J., Pliota, P., Kargl, D., Badurek, S., Kraitsy, K., Rassoulpour, A. et al. (2018). Dorsal tegmental dopamine neurons gate associative learning of fear. *Nat. Neurosci.* **21**, 952-962. doi:10.1038/s41593-018-0174-5

Guven-Ozkan, T. and Davis, R. L. (2014). Functional neuroanatomy of *Drosophila* olfactory memory formation. *Learn. Mem.* **21**, 519-526. doi:10.1101/lm.034363.114

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat. Rev. Neurosci.* **4**, 266-275. doi:10.1038/nrn1074

Hige, T., Aso, Y., Modi, M. N., Rubin, G. M. and Turner, G. C. (2015). Heterosynaptic plasticity underlies aversive olfactory learning in *Drosophila*. *Neuron* **88**, 985-998. doi:10.1016/j.neuron.2015.11.003

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* **6**, 65-70.

Hsouna, A., Lawal, H. O., Izevbaye, I., Hsu, T. and O'Donnell, J. M. (2007). *Drosophila* dopamine synthesis pathway genes regulate tracheal morphogenesis. *Dev. Biol.* **308**, 30-43. doi:10.1016/j.ydbio.2007.04.047

Karam, C. S., Jones, S. K. and Javitch, J. A. (2020). Come Fly with Me: an overview of dopamine receptors in *Drosophila* melanogaster. *Basic Clin. Pharmacol. Toxicol.* **126**, 56-65. doi:10.1111/bcpt.13277

Kaun, K. R., Azanchi, R., Maung, Z., Hirsh, J. and Heberlein, U. (2011). A *Drosophila* model for alcohol reward. *Nat. Neurosci.* **14**, 612-619. doi:10.1038/nn.2805

König, C., Schleyer, M., Leibiger, J., El-Keredy, A. and Gerber, B. (2014). Bitter-sweet processing in larval *Drosophila*. *Chem. Senses* **39**, 489-505. doi:10.1093/chemse/bju016

König, C., Khalili, A., Ganesan, M., Nishu, A. P., Garza, A. P., Niewalda, T., Gerber, B., Aso, Y. and Yarali, A. (2018). Reinforcement signaling of punishment versus relief in fruit flies. *Learn. Mem.* **25**, 247-257. doi:10.1101/lm.047308.118

König, C., Khalili, A., Niewalda, T., Gao, S. and Gerber, B. (2019). An optogenetic analogue of second-order reinforcement in *Drosophila*. *Biol. Lett.* **15**, 20190084. doi:10.1098/rsbl.2019.0084

Lammel, S., Lim, B. K., Ran, C., Huang, K. W., Betley, M. J., Tye, K. M., Deisseroth, K. and Malenka, R. C. (2012). Input-specific control of reward and aversion in the ventral tegmental area. *Nature* **491**, 212-217. doi:10.1038/nature11527

Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., Siwanowicz, I., Rubin, G. M., Preat, T. and Tanimoto, H. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* **488**, 512–5516. doi:10.1038/nature11304

McGuire, S. E., Deshazer, M. and Davis, R. L. (2005). Thirty years of olfactory learning and memory research in *Drosophila* melanogaster. *Prog. Neurobiol.* **76**, 328-347. doi:10.1016/j.pneurobio.2005.09.003

Meder, D., Herz, D. M., Rowe, J. B., Lehéricy, S. and Siebner, H. R. (2019). The role of dopamine in the brain - lessons learned from Parkinson's disease. *Neuroimage* **190**, 79-93. doi:10.1016/j.neuroimage.2018.11.021

Menegas, W., Akiti, K., Amo, R., Uchida, N. and Watabe-Uchida, M. (2018). Dopamine neurons projecting to the posterior striatum reinforce avoidance of threatening stimuli. *Nat. Neurosci.* **21**, 1421-1430. doi:10.1038/s41593-018-0222-1

Michels, B., Saumweber, T., Biernacki, R., Thum, J., Glasgow, R. D. V., Schleyer, M., Chen, Y.-C., Eschbach, C., Stocker, R. F., Toshima, N. et al. (2017). Pavlovian conditioning of larval *Drosophila*: an illustrated, multilingual, hands-on manual for odor-taste associative learning in maggots. *Front. Behav. Neurosci.* **11**, 45. doi:10.3389/fnbeh.2017.00045

Monier, M., Nöbel, S., Danchin, E. and Isabel, G. (2019). Dopamine and serotonin are both required for mate-copying in *Drosophila* melanogaster. *Front. Behav. Neurosci.* **12**, 334-335. doi:10.3389/fnbeh.2018.00334

Neckameyer, W. S. (1996). Multiple roles for Dopamine in *Drosophila* development. *Dev. Biol.* **176**, 209-219. doi:10.1006/dbio.1996.0128

Neckameyer, W. S. (1998). Dopamine and mushroom bodies in *Drosophila*: experience-dependent and - independent aspects of sexual behavior. *Learn. Mem.* **5**, 157-165.

