The content of olfactory memory in larval *Drosophila*

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Die vorgelegte Dissertation besteht aus drei Publikationen und zwei für die Veröffentlichung vorbereiteten Manuskripten, sowie einer zusätzlichen „Allgemeinen Einleitung und Diskussion“. Die Mitwirkung der Co-Autoren jeder Publikation werden auf den folgenden Seiten dargestellt.

Ich versichere, dass ich diese Dissertation in allen Teilen selbständig angefertigt habe und dazu keine anderen als die angegebenen Hilfsmittel benutzt habe.

Alle aus der Literatur entnommenen Textstellen sind als solche gekennzeichnet.

Die vorliegende Arbeit wurde weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt. Zuvor habe ich keine akademischen Grade erworben oder versucht zu erwerben.

Würzburg, den

Dushyant Mishra Prof. Dr. Bertram Gerber

Structure of the thesis

This thesis consists of 2 parts. Part I deals with a behavioural account of how intensity and quality, the two defining features of an odour, are perceived in larval *Drosophila.* It contains three chapters, corresponding to one manuscript prepared for publication and two publications. The manuscript deals with odour intensity learning, the first publication provides a behaviour based estimate of odour similarity in larval *Drosophila*, and the second publication shows for two odours (3-octanol and 1-octene-3-ol) that perceptual differences between these odours can either be ignored after non-discriminative training (generalization), or accentuated by odour-specific reinforcement (discrimination).

Part II contains two chapters, corresponding to one manuscript prepared for publication and one publication. The manuscript studies cognitive- enhancement in larval *Drosophila* where I show that food supplementation with dried roots of *Rhodiola rosea* dose-dependently improves odour- reward associative learning in larval *Drosophila*. The publication deals with local loss of function of Synapsin and its consequences for associative plasticity of larval *Drosophila*.

In addition, I present a 'General Introduction and Discussion' to give the reader an overview of the background and implications of this thesis. Also included at the end is a brief summary of the studies presented in this thesis.

This work would not have been possible without the effort of many people, and the supervision of my mentor Dr. Bertram Gerber. I sincerely acknowledge the co- authors of each manuscript, whose contributions are explicated below.

Chapter I.1

Mishra D., Chen Yi., Yarali A., Gerber B.: Olfactory memories can be intensity- specific in larval *Drosophila*.

DM, BG and AY conceived the research and designed the experiments. DM and YC performed the experiments and DM analyzed the data. DM, AY and BG wrote the paper.

Chapter I.2

Chen Yi., Mishra D., Schmitt L., Schmuker M., Gerber B. (2010): A behavioural odour-similarity 'space' in larval *Drosophila, Chemical Senses, 36(3), 237- 249.*

YC, DM and BG conceived the research and designed the experiments. YC, LS and DM performed the behaviour experiments. Specially, DM performed the experiments to describe the dose effect curves for different odours which subsequently lead to adjust the odour dilutions for equal learnability. YC, MS and BG analyzed the data. YC and BG wrote the paper.

Chapter I.3

Mishra D., Louis M., Gerber B., (2010): Adaptive adjustment of the generalization-discrimination balance in larval *Drosophila*, *J. Neurogenetics*, *24(3), 168-175.*

DM, and BG conceived the research. DM and BG designed the experiments. DM performed the experiments and analyzed the data. DM and BG wrote the paper with inputs from ML.

Chapter II.1

Lushchak O.[=], Mishra D.[=], Haberkern H., Eschbach C., Lushchak V., Niewalda T., Gerber K., Koblowsky M., Gerber B.: 'Cognitive enhancement' of associative function in larval *Drosophila* by *Rhodiola rosea* food supplementation.

OL, DM, VL and BG conceived the research. OL, DM and BG designed the experiments. OL, DM, KG, MK performed the learning experiments concerning the larval *Drosophila* and their controls. KG and TN helped to make the *Rhodiola* enriched fly food vials. CE and HH performed and analyzed the larval tracking experiments. DM analyzed the data. DM and BG wrote the paper.

Chapter II.2

Michels B., Chen Yi., Tanimoto H., Mishra D., Engmann O., Saumweber T., Gerber B., (2011): Site and mode of Synapsin action in associative learning, *Learn. Mem. 18(5):332-44.*

BM, HT and BG conceived the research and designed the experiments. BM, OE and TS performed the Synapsin rescue experiments. YC performed behavioural experiments with PKA-site mutated transgenes. DM performed behavioural and immunohistochemical analysis with Synapsin- RNAi lines. BM and BG wrote the paper.

Wuerzburg, den

Dushyant Mishra Prof. Dr. Bertram Gerber

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II.2. Loss of function of Synapsin

General introduction & discussion

A stimulus percept is born when the brains try to make "sense" of what the senses are telling them (e.g. identity and intensity of an odour). A common problem among all sensory systems is how do brains form such percepts by processing relevant stimulus information? Our current understanding of such processing is far from complete.

Studying this problem in the realm of olfaction makes it even more interesting given the central role smells play in the life of animals as they, e.g., direct the search for food or mates, signal conspecifics or predators, and mark territory. How do brains deal with smells to generate the appropriate behaviour? The common principles of neuronal olfactory processing across species (Ache and Young, 2005; Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999) suggest that nature has found an optimal and unique solution. This solution, once revealed in detail, can also be used for processing data from artificial electronic noses, which would act as systems for automated detection and classification of odours providing a wide array of applications in quality control, formulation and reformulation of products, in robotics, medical diagnostics, detection of counterband, and unfortunately also in warfare. On a basic research level, however, a better understanding of stimulus perception and processing can also be a study case of psychological processes such as memory or expectation and their underlying neurobiology.

I am specifically interested to study the principles of olfactory processing in the larvae of the fruit fly, *Drosophila melanogaster*. This model beautifully combines the genetic accessibility of adult *Drosophila* with a substantially lower level of neuronal complexity. Specifically, the larval olfactory system implements the same general layout (Gerber et al., 2008) as its adult counterpart but with fewer neurons (Stocker, 2001; Python and Stocker, 2002a). Studying this 'minimal' olfactory system thus gives us the chance to understand the essence of olfactory processing, useful for technical applications.

In terms of evolutionary conservation (Silbering et al., 2010; Ache and Young, 2005; Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999) between mammals and insects (not necessarily in terms of their common ancestory but most likely in terms of

both phyla facing similar functional constraints leading to similar olfactory circuit design) may lead one to the principles that can find parallels between *Drosophila* larvae and likely also in higher olfactory systems.

In this introduction to my thesis, I first briefly go over the larval olfactory pathway and explain how it supports olfactory associative learning; and then show how such associative learning can be used to "spy on" the larvae's olfactory percepts by focusing on three questions which also form the first part of this thesis:

(1) How is the odour intensity integrated into the olfactory percept?

(2) How do the physico- chemical properties of odours, the way they activate the olfactory pathway and finally the qualitative percepts they induce relate to each other?

(3) Can olfactory percepts be adjusted adaptively?

To understand the mechanisms of olfactory processing one needs an understanding of the underlying neural circuitry. The olfactory system of *Drosophila* larvae has been studied extensively and has been described in a lot of detail in terms of neural circuits and processing of odours. The main "noses" of *Drosophila* larvae are the bilateral dorsal organs in the head housing 21 olfactory sensory neurons (OSN) on each side. Each OSN expresses one of 25 identified ligand specific olfactory receptor genes (Or). What is note worthy here is that probably the reason why the number of ORs exceeds the total number of OSNs is because even though the majority of the neurons express one Or gene, two OSNs were shown to express two Or gene pairs Or33b/ Or47a and Or94a/ Or94b (Fishilevich et al., 2005). This expression of ORs determines the OSN's olfactory receptive field. It is thus important to note that the ligand profiles of ORs are thought to be relatively broad and overlapping between OSNs i.e. the ORs can show a wide variety to responses from being narrowly tuned to specific odourants to being broadly tuned to structurally similar odourants, in addition they can show both excitatory and inhibitory responses to various odourants (Hallem et al. 2006). All insect OSNs express the co-receptor Or83b, which is necessary for trafficking the ORs to the sensory dendrites (Larsson et al., 2004; Neuhaus et al., 2005; Benton et al., 2006). A striking difference of the insect ORs to that of mammalian ORs is that while the mammalian receptors are

mostly G- protein coupled receptors (GPCRs) and uses the cAMP second messenger signaling pathway, the insect odourant receptors are not related to mammalian GPCRs in terms of their genetic sequences and have an inverted membrane topology with respect to mammalian receptors. In addition they mainly function via an ionotrophic mechanism by forming an odour- gated ion channel with Or83b dimerizing with the odourant receptor (Silbering et al. 2010; Sato et al. 2008; Wicher et al. 2008).

Each of the 21 OSNs project to a single stereotypical glomerulus in the antennal lobe, giving output to one or a few stereotypical projection neuron(s) (PN) (Python and Stocker, 2002a; Marin et al., 2005). Each of these ~ 21 PNs in turn receives input at a single glomerulus (Ramaekers et al., 2005). Superimposed on this almost one-to-one OSN-PN connectivity, lies a multi-glomerular network of GABAergic inhibitory local interneurons (LNs) (Python and Stocker, 2002b; while there is no clear evidence of excitatory LNs in the larvae there is clear evidence of existence of both inhibitory GABAergic and excitatory cholinergic LNs in the adult *Drosophila,* Asahina et al. 2009; Shang et al. 2007; Ng et al. 2002). PNs' olfactory receptor fields are thus shaped by the direct excitatory inputs from the respective OSNs and by the lateral inhibition from other LNs. Each PN projects through bifurcating axons on the one hand to a stereotyped region of the lateral horn (LH) and on the other hand to one or two of the ~ 35 glomeruli in the mushroom body (MB) calyx. One calycal glomerulus in turn receives input from one or few PNs. Therefore in general the PNs establish a one-to-one connection between antennal lobe glomeruli and calycal glomeruli. At each calycal glomerulus then, 30- 180 of the 600 mushroom body Kenyon cells (KC) receive input from a given PN (for a calculation of this estimate see Gerber and Stocker, 2007). Thus, looking from the PNs' point of view, PN-KC connectivity is divergent. On the other hand, each KC innervates either a single or a set of randomly selected ~ 6 calycal glomeruli (Masuda-Nakagawa et al., 2005; Ramaekers et al., 2005). Therefore, from the KCs' point of view, PN-KC connectivity is convergent. Altogether, calyx houses an intricate mixture of convergence and divergence from the PNs to the KCs. How does olfactory associative learning come about in such a network?

As we just saw in the previous paragraphs that the odour- induced pattern of activity gets transformed at each step of processing, our knowledge of these transformations is far from complete; furthermore we know very little about the processing within the LH and

downstream of the KCs. Even if we did have a complete account of these, however, we would be wrong to call the odour- induced pattern of activity along the olfactory pathway as that odour's percept, since the context larvae find themselves in, as well as their needs and goals surely also come into play. Thus, the larvae's olfactory behaviour, rather than physiology, gives us a handle on their perception of the odours. Indeed, various kinds of questions pertaining to larval odour percepts have been addressed using behavioural paradigms (eg: cross adaption: Boyle and Cobb 2005; odour masking: Kreher et al 2008). Here in this thesis I focus on the use of associative learning to 'spy' on the odour percept of the larvae. A particularly well-established paradigm to study the larval odour percept is the odour- reward associative learning (Hendel et al., 2005; Neuser et al., 2005; Scherer et al., 2003), which uses a reciprocal training design: One group of animals receives an odour A with sugar as reward, whereas they experience another odour, B, without reward (A+/ B). A second group of animals are trained reciprocally as A/ B+. After training, each group is given the choice between the two odours, A and B. For each group, a preference score (PREF) is calculated as

$$PREF = (\#_A - \#_B) / \#_{Total} \quad (1)$$

where # designates the number of larvae which chose the corresponding odour. PREF values thus range from -1 to 1; positive values indicate approach towards odour A, negative ones reflect avoidance from A. An associative performance index (PI) is then calculated based on the difference in odour preference between the reciprocally trained groups:

$$PI = (PREF_{A+ / B} - PREF_{A / B+}) / 2 \quad (2)$$

In this equation, the subscripts of PREF indicate the respective training regimen. PI values range from -1 to 1, positive values indicating conditioned approach, whereas negative values indicate conditioned avoidance. Importantly, for either reciprocal regimen, the sequence of rewarded and non-rewarded odours is balanced across repetitions of the experiment.

Using the same reciprocal design, larvae can also be trained to avoid an odour, which during training was paired with bitter or strongly salty taste (Gerber and Hendel, 2006), or electric shock (Khurana et al., 2009, Pauls et al., 2010). Importantly, in the case of aversive gustatory reinforcers (quinine or HIGH salt) aversive olfactory memories

translate into larval behaviour only in the presence of the trained reinforcer or something at least equally bad (Gerber and Hendel, 2006).

Saumweber et al., 2011 have shown that such a two-odour reciprocal design might not be a best choice to assess the learnability of individual odours specifically for experiments concerning odour generalization where one odour has to be trained and a non-trained odour has to be tested or for intensity generalization type of experiments where one odour at a given intensity is trained and during the test the very same odour but at a different intensity is used. To overcome these handicaps, a one- odour version of such reciprocal training regime is introduced.

In addition to these reciprocal learning paradigms, there are those that use non- reciprocal training regimes (Honjo and Furukubo- Tokunaga, 2005). These however may under certain circumstances be confounded because the performance scores could be influenced by effects like habituation, adaption, changes in odour concentrations and animal motivation.

The molecular and neuronal mechanisms of such larval olfactory learning are beginning to emerge. Namely, in addition to being the last station along the odour processing pathway the mushroom body KCs also receive aminergic reinforcement signals (*Drosophila* larvae: Selcho et al., 2009; Schroll et al., 2006; adult *Drosophila*: Riemensperger et al., 2005; for honeybees see: Hammer et al., 1993; Hammer et al., 1998). Specifically these studies showed that octopaminergic/ tyrminergic neurons are carriers of information for appetitive reinforcement while dopaminergic neurons carry information for aversive reinforcements. Given this convergence of olfactory information via the olfactory network and reinforcement information via the aminergic reinforcement neurons, MB KCs seems to act as coincidence detectors for these two types of stimuli. The KCs in turn form synapses with neurons that are concerned with premotor areas. It has been argued that the learned olfactory responses of the larvae take place via this network (Riemensperger et al. 2011; Pauls et al., 2010; Gervasi et al., 2010; Aso et al. 2010).

Studying these mechanisms of learning and memory forms the second part of my thesis which deals with the following two topics:

(1) How and why does cognitive enhancement in larval *Drosophila* occur by feeding *Rhodiola rosea* roots?

(2) How does the local loss of function of the pre- synaptic protein Synapsin affect olfactory memory and learning?

Olfactory percepts of larval *Drosophila*:

How is odour intensity integrated into the odour percept?

Olfactory discriminative abilities of an animal can in principle rely on either or both of two primary properties of the odour namely its identity and its intensity. While the coding of odour identity is often proposed to be combinatorial across the range of activated OSNs and the ensuing activity patterns along the olfactory pathway, the question remains how the intensity of an odour is coded? In larvae, intensity clearly affects innate behaviour towards odours, as was shown by Louis et al. (2008), where they showed that in an odour gradient *Drosophila* larvae are able to compute local odour intensities in terms of both space and time and subsequently orient themselves towards the odour source.