Neckameyer, W. S. and White, K. (1993). *Drosophila* tyrosine hydroxylase is encoded by the pale locus. *J. Neurogenet.* **8**, 189-199. doi:10.3109/01677069309083448

Nichols, C. D. (2006). *Drosophila* melanogaster neurobiology, neuropharmacology, and how the fly can inform central nervous system drug discovery. *Pharmacol. Ther.* **112**, 677-700. doi:10.1016/j.pharmthera.2006.05.012

Oishi, Y. and Lazarus, M. (2017). The control of sleep and wakefulness by mesolimbic dopamine systems. *Neurosci. Res.* **118**, 66-73. doi:10.1016/j.neures.2017.04.008

Ojelade, S. A., Butts, A. R., Merrill, C. B., Champaloux, E. P., Aso, Y., Wolin, D., Cofresi, R. U., Gonzales, R. A., Rubin, G. M., Venton, B. J. et al. (2019). Dopaminergic learning and arousal circuits mediate opposing effects on alcohol consumption in *Drosophila*. *bioRxiv* 624833. doi:10.1101/624833

Owald, D. and Waddell, S. (2015). Olfactory learning skews mushroom body output pathways to steer behavioral choice in *Drosophila*. *Curr. Opin. Neurobiol.* **35**, 178-184. doi:10.1016/j.conb.2015.10.002

Paisios, E., Rjosk, A., Pamir, E. and Schleyer, M. (2017). Common microbehavioral "footprint" of two distinct classes of conditioned aversion. *Learn. Mem.* **24**, 191-198. doi:10.1101/lm.045062.117

Pandey, U. B. and Nichols, C. D. (2011). Human disease models in *Drosophila* melanogaster and the role of the fly in therapeutic drug discovery. *Pharmacol. Rev.* **63**, 411-436. doi:10.1124/pr.110.003293

Perisse, E., Yin, Y., Lin, A. C., Lin, S., Huetteroth, W. and Waddell, S. (2013). Different kenyon cell populations drive learned approach and avoidance in *Drosophila*. *Neuron* **79**, 945-956. doi:10.1016/j.neuron.2013.07.045

Pyakurel, P., Shin, M. and Venton, B. J. (2018). Nicotinic acetylcholine receptor (nAChR) mediated dopamine release in larval *Drosophila* melanogaster. *Neurochem. Int.* **114**, 33-41. doi:10.1016/j.neuint.2017.12.012

Quinn, W. G., Harris, W. A. and Benzer, S. (1974). Conditioned behavior in *Drosophila* melanogaster. *Proc. Natl. Acad. Sci. USA* **71**, 708-712. doi:10.1073/pnas.71.3.708

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., Iché-Torres, M., Cassar, M., Strauss, R., Preat, T. et al. (2011). Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. *Proc. Natl. Acad. Sci. USA* **108**, 834-839. doi:10.1073/pnas.1010930108

Rohwedder, A., Wenz, N. L., Stehle, B., Huser, A., Yamagata, N., Zlatic, M., Truman, J. W., Tanimoto, H., Saumweber, T., Gerber, B. et al. (2016). Four

individually identified paired dopamine neurons signal reward in larval *Drosophila*. *Curr. Biol.* **26**, 661-669. doi:10.1016/j.cub.2016.01.012

Ryvkin, J., Bentzur, A., Zer-Krispil, S. and Shohat-Ophir, G. (2018). Mechanisms underlying the risk to develop drug addiction, insights from studies in *Drosophila* melanogaster. *Front. Physiol.* **9**, 327. doi:10.3389/fphys.2018.00327

Saumweber, T., Husse, J. and Gerber, B. (2011). Innate attractiveness and associative learnability of odors can be dissociated in larval *Drosophila*. *Chem. Senses* **36**, 223-235. doi:10.1093/chemse/bjq128

Scaplen, K. M. and Kaun, K. R. (2016). Reward from bugs to bipeds: a comparative approach to understanding how reward circuits function. *J. Neurogenet.* **30**, 133-148. doi:10.1080/01677063.2016.1180385

Scherer, S., Stocker, R. F. and Gerber, B. (2003). Olfactory learning in individually assayed *Drosophila* larvae. *Learn. Mem.* **10**, 217-225. doi:10.1101/lm.57903

Schleyer, M., Reid, S. F., Pamir, E., Saumweber, T., Paisios, E., Davies, A., Gerber, B. and Louis, M. (2015). The impact of odor-reward memory on chemotaxis in larval *Drosophila*. *Learn. Mem.* **22**, 267-277. doi:10.1101/lm.037978.114

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr. Biol.* **16**, 1741-1747. doi:10.1016/j.cub.2006.07.023

Schultz, W. (2007). Multiple dopamine functions at different time courses. *Annu. Rev. Neurosci.* **30**, 259-288. doi:10.1146/annurev.neuro.28.061604.135722