Here I want to answer a more interesting question and ask whether larvae recognize a particular odour intensity as such and consequently form intensity-specific memories? As a prelude to such a study, using appetitive odour- taste associative learning in larval *Drosophila*, I first describe the dose- effect curves of learnability across odour intensities for four different odours (*n*-amyl acetate, 3-octanol, 1-octene-3-ol and benzaldehyde). Odour intensities are then chosen such that larvae are trained at intermediate odour intensity, but are tested for retention with either that trained intermediate odour intensity, or with respectively HIGHer or LOWer intensities. I observe a specificity of retention for the trained intensity for all four odours used. This study follows the same experimental strategy advocated by Yarali et al., 2009 for adult *Drosophila*. In that study, using odour-shock learning with four kinds of odours (3-octanol, n-amylacetate, 4-methylcycohexanol and benzaldehyde), the authors showed for three of the four odours that there was a specificity of retention for the trained intensity. For benzaldehyde they could not find any intensity specific learning. Thus, in general, both adult and larval *Drosophila* recognizes particular odour intensities as such, and form odour intensity-specific associative

memories. The discrepancy between the two developmental stages with respect to the odour benzaldehyde awaits explanation. Also the odour 4- methylcyclohexanol was shown to be learned well in case of adult *Drosophila* (Yarali et al., 2009) while larvae seemed to be behaviourally little responsive towards this odour (Chapter I.1 Fig. S2C). Contemplating these discrepancies in general tempts one to speculate that these could arise based on different receptor repertoires of the two life stages (Hallem et al., 2006, Kreher et al., 2008) given the fact that the general olfactory circuit architecture is rather similar between the larvae and the adult flies (Gerber et al., 2008).

There has been other studies in adult *Drosophila* which looked at the intensity learning using differential training regimes (for a discussion see Yarali et al. 2009; Xia & Tully 2007; Masek & Heisenberg 2008; see also Borst 1981 with respect to sugar reward learning) . Essentially what was done in these studies was that one group of flies received during training a low intensity of the odour with shock, whereas a high odour intensity was without shock (low- shock / high); another group of flies was trained reciprocally as high- shock / low. Both groups were then tested for their preference between the low and the high odour intensities. As the flies trained high- shock / low avoided the high intensity more than those trained as low- shock / high, the authors concluded that intensity-learning had taken place. Such interpretation may be confounding: That is, flies from both groups may simply avoid the high intensity during the test according to the strength of the memory that has been formed during training. Now when one looks at the case of benzaldehyde the odour which did not show intensity specific learning in adult *Drosophila* for example (Yarali et al. 2009, see Fig. 4 in that paper), training with a low odour intensity results in weaker memory than training with a high odour intensity, the preferences of the two reciprocally trained groups would then be different, leading to an incorrect conclusion regarding intensity learning. Thus, differential training- differential testing designs may be misleading in terms of intensity learning.

A behavioural odour-similarity 'space'

From a biological perspective, maybe the most general statement about odour perception is that it must be ABOUT the odour. That is, there must be a biologically meaningful relation between the physico- chemical properties of the odours and the behavioural tendencies they impose (as well as the psychological experiencing of this relation). The

organ by which this relation is ensured is the brain, and although the pattern of activity the odour induces along the olfactory pathway IS not the percept of that odour, perception must be BASED on it. Therefore, it is reasonable to look for the relation between the physico- chemical properties of odours and olfactory behaviour, and to try to describe the way this is brought about in the brain. Contrast the question "How do you perceive this odour?" a question you cannot reasonably ask in animal studies, with the question "Do you regard this odour AS THE SAME AS the one you were trained with?" Keeping this view in mind I joined hands with Yi- chun Chen to answer these question.

A major distinguishing feature in this study is that learnability of the odours used was adjusted i.e. I adjusted odour dilutions for equal learnability based on a behavioural rather than a physical basis contrary to what is mentioned in other studies (Cobb and Domain 2000; Boyle and Cobb 2005; Kreher et al. 2008) where they choose the same odour dilution for all the odours based on physical and chemical properties of the odours. Here I adjusted the odour dilutions for equal learnability based on the dose effect curves for these odours. This is an important aspect for such an analysis because asymmetric measurements of odour perception would leave these results to be incomparable to physico- chemical based measurements of odour molecules.

To provide a behaviour- based estimate of odour similarity we used four types of experiments:

(i) We trained the larvae to associate an odour with food, and then test whether they would regard another odour as the same as the trained one (generalization).

(ii) We trained the larvae to associate an odour with food, and test whether they prefer the trained odour against a novel, non-trained one.

(iii) We trained the larvae differentially to associate one odour with food, but not the other one, and test whether they prefer the rewarded against the non- rewarded odour.

(iv) In an experiment like (iii), we tested the larvae after a 30 min-break.

This yielded a combined, task-independent estimate of perceived difference between odour-pairs. Comparing these perceived differences to published measures of physico-chemical difference (Schmuker and Schneider 2007; Haddad et al. 2008) revealed a weak correlation. The exceptions are 3-octanol and benzaldehyde, which are distinct in

published accounts of chemical similarity, and in terms of their published sensory representation, but are consistently regarded as the most similar of the ten odour pairs employed here. It thus appears as if at least some aspects of olfactory perception are 'computed' in post-receptor circuits on the basis of sensory signals.

Indeed, regarding the brain as the mediator of the relation between physico- chemical and similarity judgements, For instance in honeybees, Guerrieri et al., 2005 have tried to draw parallels between odour similarity and chemical characteristics of an odour such as functional chemical group or the carbon chain length and showed that a honeybee's olfactory space codes for such physical dimensions of an odourant. Further they showed that the perceptual distances in honeybees correlate well with the physiological distances determined by optophysiological recordings of the AL.

In adult *Drosophila,* too, Niewalda et al. 2011 (in prep.) have shown regarding four odours benzaldehyde (B), 3-octanol (O), 4-methylcyclohexanol (M), *n*-amylacetate (A) that O and A are the most similar odours in terms of their perceptual distances (this was revealed by a normalized measure of four different behavioural tasks thus making these percept distances task independent) and their psysico- chemical properties. Further, when they compared these perceptual distance scores for these odours with calcium imaging analysis they did not observe any correspondence at the level of sensory neurons but did see a correspondence at the level of projection neurons (for odours O and A), this revealed a novel role of the AL in odour categorization. This study in the larvae thus compliments the study done by Niewalda et al. 2011 (in prep.) in adult *Drosophila.*

Odour percepts can be adaptively adjusted

As the perception serves the purpose of organizing the appropriate kind of behaviour, it is reasonable to ask whether it changes according to the demands of the behavioural task at hand. I provide a case of such adaptiveness of olfactory perception in *Drosophila* larvae. In particular I focus on whether the larval olfactory system is flexible enough to either generalize or discriminate between two odours, dependent on the task. To test this hypothesis I used two odourants 1-octen-3-ol (1-OCT-3-OL) and 3-octanol (3-OCT) which have been shown to activate a similar subset of larval ORs (Kreher et al. 2008). At high odour concentrations five broadly tuned ORs get activated by these odours (the

receptors encoded by the Or13a, Or35a, Or45a, Or47a and Or85c genes). Among these, two displays a particular affinity for both of these odours namely the receptors encoded by Or13a and Or85c genes. What is interesting in these receptors is that the Or13a encoded OR has higher affinity for 1-OCT-3-OL than 3-OCT while the Or85c encoded OR has higher affinity for 3-OCT than for 1-OCT-3-OL.

Using the odour- sugar reward learning paradigm for larval *Drosophila* I show for these two odours that depending on the task larvae do or do not make a difference between these odours i.e. perceptual differences between these odours can either be ignored after non-discriminative one- odour training (generalization), or if the larvae are trained to specifically make a difference between the two odours by discriminative training they are able to show conditioned preference for the rewarded odour with respect to the un-rewarded odour. On the other hand if the larvae are trained non- discriminatively but during the test asked to choose between the odours, no conditioned behaviour is observed. Therefore it could be concluded that only discriminative training confers an odour-specific memory trace, where as one- odour training does not.

Contemplating these findings leads one to think that if both these odours would have induced exactly the same kind of peripheral activity in the OSNs there would not be any difference between them by discriminative reinforcement, leading to the conclusion that by default there is a small but salient difference in the processing of these two odours. I also observed that anosmic $Or83b^{1}$ mutants have lost these faculties, indicating that this adaptive adjustment between generalization- discrimination is taking place downstream of Or83b expressing sensory neurons. In future it would be interesting to observe how the difference in processing of these two odours at the level of OSNs leads to the observed adaptive adjustment of the generalization- discrimination balance.

Mechanisms of learning and memory in larval *Drosophila*:

'Cognitive-enhancement' in larval Drosophila

Here I look at possible cognitive enhancing effects of a plant root called *Rhodiola rosea*. Traditionally this plant's roots have been used by humans as a means to enhance memory and remedy age related memory decline. It has been previously shown in *C. elegans* that

food supplementation with *Rhodiola rosea* preparations promotes resistance to stress and increases life span (Wiegant et al., 2009) and in rats it has been shown to increase mnemonic function (Petkov et al., 1986). I show here that food supplementation with dried roots of *Rhodiola rosea* dose- dependently improves odour- reward associative learning in larval *Drosophila*. Supplementing fly food with commercially available tablets or extracts, however, does not have a 'cognitive enhancing' effect, enabling us to differentiate between the effective substances in the root versus these preparations. It is noteworthy that this improvement is neither due to alterations in those sensory and motor functions that are relevant for the employed odour- reward learning task, nor to alterations in general locomotor parameters, nor to alterations induced by reward- or odour- exposure *per se*.

The ability to learn and remember provides obvious advantages to the animal. But it is a costly affair to maintain the molecules and cellular structures needed for learning and memory. In addition, in nature one also finds enough genetic variability to allow for evolution of abilities related to associative function (*Drosophila*: Mery and Kawecki, 2002; blow flies: McGhuire et al. 1977; honey bees: Chandra et al. 2000). Thus it is plausible that the abilities to learn and remember do not function at its optimal but function at a sub- optimal level which confers the animal with enough evolutionary fitness and also lower costs of maintaining the memory molecules. Indeed in fruit flies such costs of better learnability have been previously observed in a series of experiments done by Mery and Kawecki. In one of their studies they artificially selected for fly lines with increased learning scores based on aversive learning of oviposition substrate choice (Mery and Kawecki, 2002) and observed that the larvae of these lines showed reduced competition under low food availability conditions (Mery and Kawecki, 2003). In another study they showed that when these enhanced learning fly lines under mild food limiting conditions are subjected to alternating- conditioning treatments which require learning for substrate choice, the fly line with improved learning ability exposed to such conditioning had lower egg- laying rates than the unselected flies and also to the improved learning lines not exposed to such conditioning regimen (Mery and Kawecki, 2004). Finally, they showed that flies trained to produce long- term memory died sooner in the absence of food and water than the control flies. All these experiments thus suggest that learning indeed comes with a fitness cost relating to energy expenditure. In these circumstances the roots of *Rhodiola* could confer the animal with increased associative function on the one

hand by lowering the costs of maintaining memory molecules and/ or by helping in terms of improving the fitness costs; for example by providing longer life spans (Burger et al. 2008), increasing larval competition under food limiting conditions or last but not the least allowing the flies to lay more number of eggs. A possible downside of an enhanced associative function offered by *Rhodiola* could be that this enhanced learning ability distorts the balance between learning and forgetting and thus lead to superstitious behaviour on the part of the organism e.g. learning non- context related cues. Thus it might be interesting to devise experimental procedures which enable us to measure the memory decay kintetics of *Rhodiola* treated *Drosophila* larvae to see if such balance between learning and forgetting is indeed distorted.

In future it would be interesting to uncover genetic approaches which allow for gain-of-function to be detected in mutants as these could reveal neural pathways necessary for such increase in associative functions. *Drosophila* as a genetically accessable organism should now allow accelerated analyses of the molecular mechanism(s) that underlie this 'cognitive enhancement'. Lastly, it should be interesting to see whether *Rhodiola* roots are able to compensate ageing- related or pathological weaknesses of associative function in flies.

Local loss of function of Synapsin

I joined the project which seeks to investigate where and how Synapsin, an evolutionarily conserved presynaptic phosphoprotein which is associated with synaptic vesicles, functions in associative plasticity of larval *Drosophila*. Synapsins have been known to regulate the neurotransmitter release by controlling the number of synaptic vesicles available to release the neurotransmitter (Hilfiker et al., 1999). This is done by association of Synapsin with the cytoskeleton actin mesh and the synaptic vesicle, an arrangement that thus gives rise to the so called “reserve pool” of synaptic vesicles. Upon phosphorylation, the synaptic vesicles are released by Synapsin from this reserve pool and translocated to the active zone where these vesicles can release the neurotransmitter upon subsequent activation of the cell (Hosaka et al., 1999; Chi et al., 2001; Menegon et al., 2006; Gitler et al., 2008). It has already been shown that a lack of Synapsin in the syn^{97} deletion mutant entails a 50 % defect in associative learning in larval *Drosophila* (Michels et al., 2005; for a corresponding phenotype in adult flies see Godenschwege et. al. 2004;

Knapek et al. 2010). In this endeavour we showed that a Synapsin- dependent memory trace can be pinpointed to the mushroom bodies of larval *Drosophila*. On the molecular level, our data assigned Synapsin as a behaviourally- relevant effector of the AC-cAMP-PKA cascade. My contribution here was to show that an RNAi-mediated reduction of Synapsin leads to an impairment in learning of *Drosophila* larvae. I used a combined behavioural and genetic approach to achieve this. As a tool to see this RNAi mediated reduction I used the GAL4- UAS ectopic expression system (Brand and Perrimon, 1993). The system in principle allows the expression of any transgene, in any cellular pattern, at any time (for review Brand et al. 1995). Here, I used a UAS-RNAi-SYN II.6 strain (B. Michels, Universität Würzburg) to see whether it supports a knock-down of the Synapsin protein when crossed with an appropriate Gal4-driver line. Indeed, by a pan- neuronal knock- down of Synapsin protein the experimental larvae performed poorly in comparison to control larvae in the learning task. Thus the associative defect in the syn^{97}-mutant (Fig. 1K; Michels et al. 2005) could be phenocopied by an RNAi- mediated knock- down of Synapsin.

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Part I.

Olfactory percepts of larval *Drosophila*

Chapter I.1

How is odour intensity integrated into the odour percept?

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Olfactory memories are intensity-specific in larval *Drosophila*

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Running head

Odour-intensity learning

Key Words

Learning, Olfaction, Taste, Odour Intensity, *Drosophila*

ABSTRACT

Learning can rely on stimulus quality, stimulus intensity or a combination of these. Regarding olfaction, the coding of odour quality is often proposed to be combinatorial along the olfactory pathway, and working hypotheses are available concerning short-term associative memory trace formation of odour quality. However, it is less clear how odour intensity is coded and whether olfactory memory traces include information about the intensity of the learnt odour. Using odour-sugar associative conditioning in larval *Drosophila*, we first describe the dose-effect curves of learnability across odour intensities for four different odours (*n*-amyl acetate, 3-octanol, 1-octene-3-ol, benzaldehyde). We then choose odour intensities such that larvae are trained at intermediate odour intensity, but are tested for retention with either that trained intermediate odour intensity, or with respectively HIGHer or LOWer intensities. We observe a specificity of retention for the trained intensity for all four odours used. This adds to appreciate the richness in 'content' of olfactory memory traces, even in a system as simple as larval *Drosophila*, and to define the demands on computational models of associative olfactory memory trace formation.

INTRODUCTION

Stimuli can differ in kind and/or intensity. On the sensory level, stimulus kind could be coded by the kind of receptor activated, and the level of activation of the receptor could code for the intensity of the stimulus. If so, processing of stimulus kind and stimulus intensity would be entangled: One cannot conceive of a receptor that *is* activated, but at *no particular level*. In turn, a given level of activation must always be a *particular receptors´* level of activation. To complicate matters, there are two fundamental ambiguities at the level of receptor activation: First, a particular sub-maximal level of receptor activation may mean that the given stimulus does have e.g. the wavelength preferred by this receptor, but that its intensity is low, or that the intensity is high, but the wavelength is not the preferred one. Second, even if intensity differences were not playing a role, the typical bell-shaped tuning curve of photoreceptor activation across wavelengths would limit discerning whether a particular level of receptor activation relates to a wavelength shorter-than or longer-than the preferred one.

On the perceptual level, however, we are able to distinguish between deviations towards shorter from deviations towards longer wavelength of a visual reference. Also, the entanglement of quality and intensity can to some extent be resolved: It is possible to refer to the grass as just 'green' without specifying the intensity of the visual impression, or to regard downtown New Delhi as just 'loud', without specifying the kind of the auditory impression. Clearly, both the disambiguation of stimulus parameters (shorter *versus* longer wavelength), and the disentanglement of intensity from quality are features of perception, coming about by post-receptor computations. It is one of the more challenging tasks to understand these computations neurobiologically.

In this context, we decided to study intensity-processing in olfactory associative function. That is, olfactory discrimination learning can rely either on intensity differences, quality differences, or both. While the coding of odour quality is often proposed to be combinatorial along the olfactory pathway (see Discussion), and although a fairly explicit working hypothesis about short-term odour-quality memory trace formation is available (see Discussion), it is less obvious how odour intensity is treated. In the present paper, we focus on the question whether odour-intensity information is included in olfactory memory traces.

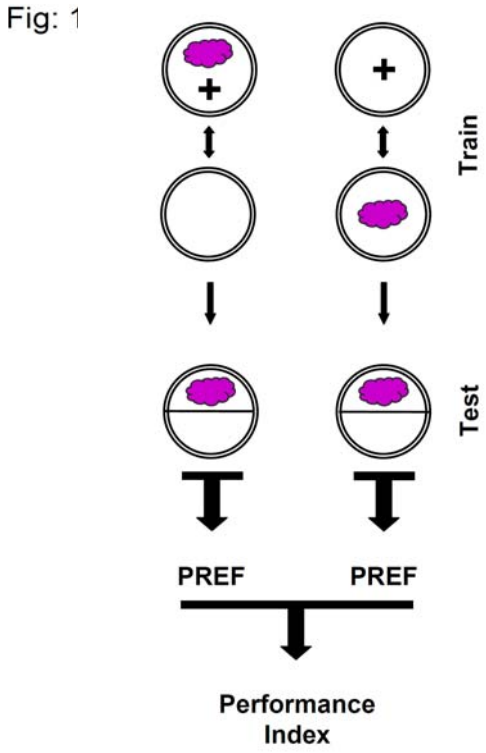


Figure 1

Learning assay

Larvae are trained and tested in groups of 30, using a reciprocal training regimen. At the beginning of training, odour (purple cloud) is presented throughout a Petri dish containing agarose, added with fructose (+). After 5 min, larvae are removed to another dish containing no odour and filled with only agarose, where they also spend 5 min. This cycle of training is repeated three times, using fresh dishes each time. For the test, larvae are placed in the middle of a dish filled with only agarose; on one side, odour is presented, and on the other side no odour is presented. After 3 min, larvae on each half of the dish are counted. Alternately, we train larvae reciprocally, by unpaired presentation of fructose and odour. This then allows subsequent calculation of a performance index (PI) comparing the preference values between the reciprocally trained groups. Note that the sequence of training trials within groups as well as the sidedness of placing these containers is balanced across repetitions of the experiment.

We tackle this issue using odour-sugar associative conditioning in larval *Drosophila* (Fig. 1) (Scherer et al, 2003; Neuser et al, 2005; Saumweber et al, 2011a, b; for review Gerber & Stocker, 2007; Gerber et al, 2009). This is a suitable system for such a study due to its simplicity in terms of cell number, its genetic tractability and the robustness of the paradigm.

Last, but not least, the circuit architecture of the olfactory pathway of the larva (as of insects in general) is functionally analogous to the one in vertebrates (for comparative reviews see Hildebrand & Shepherd, 1997; Strausfeld & Hildebrand, 1999; Korsching 2002; Davis 2004; Ache & Young, 2005; Bargmann 2006; Wilson, 2008, Galizia &

Rössler, 2010), rendering experimental as well as computational studies of insect olfaction potentially inspiring at a broader scale.

Fig: 2

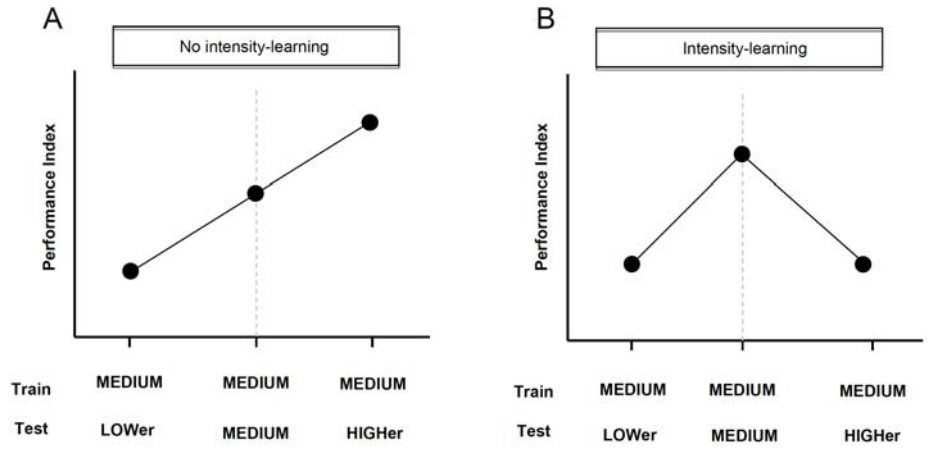


Figure 2

Rational for intensity learning

We train larvae with a MEDIUM odour intensity and during the subsequent test different odour intensities are offered to independent sets of larvae i.e. either the same trained MEDIUM intensity, or a LOWer, or a HIGHer odour intensity. (A) No intensity learning: Accoding to this scenario we would find increased levels of conditioned behaviour when the test intensity is HIGHer than in training, indicating that the intensity parameter is not included in their memory trace. (B) Intensity learning: In this scenario we expect that only when training and testing odour intensities are matching we see the full level of conditioned behaviour indicating that the intensity parameter is included in the memory trace.

Our approach follows the one advocated for adult flies (Yarali et al, 2009; that paper also includes a discussion of alternative approaches by DasGupta & Waddell 2008; Masek & Heisenberg 2008; Xia & Tully, 2007). A distinguishing feature of this approach is that, for each of four different odours, we first describe the dose-effect curves of learnability. This allows choosing odour intensities appropriate for an intensity-generalization type of experiment (see Fig. 2). That is, we train larvae to a MEDIUM intensity, but test them with either a LOWer or a HIGHer intensity of the trained odour. The rational of this experimental design is that if associative testing scores turn out to increase when the testing intensity is HIGHer than the training intensity, this must be because a HIGHer intensity is judged by the larvae as 'more of the trained' odour (Fig. 2A). If, in contrast, the larvae regard a HIGHer intensity as 'something different', we should observe a generalization-decrement for the HIGHer testing condition (Fig. 2B). This latter result

would argue that the memory trace established by the larvae during training is parametrically specific for the trained intensity of the odour.

MATERIALS AND METHODS

Flies

Third-instar, feeding-stage *Drosophila* larvae (5 days after egg laying) of the Canton Special wild type strain are used. The flies are kept in mass culture under a 14-10 h light-dark cycle at 25 ^{o}C and 60-70 % relative humidity. For the learning assay, a spoon-full of medium containing larvae is taken into an empty Petri dish, 30 larvae are collected and washed in distilled water.

Petri dishes

One day prior to the experiment, Petri dishes of 85 mm inner diameter (Sarstedt, Nümbrecht, Germany) are filled either with a solution of 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany), or with 1 % agarose added with 2 M fructose (Roth, Karlsruhe, Germany). Once the agarose has solidified, dishes are covered with their lids and left until the following day.

Learning assay

Learning assays are performed under a fume hood at 21-26 ^{o}C, under room-light from a fluorescent lamp. Larvae are trained and tested in groups of 30, using either of two reciprocal training regimen (for a sketch see Fig. 1). For each regimen, the sequence of training trials is balanced across repetitions of the experiment. For example, at the beginning of training, two odour-filled Teflon containers are placed at opposite sides of a Petri dish containing agarose, added with fructose (Odour +). Larvae are placed in the middle of this dish and left crawling for 5 min. They are then removed to another Petri dish containing two empty Teflon containers (EM) and filled with only agarose, where they also spent 5 min. This cycle of Odour + / EM training is repeated three times, using

fresh Petri dishes each time. At the end of training, larvae are placed in the middle of a Petri dish filled with only agarose. On opposing sides, Teflon containers are placed, one filled with the odour and one empty container; the sidedness of placing these containers is balanced across repetitions of the experiment. After 3 min, larvae on each half of the Petri dish are counted to calculate a preference index (PREF) as:

(1) $$PREF = (\#Odour - \#EM) / \#Total$$

In this formula, # designates the number of larvae on the corresponding side of the dish. PREF values range from -1 to 1; negative values indicate avoidance of the odour, positive values reflect approach. The PREF scores for all experiments are documented in the Supplement.

Alternately, we train larvae reciprocally, that is by unpaired presentations of odour and reward (Odour / EM +). An associative performance index (PI) can then be calculated based on the difference in odour preference between these two reciprocally trained groups (Saumweber, 2007; Selcho et al, 2009; Saumweber et al, 2011a, b):

(2) $$PI = (PREF_{Odour + / EM} - PREF_{Odour / EM +}) / 2$$

The subscripts of PREF indicate the respective training regimen. These associative performance indices thus range from -1 to 1, positive values indicating conditioned approach (appetitive learning); whereas negative values indicate conditioned avoidance (aversive learning).

Odours

As odours, we use 3-octanol (3-OCT), *n*-amyl acetate (AM), 1-octene-3-ol (1-OCT-3-OL), linalool (LIN), 1-octanol (1-OCT) (all from Merck, Darmstadt, Germany; CAS: 589-98-0, 628-63-7, 3391-86-4, 78-70-6, 111-87-5), hexyl acetate (HA), benzaldehyde (BA)

and 4-methylcyclohexanol (MCH) (from Sigma-Aldrich, Steinheim, Germany; CAS: 100-52-7, 589-91-3, 142-92-7). Odours are diluted in paraffin oil (Merck, Darmstadt, Germany) to the final concentrations mentioned in the Results section. In each case, 10 µl of odour-solution is applied into custom-made Teflon containers with an inner diameter of 5 mm, and a perforated cap with 7 holes of 0.5 mm diameter, each.

Statistics

Data is collected in parallel for all the groups to be statistically compared, using non-parametric analyses throughout. Kruskal-Wallis (KW) tests are used to compare across multiple groups; in case of significance, we then separately test the scores of single groups against zero using one-sample sign tests (OSS). The significance level for these tests is set to 0.05, maintaining an experiment-wide error rate of 5 % by a Bonferroni correction. That is, in a case where e.g. five groups are to be compared individually to zero, the critical P-level is set to 0.05/5= 0.01. The Mann- Whitney U test (MWU) along with the Bonferroni correction is employed to compare two groups with each other. All statistical analyses are performed with Statistica (version 8.0, StatSoft Inc., Tulsa, OK, USA) on a PC.

Performance indices are presented as box plots with the median as mid-line, box boundaries as the 25 /75 % quantiles and whiskers as the 10 /90 % quantiles. Sample sizes are given within the Figures.

RESULTS

Memory is intensity-specific for n*-amyl acetate, 3-octanol and 1-octen-3-ol*

Using AM as odour, we find an optimum-function for associative performance indices across odour intensities (Fig. 3Ai: KW: H= 47.4, df= 7, P< 0.05). Specifically, at intermediate intensities significant associative scores are obtained, whereas the lowest intensity used is apparently not learnable; notably, also at the highest intensity performance indices do not formally differ from chance (Fig. 3Ai: OSS tests at P<>

0.05/8). This likely is because at such high intensity the relatively strong innate preference for AM hinders revealing an associative memory (see Fig. S1A). We therefore restrict our choice of odour intensities to lower than the 1:10 dilution.

To probe for a possible intensity-specificity of the AM-memory trace, we use an intensity that supports about half-maximal associative performance indices (Fig. 3Aii), allowing us to detect both increases and decreases in scores. Specifically, we choose $1:10^4$ as the MEDIUM intensity for training, and then test larvae either at LOWer ($1:10^5$, $1:10^6$) or HIGHer ($1:10^3$; $1:10^2$, 1:10) intensities. It turns out that as the testing intensities deviate from the training intensity towards either HIGHer or LOWer intensities, performance indices approach zero (Fig. 4A: OSS tests at $P<> 0.05/6$; the Kruskal-Wallis test across all groups yields $P< 0.05$, $H= 29.4$, $df= 5$). Thus, in order to support full retention, the testing intensity needs to match the training intensity; this follows scenario B in Figure 2.

Fig: 3A (i)

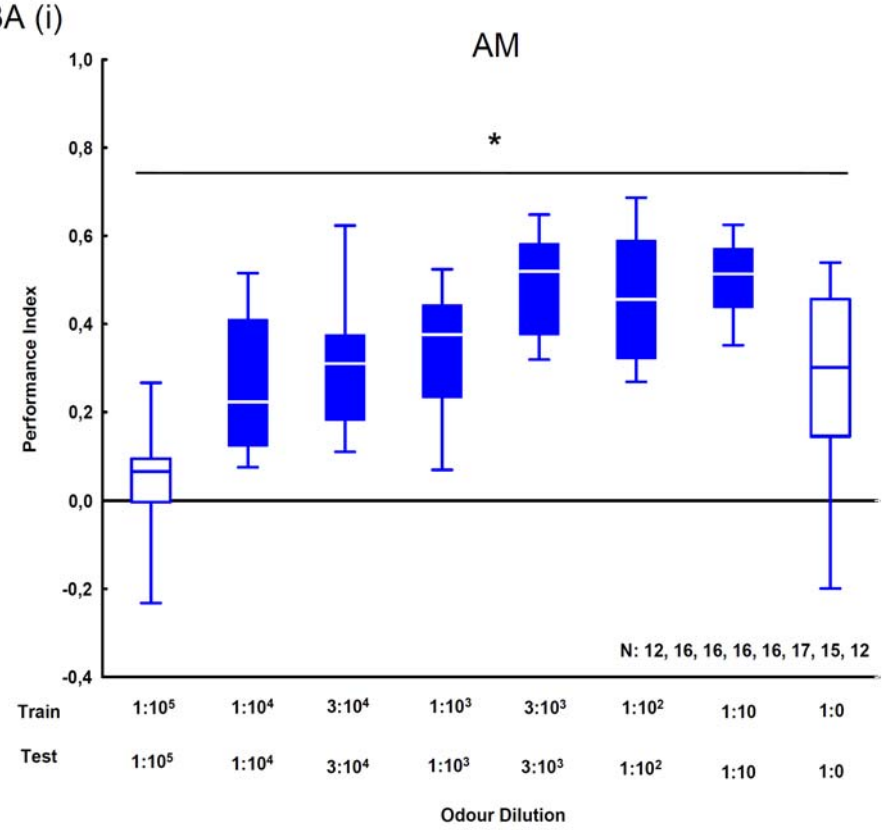


Fig: 3A (ii)