Schultz, W. (2015). Neuronal reward and decision signals: from theories to data. *Physiol. Rev.* **95**, 853-951. doi:10.1152/physrev.00023.2014

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. and Heisenberg, M. (2003). Dopamine and Octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J. Neurosci.* **23**, 10495-10502. doi:10.1523/JNEUROSCI.23-33-10495.2003

Selcho, M., Pauls, D., Han, K.-A., Stocker, R. F. and Thum, A. S. (2009). The role of dopamine in *Drosophila* larval classical olfactory conditioning. *PLoS ONE* **4**, e5897. doi:10.1371/journal.pone.0005897

Seugnet, L., Suzuki, Y., Vine, L., Gottschalk, L. and Shaw, P. J. (2008). D1 receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in *Drosophila*. *Curr. Biol.* **18**, 1110-1117. doi:10.1016/j.cub.2008.07.028

Srivastava, D. P., Yu, E. J., Kennedy, K., Chatwin, H., Reale, V., Hamon, M., Smith, T. and Evans, P. D. (2005). Rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. *J. Neurosci.* **25**, 6145-6155. doi:10.1523/JNEUROSCI.1005-05.2005

Thane, M., Viswanathan, V., Meyer, T. C., Paisios, E. and Schleyer, M. (2019). Modulations of microbehaviour by associative memory strength in *Drosophila* larvae. *PLoS ONE* **14**, e0224154. doi:10.1371/journal.pone.0224154

Thum, A. S. and Gerber, B. (2019). Connectomics and function of a memory network: the mushroom body of larval *Drosophila*. *Curr. Opin. Neurobiol.* **54**, 146-154. doi:10.1016/j.conb.2018.10.007

Tomita, J., Ueno, T., Mitsuyoshi, M., Kume, S. and Kume, K. (2015). The NMDA receptor promotes sleep in the fruit fly, *Drosophila* melanogaster. *PLoS ONE* **10**, e0128101. doi:10.1371/journal.pone.0128101

Ueno, T. and Kume, K. (2014). Functional characterization of dopamine transporter in vivo using *Drosophila* melanogaster behavioral assays. *Front. Behav. Neurosci.* **8**, 303. doi:10.3389/fnbeh.2014.00303

Verlinden, H. (2018). Dopamine signalling in locusts and other insects. *Insect Biochem. Mol. Biol.* **97**, 40-52. doi:10.1016/j.ibmb.2018.04.005

Waddell, S. (2013). Reinforcement signalling in *Drosophila*; dopamine does it all after all. *Curr. Opin. Neurobiol.* **23**, 324-329. doi:10.1016/j.conb.2013.01.005

Wang, Y., Pu, Y. and Shen, P. (2013). Neuropeptide-gated perception of appetitive olfactory inputs in drosophila larvae. *Cell Rep.* **3**, 820-830. doi:10.1016/j.celrep.2013.02.003

Weiglein, A., Gerstner, F., Mancini, N., Schleyer, M. and Gerber, B. (2019). One-trial learning in larval *Drosophila*. *Learn. Mem.* **26**, 109-120. doi:10.1101/lm.049106.118

Wicker-Thomas, C. and Hamann, M. (2008). Interaction of dopamine, female pheromones, locomotion and sex behavior in *Drosophila* melanogaster. *J. Insect Physiol.* **54**, 1423-1431. doi:10.1016/j.jinsphys.2008.08.005

Widmann, A., Eichler, K., Selcho, M., Thum, A. S. and Pauls, D. (2018). Odor-taste learning in *Drosophila* larvae. *J. Insect Physiol.* **106**, 47-54. doi:10.1016/j.jinsphys.2017.08.004

Xiao, N. and Venton, B. J. (2015). Characterization of dopamine releasable and reserve pools in *Drosophila* larvae using ATP/P2X$_2$ -mediated stimulation. *J. Neurochem.* **134**, 445-454. doi:10.1111/jnc.13148

Yamamoto, S. and Seto, E. S. (2014). Dopamine dynamics and signaling in *Drosophila*: an overview of genes, drugs and behavioral paradigms. *Exp. Anim.* **63**, 107-119. doi:10.1538/expanim.63.107

Yamamoto, K. and Vernier, P. (2011). The evolution of dopamine systems in chordates. *Front. Neuroanat.* **5**, 21. doi:10.3389/fnana.2011.00021

Yellman, C., Tao, H., He, B. and Hirsh, J. (1997). Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated *Drosophila*. *Proc. Natl. Acad. Sci. USA* **94**, 4131-4136. doi:10.1073/pnas.94.8.4131

Zhang, S., Yin, Y., Lu, H. and Guo, A. (2008). Increased dopaminergic signaling impairs aversive olfactory memory retention in *Drosophila*. *Biochem. Biophys. Res. Commun.* **370**, 82-86. doi:10.1016/j.bbrc.2008.03.015