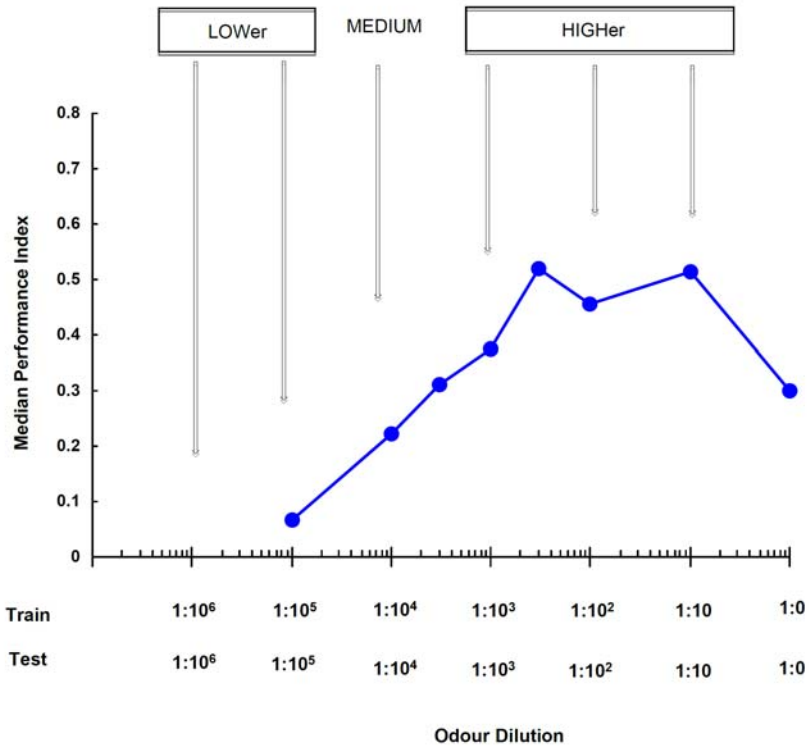
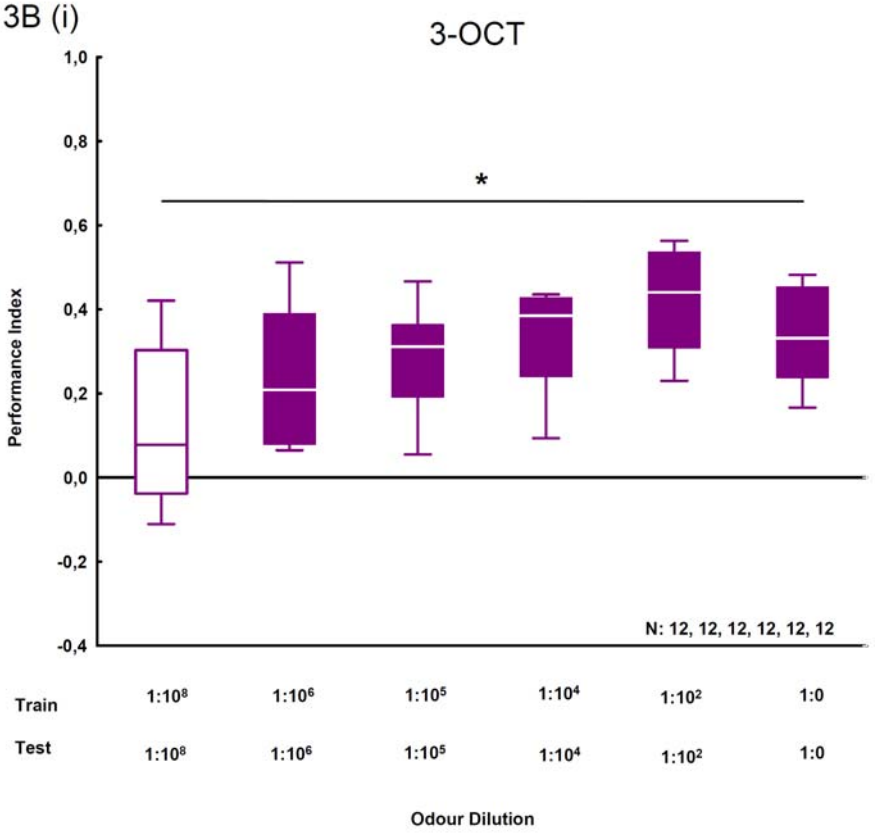


Fig: 3B (i)



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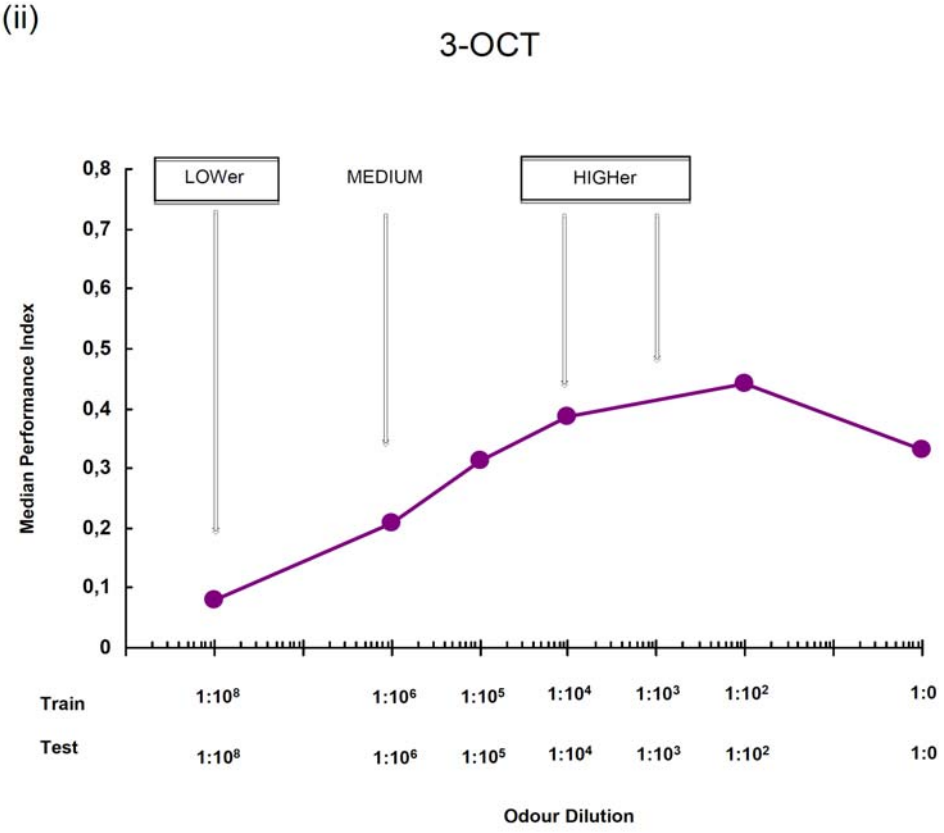

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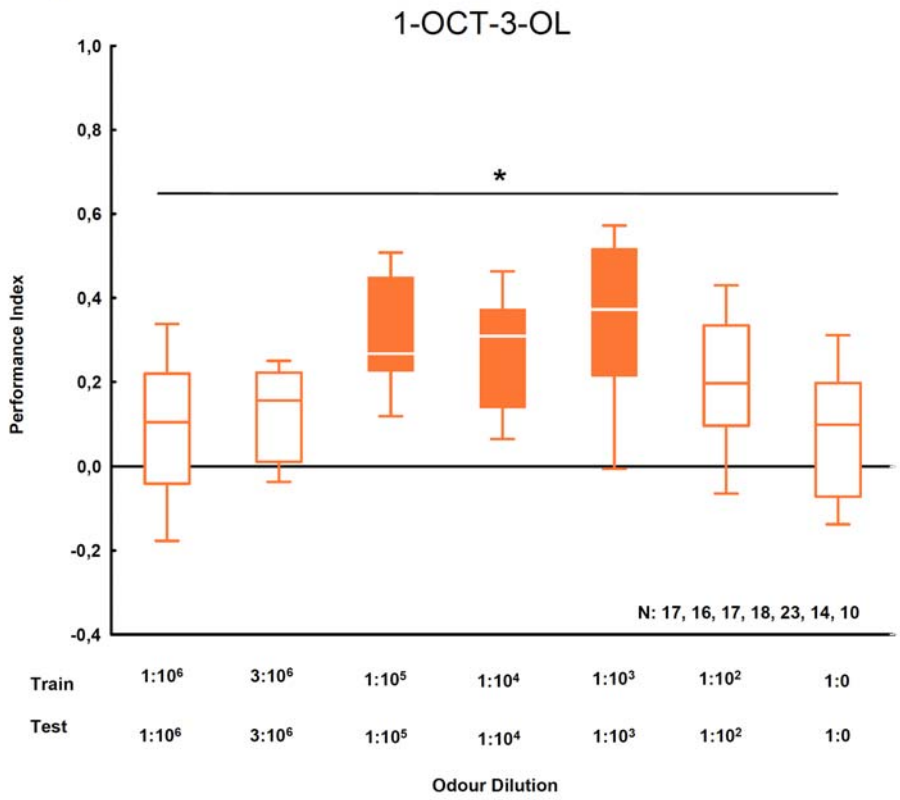

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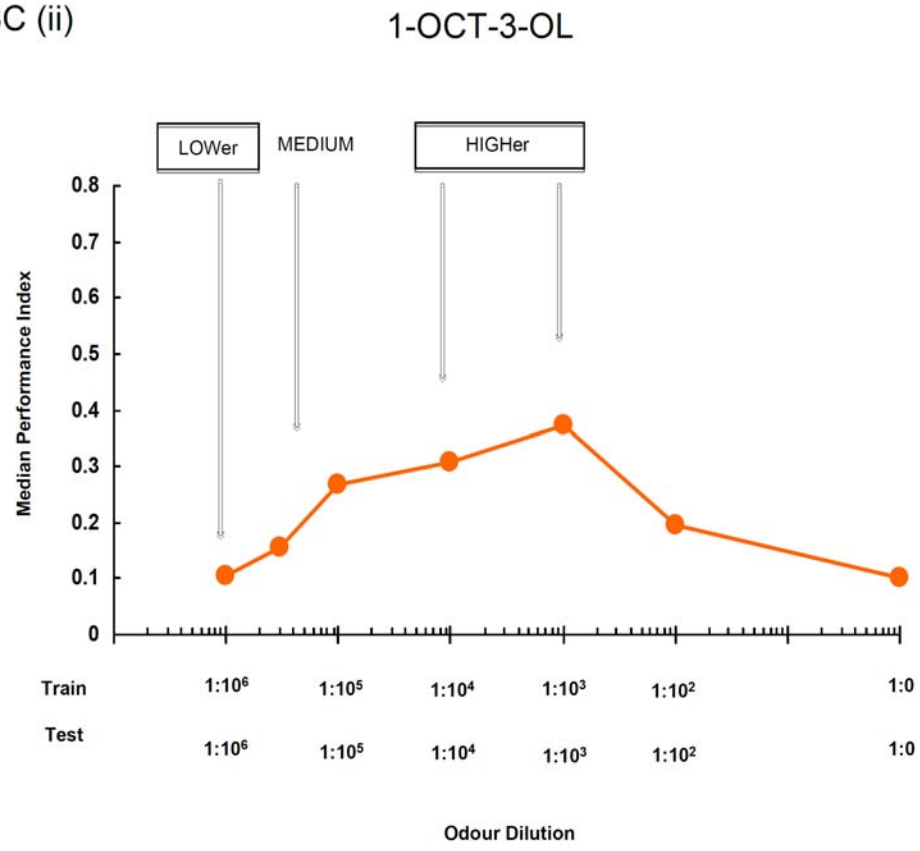

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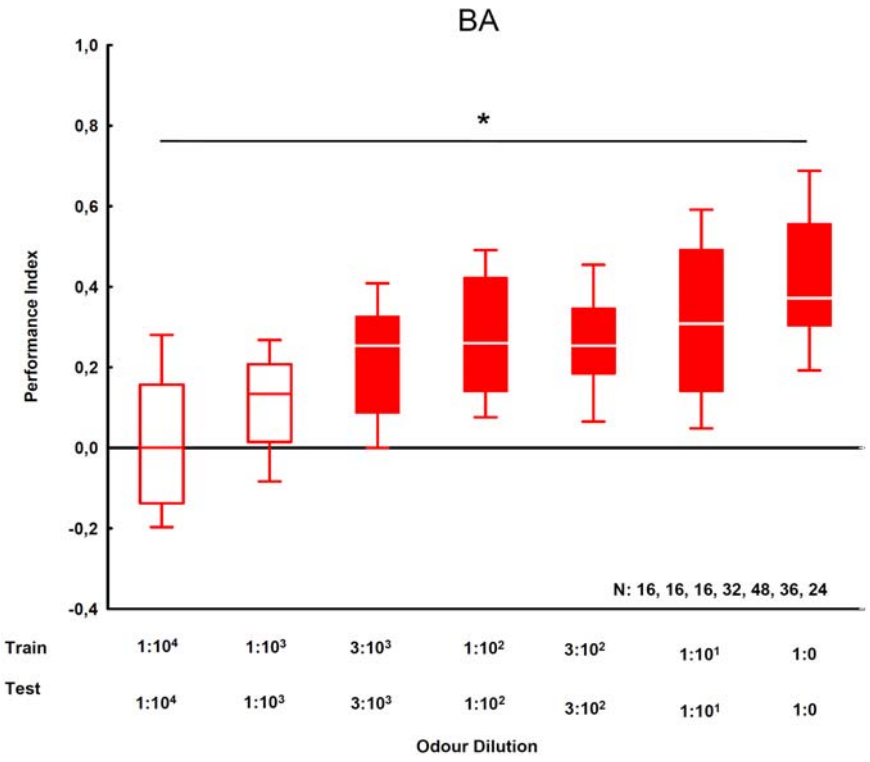

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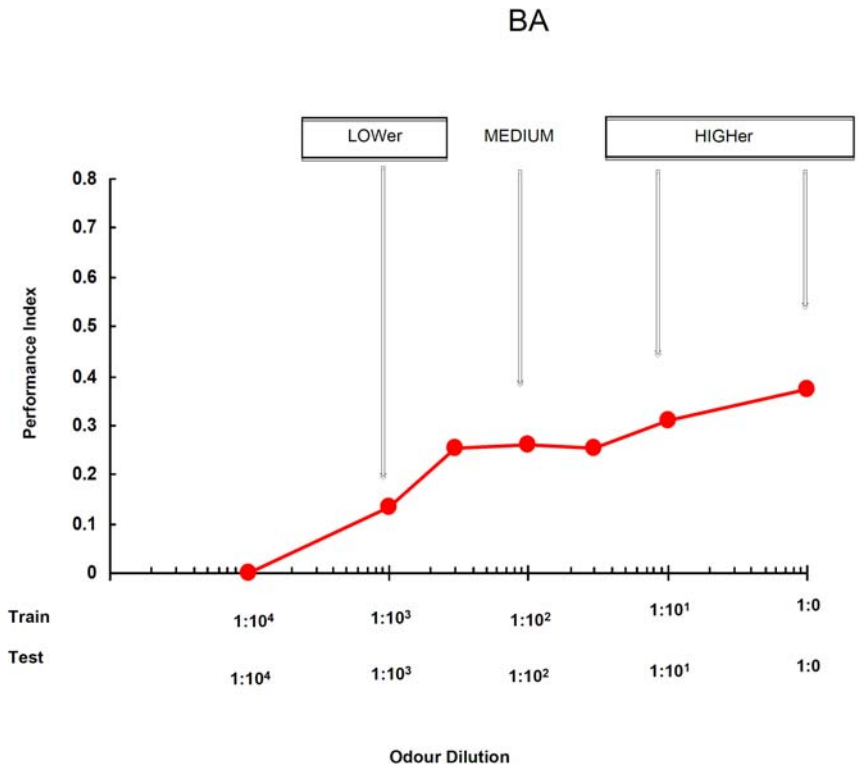


Figure 3

Dose-dependency of learnability

Dose-effect curves of learnability across odour intensities for four different odours (A, B, C, and D presenting data for n-amyl acetate, 3-octanol, 1-octene-3-ol, and benzaldehyde, respectively).

In the i-parts, data are presented as box plots (bold line: median as bold line, 25/75 % quartiles as box boundaries and the 10/90 % quantiles as whiskers). *: $P< 0.05$ refers to across-all comparisons between odour intensities in KW tests. Shading of the boxes indicates performance indices significantly different from zero (OSS tests, Bonferroni corrected). Respective sample sizes are shown at bottom corner of the graphs.

In the ii-parts, the median performance indices from (i) are plotted over odour dilution; from these curves odour dilutions for the follow-up experiment (Figure 4) are chosen such that they support about half-maximal performance indices, designated as MEDIUM intensity, as well as respectively LOWer, and HIGHer intensities.

(Ai) For AM we find an optimum-function for associative performance scores across odour intensities; from (ii) we designate $1:10^4$ as the MEDIUM intensity, $1:10^5$ as well as $1:10^6$ as LOWer, and $1:10^3$, $1:10^2$ as well as 1:10 as HIGHer intensities (for statistics see text).

(Bi) For 3-OCT, associative performance indices at very low intensity are not significantly different from zero, whereas all other groups do show significant learning scores (OSS tests at $P<> 0.05/6$) (the KW test across groups yields $H= 13.89$, $df= 5$, $P< 0.05$), although one may note a trend for decreasing performance indices for the highest intensity used. From (ii), we identify $1:10^6$ as MEDIUM intensity, $1:10^8$ as LOWer intensity, and $1:10^4$ as well as $1:10^3$ as HIGHer intensities.

(Ci) For 1-octen-3-ol, we find an optimum-function for associative performance scores across odour intensities (KW: $H= 28.1$, $df= 6$, $P< 0.05$): At very low and very high odour intensities, performance indices are not significantly different from zero, whereas the other groups do show significant associative performance scores (OSS tests at $P<> 0.05/7$). From (ii), we designate $5.6:10^6$ as MEDIUM intensity, $1:10^6$ as LOWer, and $1:10^4$ as well as $1:10^3$ as HIGHer odour intensities.

(Di) For very low intensities of BA, associative performance indices are not significantly different from zero, whereas all other groups do show significant scores (OSS tests at $P<> 0.05/7$) (the KW test across groups yields $H= 43.3$, $df= 6$, $P< 0.05$). From (ii), we designate $1:10^2$ as MEDIUM intensity, $1:10^3$ as LOWer, and $1:10^1$ as well as 1:0 as HIGHer odour intensities.

Given that for 3-OCT and 1-OCT-3-OL we obtain the same results (Fig.s 3B/4B and 3C/4C), we conclude that as a rule olfactory associative learning establishes intensity-specific memory traces in larval *Drosophila*.

Is benzaldehyde an exception?

In the adult, it has been reported that BA-memories are not intensity-specific as assayed in an odour-electric shock associative paradigm. That is, in the adult higher-than-trained BA intensities support higher associative performance indices than the actually trained intensity (Yarali et al, 2009, loc. cit Fig. 4D) (following scenario A in Figure 2). We therefore include BA in our analysis concerning the larva as well.

In the dose-effect description of the learnability of BA, associative performance indices increase as odour intensity is increased (Fig. 3Di: KW: H= 43.3, df= 6, P< 0.05). We choose HIGH, MEDIUM and LOW intensities from this dose response curve (Fig. 3Dii), and train the larvae with the MEDIUM intensity. Different groups of larvae then are tested with either the same MEDIUM, LOWer, or HIGHer intensities, respectively. As expected, when LOWER intensities are used for testing, associative performance indices are lower than when the trained MEDIUM intensity is presented at test (Fig. 4Di; MWU test: U= 30.0, P< 0.05/3). However, associative performance indices remain unaltered if MEDIUM-trained larvae are tested with HIGHer or even Much HIGHer intensities (Fig. 4Di; MWU tests: U= 90, 64.0, P= 0.51, 0.12) (the corresponding KW test yields P< 0.05, H= 13.11, df= 3). This result is not conclusive regarding the question whether BA-memory traces are intensity-specific or not (compare the data of Fig. 4Di to the two scenarios presented in Figure 2A, B).

To overcome this deadlock, we train larvae with a LOW intensity and test them with either that very same LOW intensity, or the MEDIUM, or the HIGHer odour intensiy (please note that in this experiment the latter two testing intensities are both *higher-than-trained*). We find that associative performance indices decrease as testing intensities are elevated above the trained LOW intensity (Fig. 4Dii; train LOW, test LOW *versus* the groups tested with MEDIUM [MWU test: U= 271.0, P= 0.42], HIGHer [MWU test: U= 203.0, P= 0.2], or tested with much HIGHer intensities [MWU test: U= 124.0, P< 0.05/3]) (the corresponding KW test yields: P< 0.05, H= 9.16, df= 3). Thus, also BA-memories are intensity-specific in larval *Drosophila*.

Fig: 4A

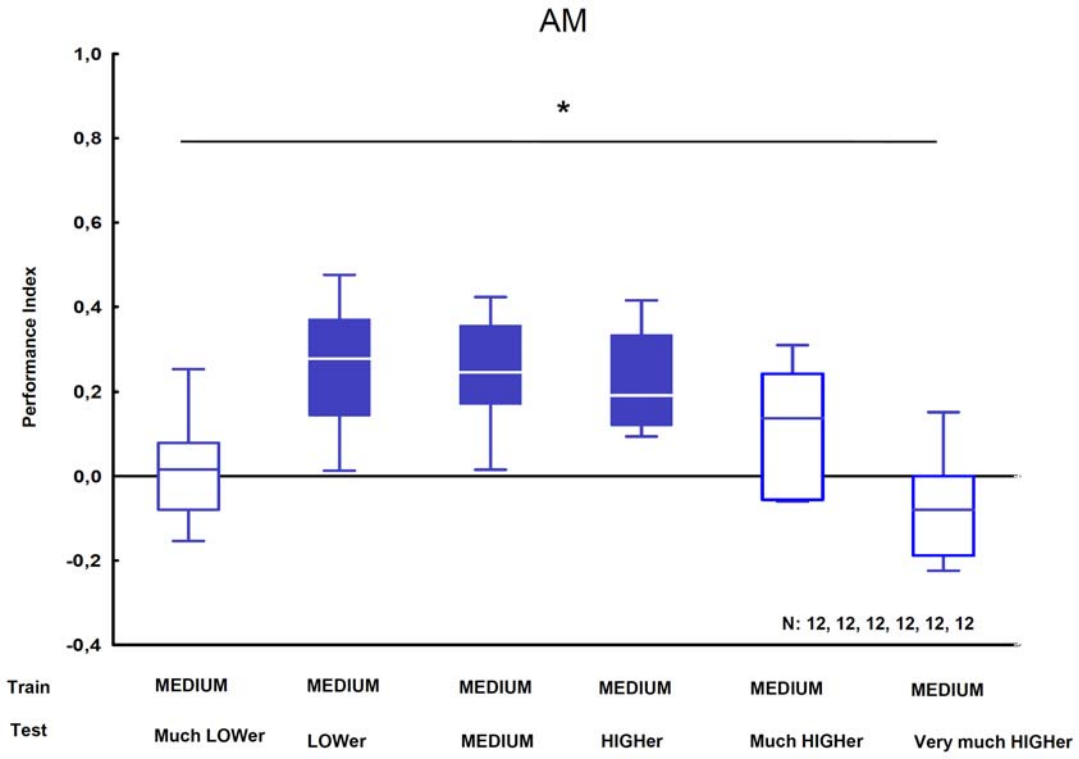

AM
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
Performance Index
*
N: 12, 12, 12, 12, 12, 12
Train
Test
MEDIUM
Much LOWer
MEDIUM
LOWer
MEDIUM
MEDIUM
MEDIUM
HIGHer
MEDIUM
Much HIGHer
MEDIUM
Very much HIGHer

Fig: 4B

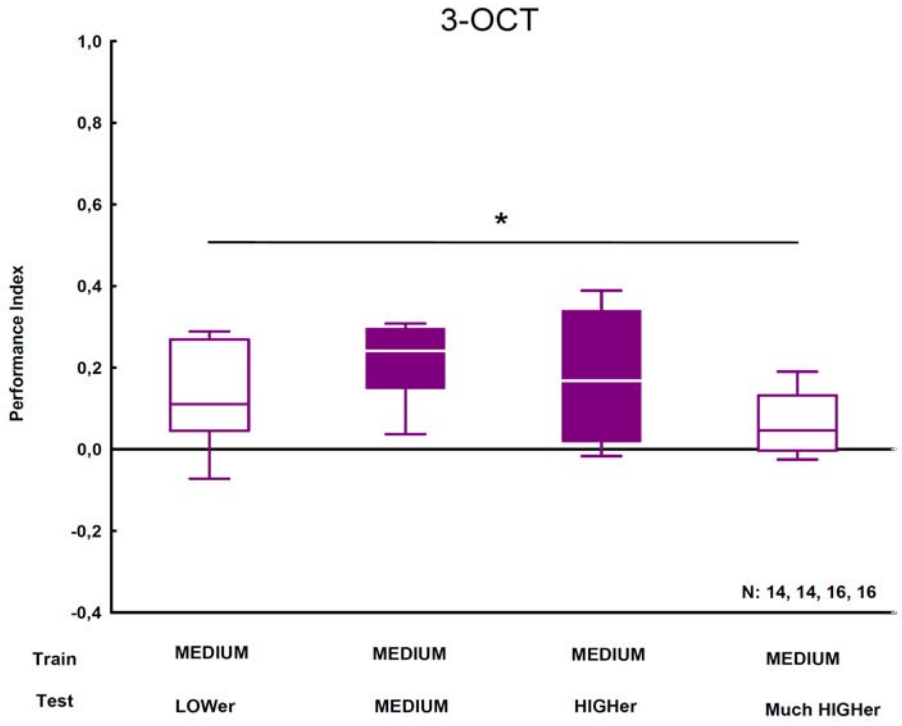
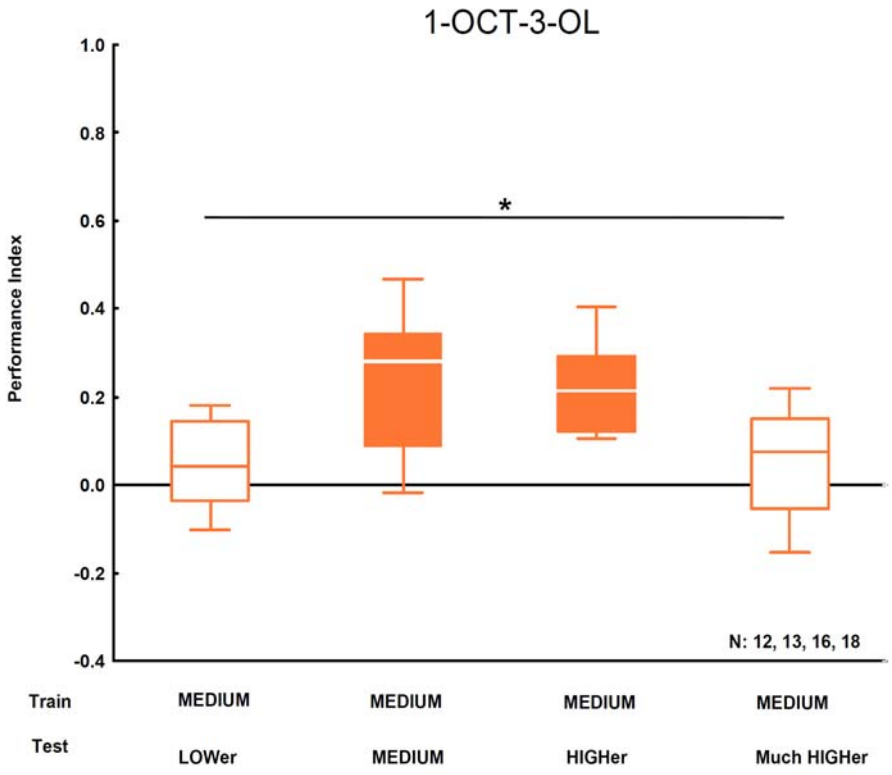

3-OCT
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
Performance Index
*
N: 14, 14, 16, 16
Train
Test
MEDIUM
LOWer
MEDIUM
MEDIUM
MEDIUM
HIGHer
MEDIUM
Much HIGHer

Fig: 4C



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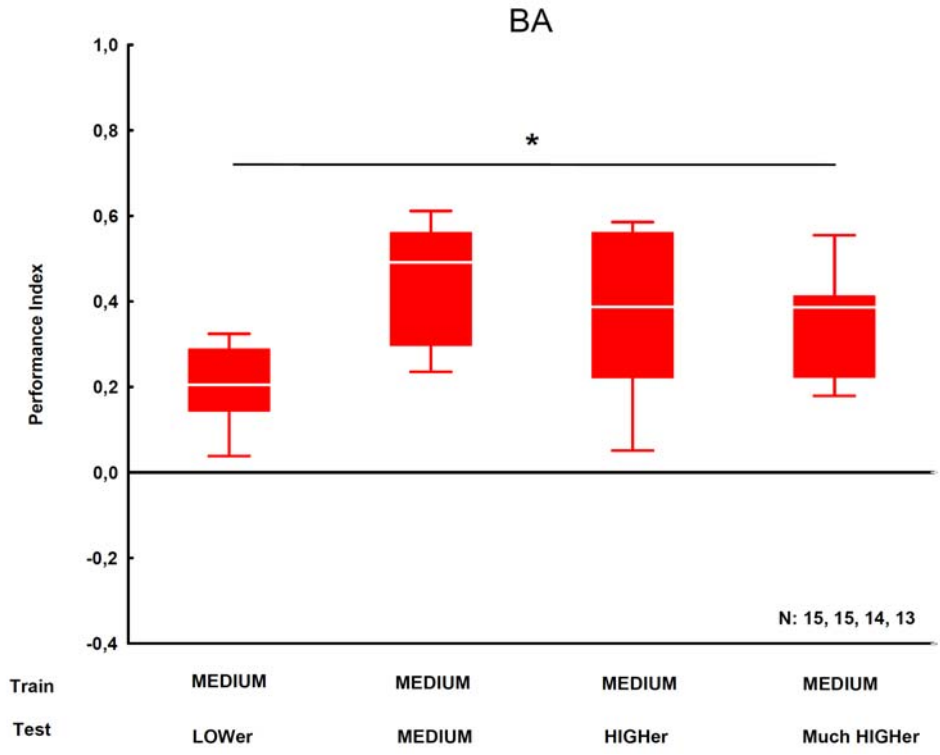

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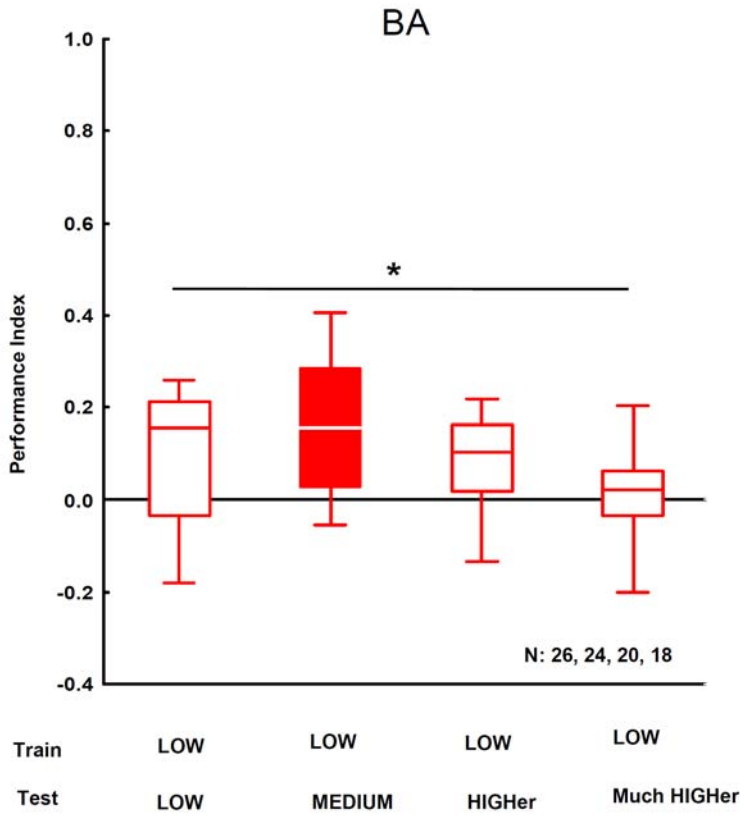


Fig: 4E

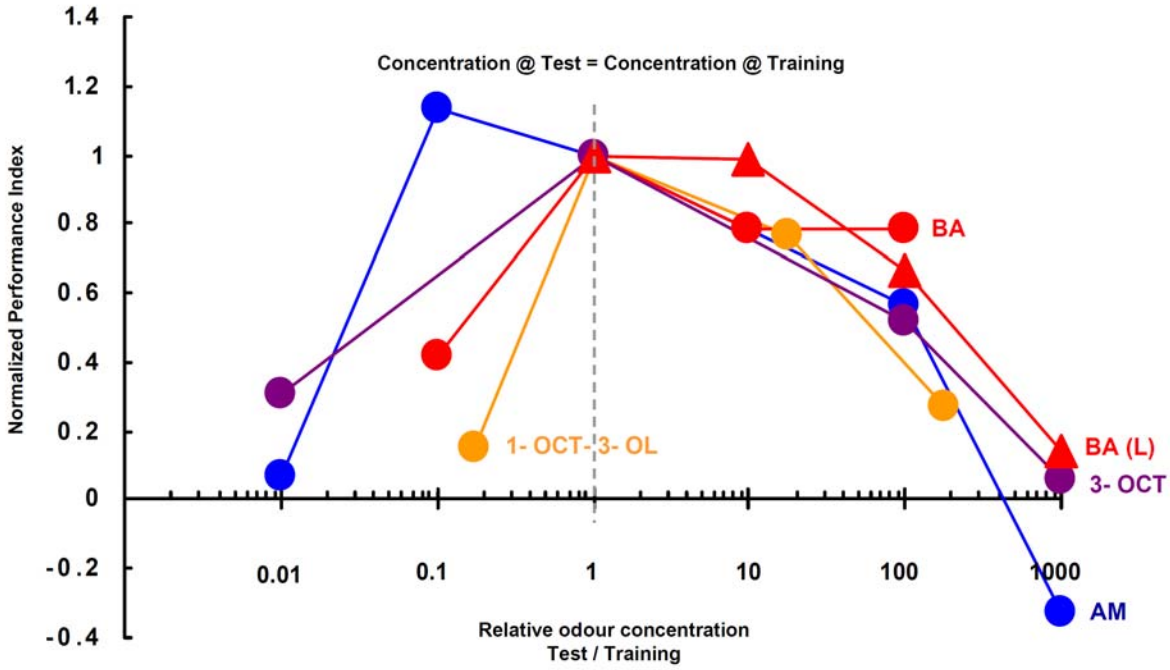


Figure 4

Memory traces are intensity-specific

Larvae are trained at MEDIUM odour intensity, but are tested for retention with either that trained MEDIUM odour intensity, or with respectively HIGHer or LOWer intensities. We observe a specificity of retention for the trained intensity for all four odours used. Other details as in Fig. 3.

(A) After training with a MEDIUM intensity of AM, associative performance indices degrade upon a mis-match between training and testing odour intensities (for statistics see text).

(B) For 3-OCT, larvae show the highest associative performance indices when the testing intensity matches the training intensity (OSS tests at P<> 0.05/4) (the respective KW test yields H= 8.5, df= 3, P< 0.05; pair-wise MW tests confirm this conclusion).

(C) For 1-octene-3-ol we also observe a loss of associative performance indices upon a mis-match between training and testing odour intensities (OSS tests at P<> 0.05/4) (the respective KW test yields H= 15.2, df= 3, P< 0.05; pair-wise MW tests confirm this conclusion).

(Di) For BA, associative performance indices decrease when the testing odour intensity is LOWer than the training intensity (MWU test, U= 30, P< 0.05/3) (scores remain significantly different from zero: OSS test: P< 0.05/4). When testing intensities are HIGHer or much HIGHer than the training intensity, scores remain formally unaltered, despite an apparent trend towards decreasing scores (MWU tests: MEDIUM versus HIGHer, U= 90, MEDIUM versus much HIGHer U= 64, P> 0.05/3 in both cases) (the respective KW test yields H= 13.1, df= 3, P< 0.05).

(Dii) When we use the LOW intensity of BA as the training intensity, associative performance indices decrease as the testing intensity is increased towards HIGHer-than-trained and much HIGHer-than-trained odour intensity (MWU tests: LOW versus MEDIUM U= 271, P> 0.05/3; LOW versus HIGHer U= 203, P> 0.05/3; LOW versus much HIGHer U= 124, P< 005/3) (the respective KW test yields H= 10.9, df= 3, P< 0.05).

(E) Semi-schematic summary of the data from (A-D). On the X-axis we use a log-scale to indicate relative odour concentrations. A value of 1 indicates that testing intensity equals training intensity; all other values indicate the fold-mismatch between training and testing intensity. On the Y-axis, for each odour we define the median associative performance index observed when training and testing intensity match as 1; all other medians regarding that odour then are plotted as normalized performance indices. For all odours used, performance scores decay upon mismatch in odour intensity between training and test.

DISCUSSION

We provide an analysis of whether intensity can be a distinctly learnable parameter of an odour. Indeed for adult flies (Xia & Tully 2007, Masek & Heisenberg 2008, Yarali et al, 2009) and bees (Bhagavan & Smith 1996; but see Pelz et al, 1997) such intensity-specificity of memory has been reported. Here, we show that in a system as simple as larval *Drosophila*, too, there is intensity learning (Fig. 4E). Interestingly, in a corresponding study in adult *Drosophila*, three of the odours used (namely AM, 3-OCT, and 4-methylcyclohexanol) support intensity-learning, but BA does not (Yarali et al, 2009). Indeed, in adult *Drosophila* the genetic and neuronal basis for BA responsiveness

seems to differ from those of other odours (Ayer & Carlson 1992, Helfand & Carlson 1989, Keene et al, 2004; see discussion in Yarali et al, 2009) while this is not apparently the case in the larva. Also, while many investigators have found that 4-methylcyclohexanol can be learned well in adults (e.g. Yarali et al., 2009 and references therein), this is not the case in larvae (Fig. S2C). Actually, larvae seem behaviourally little responsive to 4-methylcyclohexanol (Fig. S2C). Given that the general circuit architecture between larvae and adults is rather similar (Gerber et al., 2009), it is tempting to speculate that these discrepancies between larvae and adults may be based on different receptor repertoires of the two life stages (Hallem et al., 2006, Kreher et al., 2008).

Possible circuitry underlying intensity-learning

With respect to larval *Drosophila*, nothing is known as yet about the mechanisms of intensity-learning. Trivially, the recognition of a particular test-odour intensity *as being different from the trained one* is possible only if the neuronal activity induced by a given odour intensity differs at least in some regard from the activity induced by other intensities of that same odour. At which stage along the olfactory pathway may such dissociation be found? We first briefly review the architecture of the olfactory pathway (see recent reviews by Gerber & Stocker, 2007, Vosshall, 2007, Vosshall & Stocker, 2007, Stocker, 2008, Vosshall, 2008, Gerber et al., 2009, Masse et al., 2009 and references therein) and then suggest two alternative scenarios for intensity-learning.

Different odours initially activate partially overlapping subsets of olfactory sensory neurons in the olfactory organs, dependent on the ligand profile of the olfactory receptor protein expressed. In the larva, each of the 21 olfactory sensory neurons expresses but one receptor gene, and in turn each receptor gene is expressed in only one sensory neuron. The sensory neurons then innervate but one of the 21 glomeruli in the antennal lobe. In analogy to the situation in adults (Wilson, 2008), the pattern of activity in the antennal lobe likely is moulded by local interneurons. The resulting glomerular activity pattern is picked up by typically uni-glomerular projection neurons and is relayed to pre-motor centers as well as the Kenyon cells of the mushroom bodies which have access to pre-motor areas as well. Thus, dependent on the ligand profiles of the receptors and the connectivity in this system, odour quality could be combinatorially encoded along the olfactory pathway.

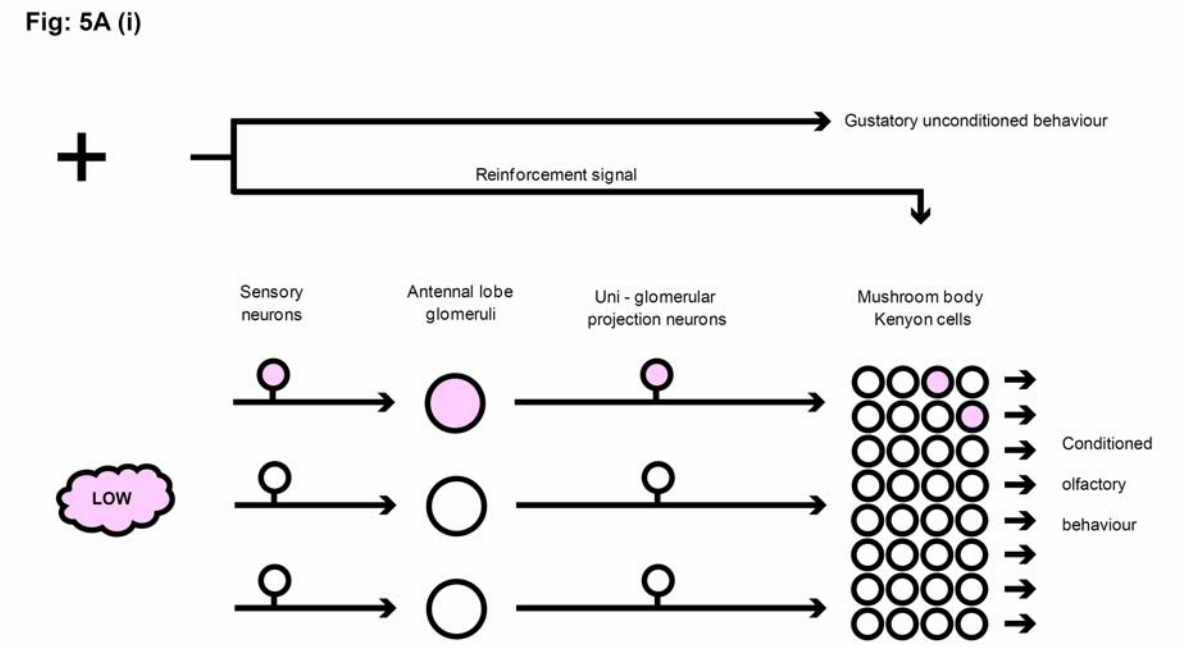
As for odour intensity, activity patterns seem to broaden with increasing intensity (larva: Asahina et al., 2009; adult: Ng et al., 2002; Wang et al., 2003; Root et al., 2007) (notably, however, at successive processing stages activity patterns become more and more intensity-invariant [Voeller, 2009]). Such nested representations clearly could not accommodate intensity-learning: Suppose that during training a memory trace were laid down in those neurons that are activated by the particular odour intensity used. In the subsequent test, a higher intensity of the same odour would activate among others always all these same neurons, likely even more strongly than the trained intensity does, hence inducing at least as strong conditioned behaviour as the trained intensity. It therefore seems unlikely that the traces of intensity memories are laid down at the level of sensory or projection neurons. At the next level of olfactory processing, mushroom body Kenyon cells show different levels of intensity-invariance in their responses (adult: Wang et al., 2004; Voeller, 2009); critically, the activity pattern evoked by a low intensity of an odour is not always nested within that evoked by a higher intensity of the same odour (e.g. for ethyl acetate, see Wang et al, 2004, loc. cit. Fig. 3). It remains unclear what kind of a connectivity scheme could transform nested representations at the projection neuron level to intensity-specific representations at the Kenyon cell level. In any case, taking this scenario to its logical extreme, training with a particular intensity lays down a memory trace in a set of Kenyon cells which, as a set, is specifically activated only by that same odour and that same intensity. Obviously, this implies an entangled storage of quality- and intensity-information in the Kenyon cells (Fig. 5A).

Alternatively, quality and intensity might be encoded separately, enabling independent learning and retrieval of each (Fig. 5B): While the quality of an odour may be coded by the unique set of Kenyon cells it activates, its intensity may be coded e.g. by the level of activity summed across all antennal lobe glomeruli, as argued by Borst (1981) with respect to adult *Drosophila* and by Sachse & Galizia (2003) with respect to the bee. Both larval (Python & Stocker, 2002ab; Asahina et al., 2009) and adult (Ng et al., 2002; Wilson, 2008) antennal lobes harbour omni-glomerular inhibitory interneurons, innervating most, if not all, glomeruli, and being activated by many different odours. Also, excitatory interneurons with similarly wide connectivity are found in the adult antennal lobe (Olsen et al., 2007; Shang et al., 2007). Finally, particular adult projections neurons with yet unknown response characteristics connect multiple glomeruli to pre-motor centers in the lateral horn (Lai et al, 2008). Any or all these multi/omni-glomerular

neurons could sum up the activity across broad aspects of / the complete antennal lobe, and might thus contribute to encoding odour intensity. Note however that even at the level of a set of omni-glomerular neurons differing in sensitivity, the representation of a low intensity would be nested within that of a higher intensity. In order to lay down an unambiguous intensity-specific odour memory trace, one would need an additional layer of neurons. These would need to receive excitatory input from e.g. a LOW-sensitivity omni-glomerular neuron and inhibitory input from a MEDIUM-sensitivity omni-glomerular neuron to become activated by specifically LOW but not MEDIUM intensity ranges (Fig. 5B). It would be in these neurons where a memory trace for specifically a LOW odour intensity could be established. Note that, at its logical extreme, this scenario implies that odour intensity is encoded entirely independent of odour quality. It is yet unclear whether or not such circuit exists, and if so whether and how such an intensity-memory trace is perceptually and behaviourally integrated with the odour-quality memory trace.

To summarize, we show that in a system as simple as the one of larval *Drosophila,* olfactory memory traces are intensity-specific. This reveals a maybe unexpected richness of olfactory processing in the larva, and defines the demands on cellular accounts and computational models of associative olfactory function.

Fig: 5A (i)



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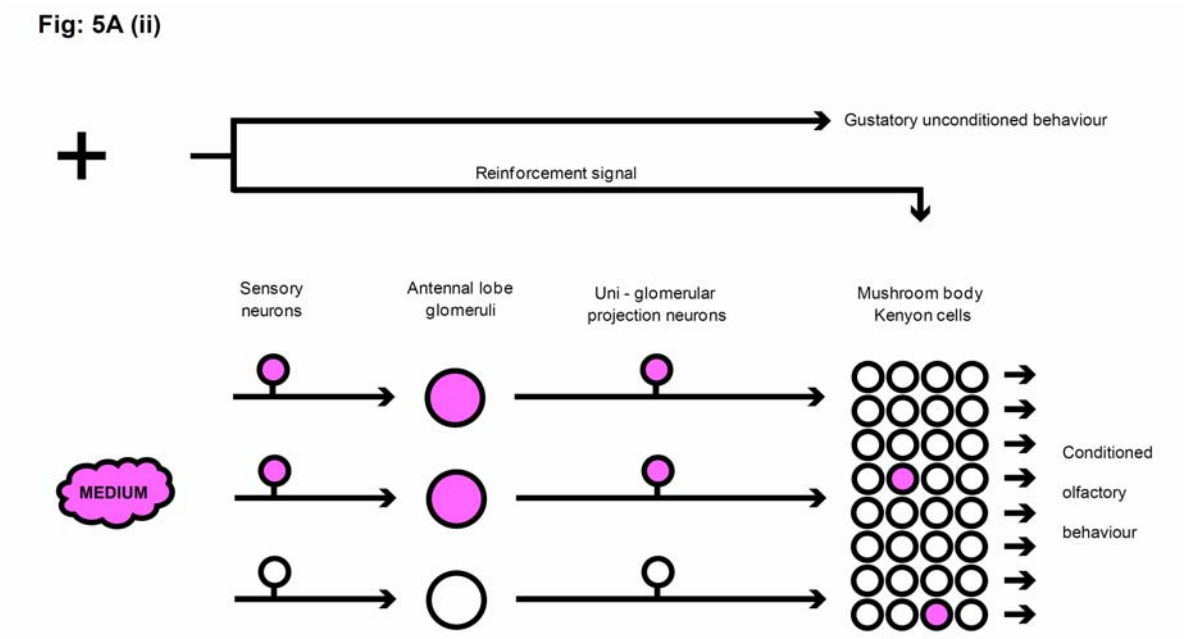
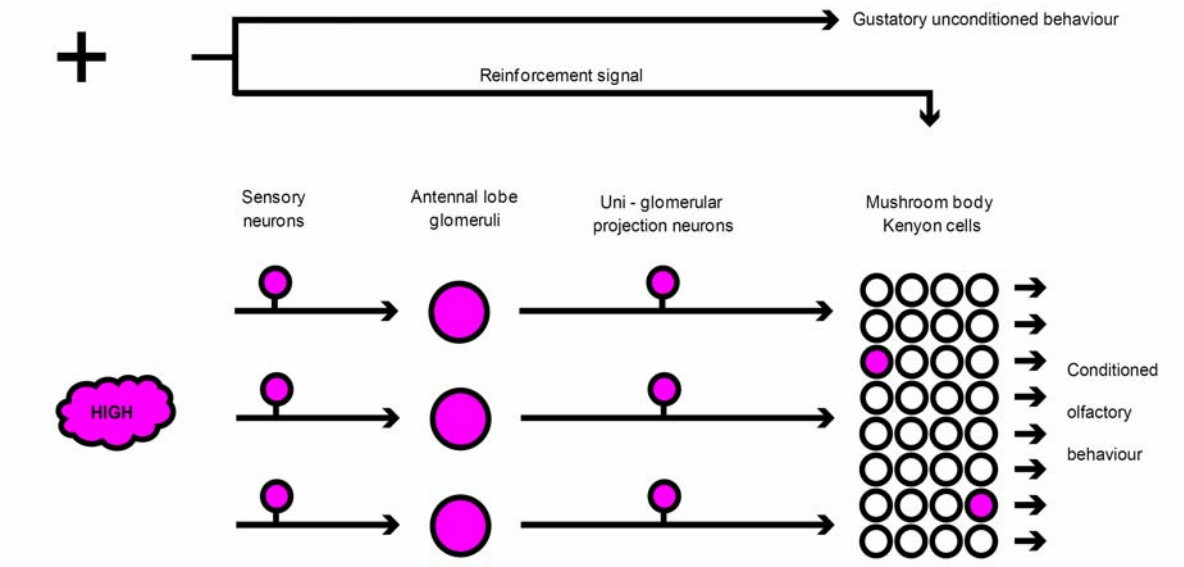
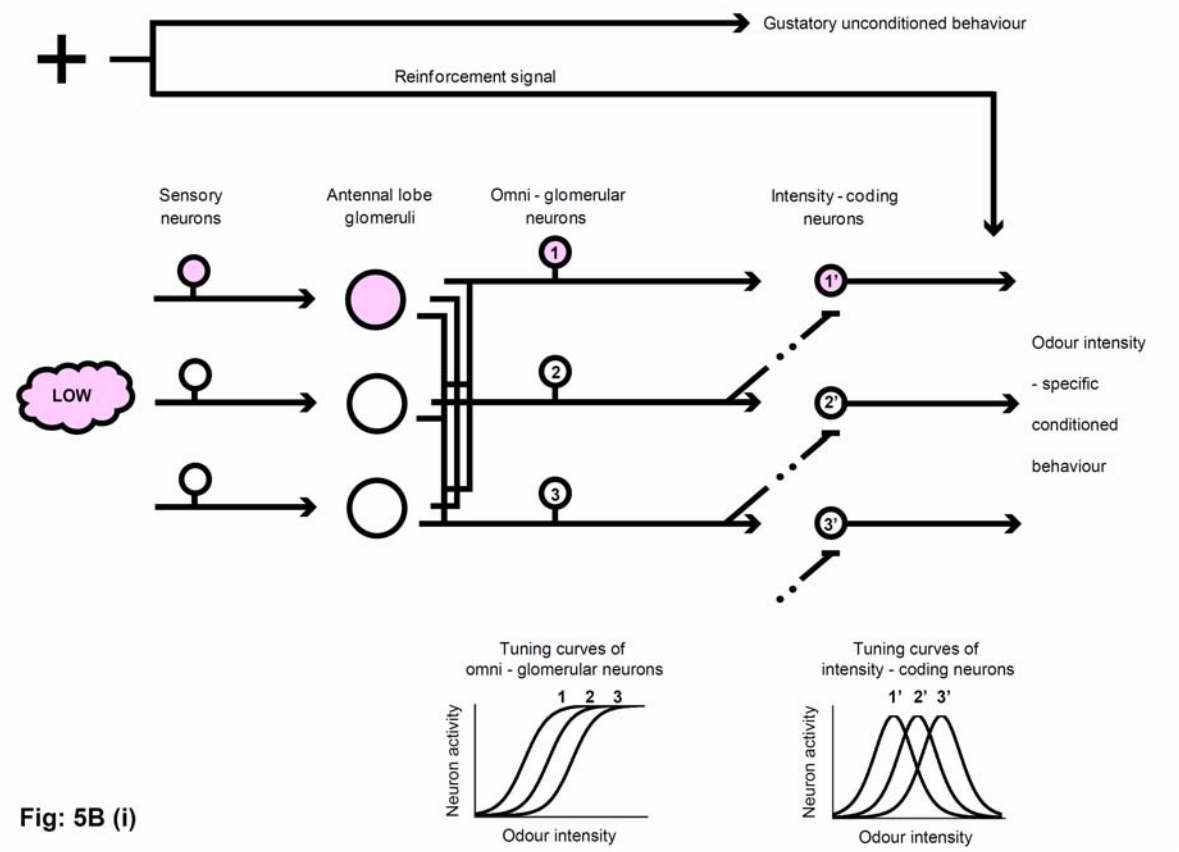


Fig: 5A (iii)





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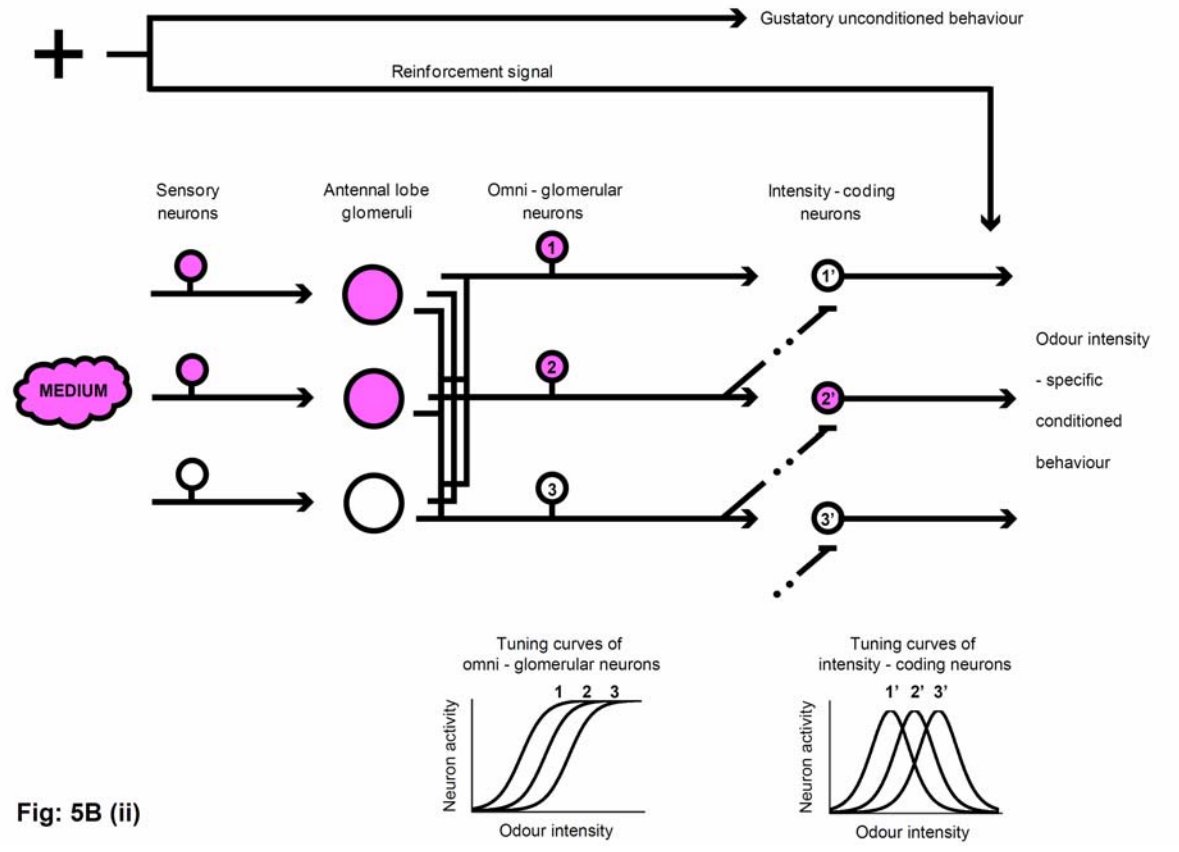

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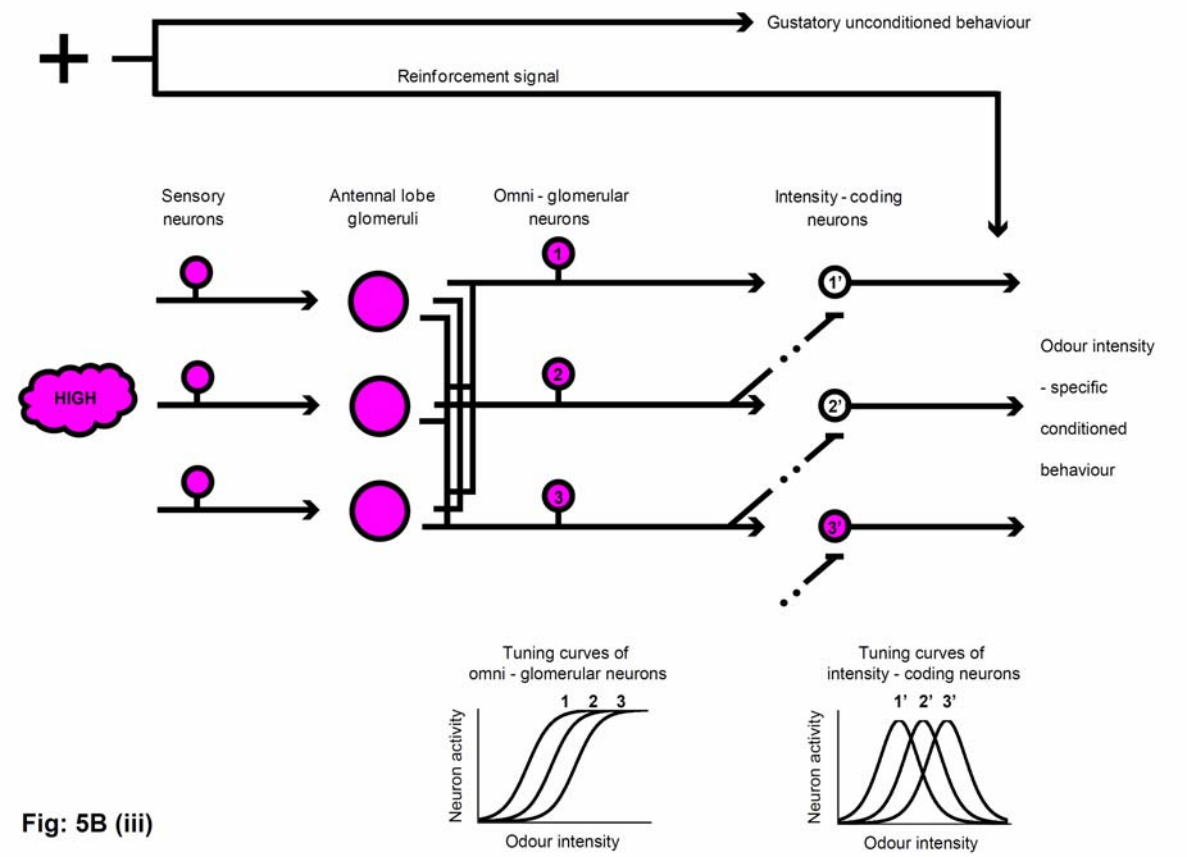

Figure 5

We sketch two logical extremes as to how odour intensity may be encoded along the olfactory pathway. For simplicity, only few units are displayed at each level of olfactory processing and the connectivity is inspired from larval fruit flies. We exemplify the encoding of three different intensities, (i) LOW, (ii) MEDIUM, and (iii) HIGH of one odour. Along the sketched olfactory pathway, those units that are activated by a particular intensity are coloured accordingly; faintest for LOW and strongest for HIGH. Arrowheads indicate excitatory outputs; blunt ends represent inhibition.

In either scenario (A and B), at the sensory neuron- and antennal lobe glomeruli-level, more units are activated with increasing odour intensity; thus, the pattern of activity for the LOW intensity is nested within that for the MEDIUM, which in turn is nested within the pattern for the HIGH.

In the first scenario (A), uni-glomerular projection neurons pick up these nested representations and relay them to the mushroom body Kenyon cells. Due to the yet unknown scheme of connectivity from the projection neurons, non-overlapping sets of Kenyon cells are activated by different odour intensities, enabling intensity-specific memories to be laid down. In the second scenario (B), omni-glomerular neurons sum up the activity over all antennal lobe glomeruli. We sketch three omni-glomerular neurons, with different sensitivities, that is, different sigmoidal tuning curves. Note that at the level of these omni-glomerular neurons, too, we obtain nested representations for different intensities as LOW < MEDIUM < HIGH. This is sorted out at the next level of neurons; namely, each of these receives excitatory input from one omni-glomerular neuron and inhibitory input from the neighbouring omni-glomerular neuron with less sensitivity, that is, with a right-shifted tuning curve. This pattern of connectivity results in bell-shaped tuning to odour intensity at this last level of neurons, enabling intensity learning.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

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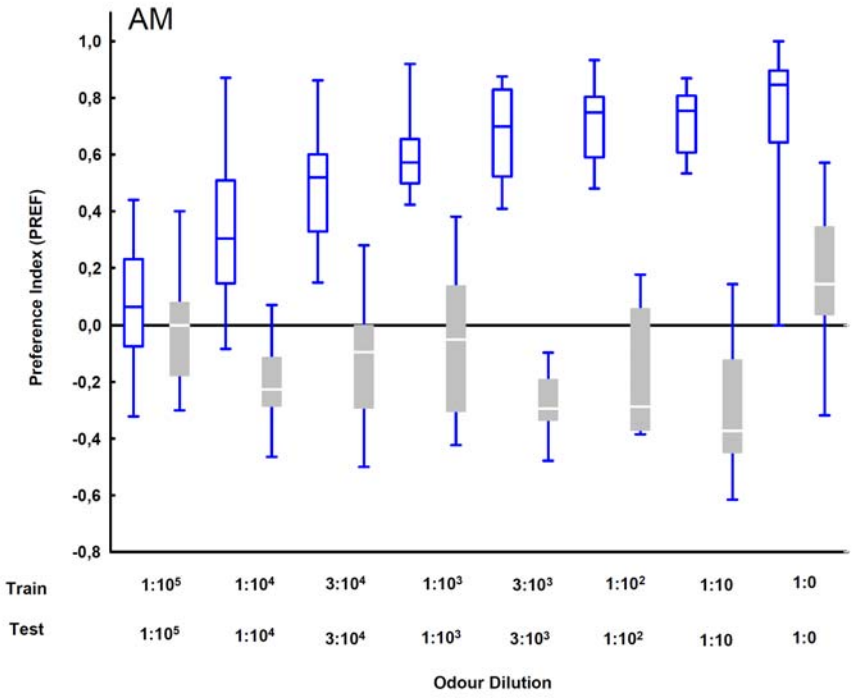

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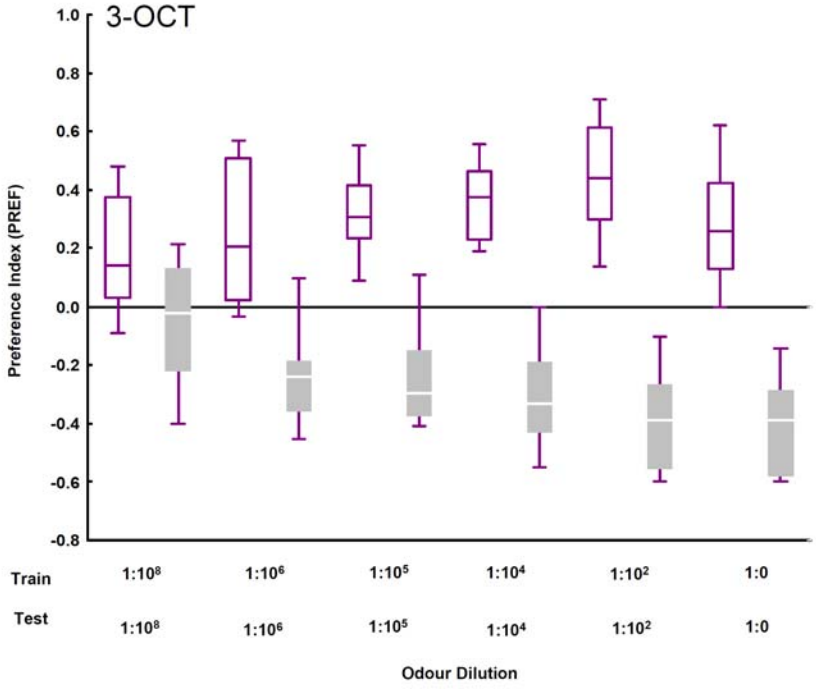

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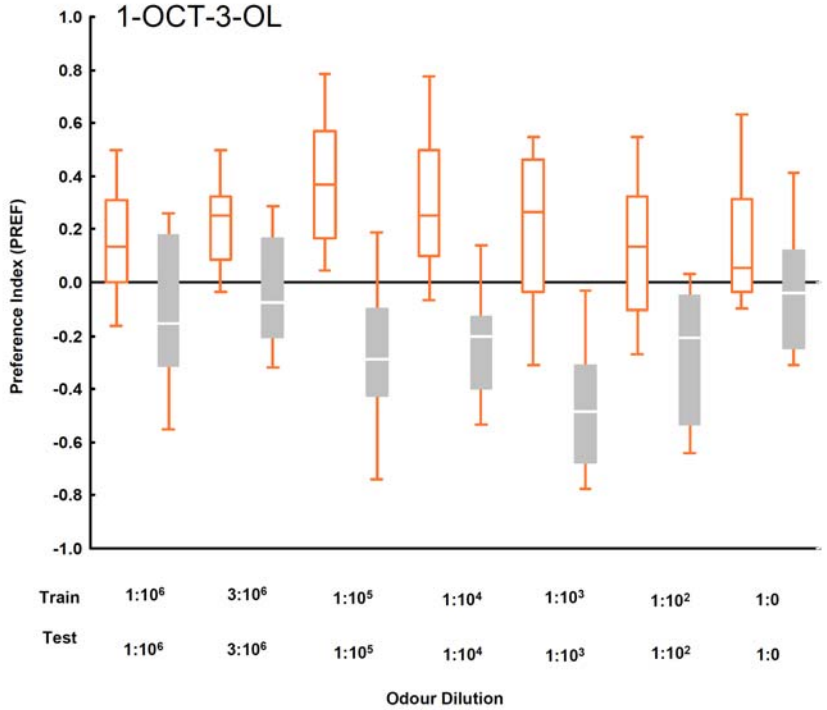

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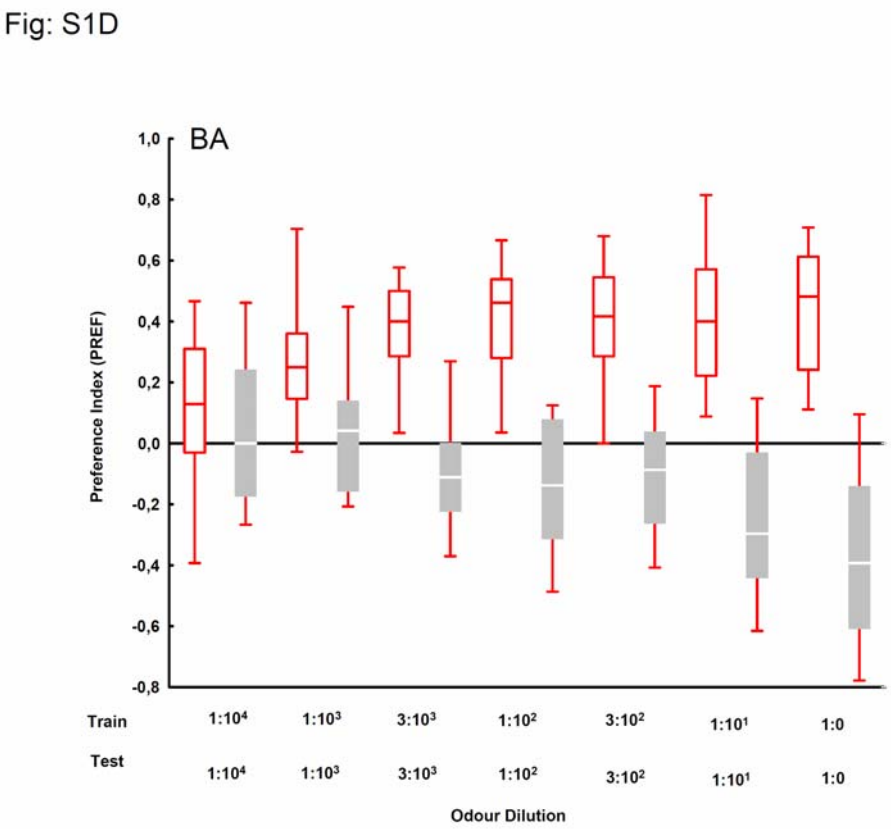
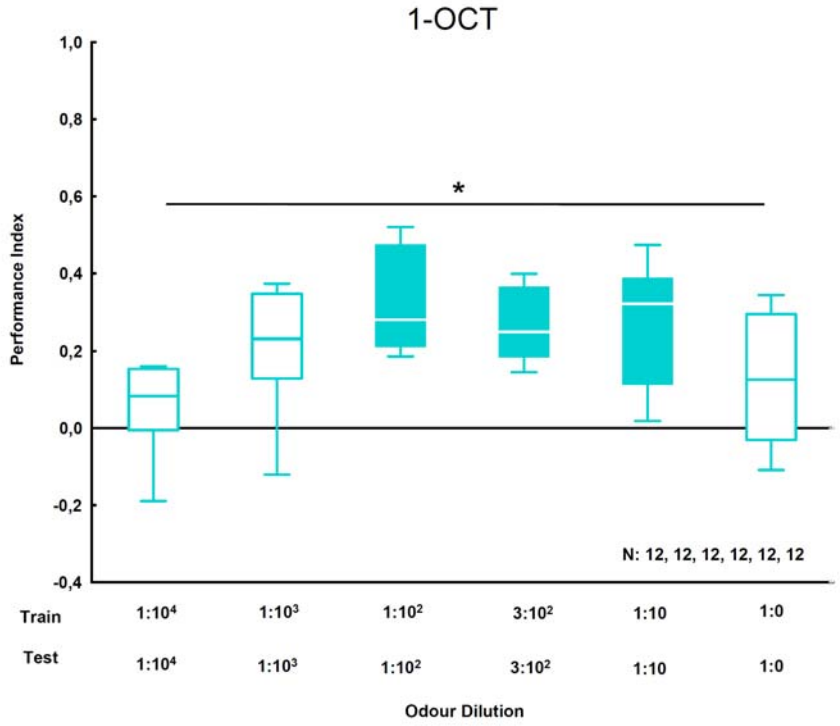


Figure S1

Preference scores for AM, 3-OCT, 1-OCT-3-OL, and BA as related to Figure 3

Shown are the preference scores (according to equation [1]) underlying the associative performance scores in Figures 3 A-D. Preference is measure after either the odour was rewarded (e.g. AM+/EM; open boxes) or the odour was non-rewarded (e.g. AM/EM+; grey boxes). This is displayed in A-D for AM, 3-OCT, 1-OCT-3-OL and BA. Within each panel, preferences scores are plotted across the indicated concentration of the respective odour used. Positive values indicate approach towards the odour and negative values odour avoidance.

Fig: S2A (i)



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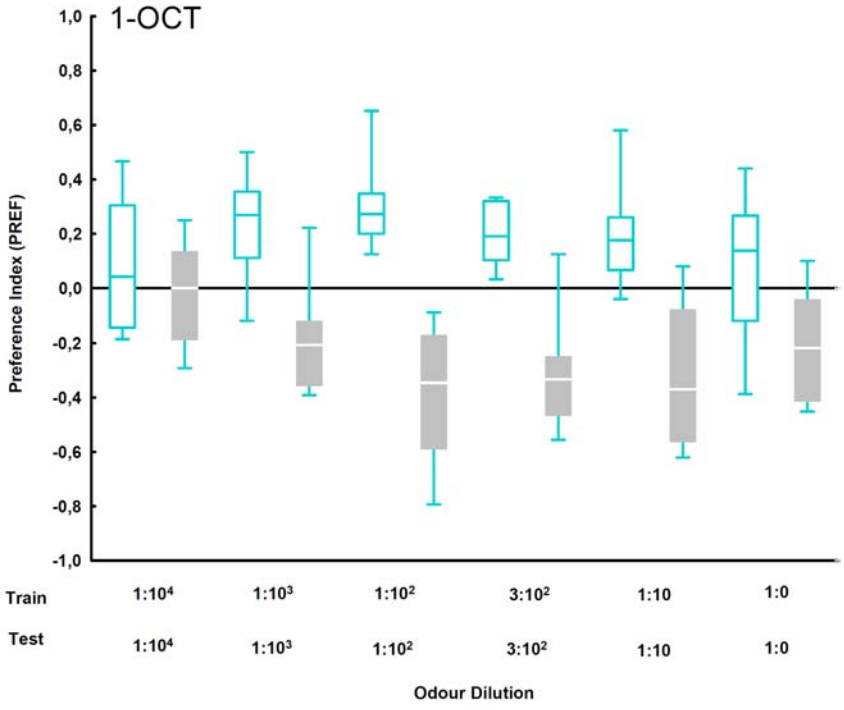

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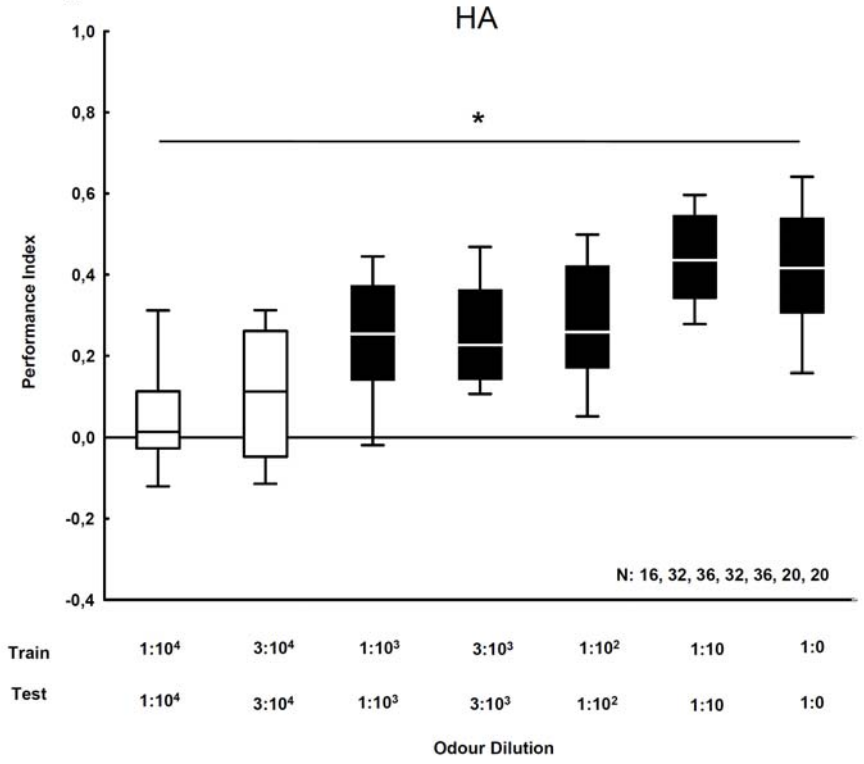

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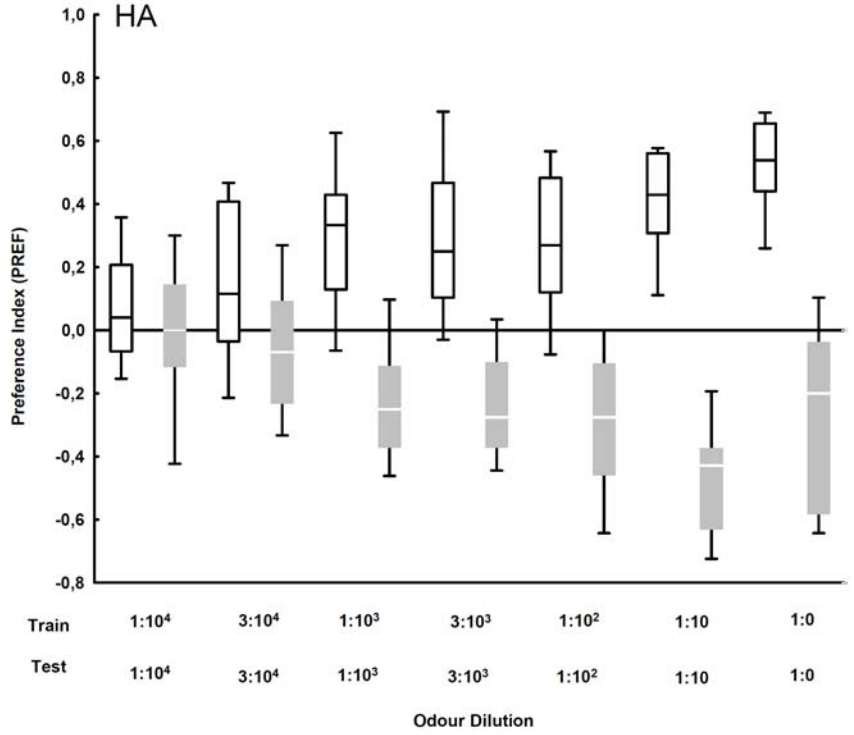
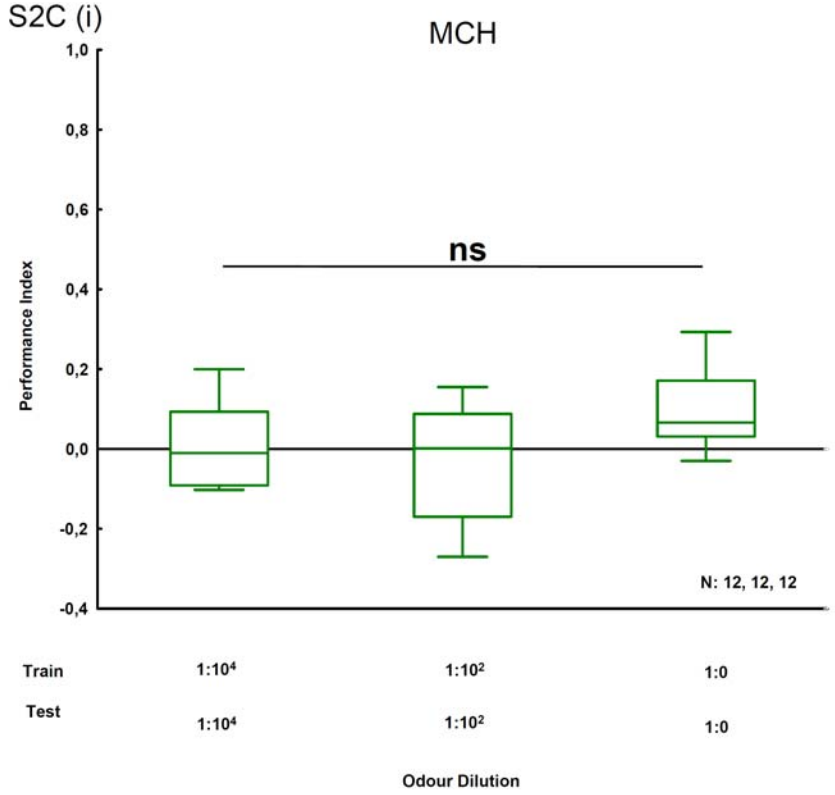


Fig: S2C (i)



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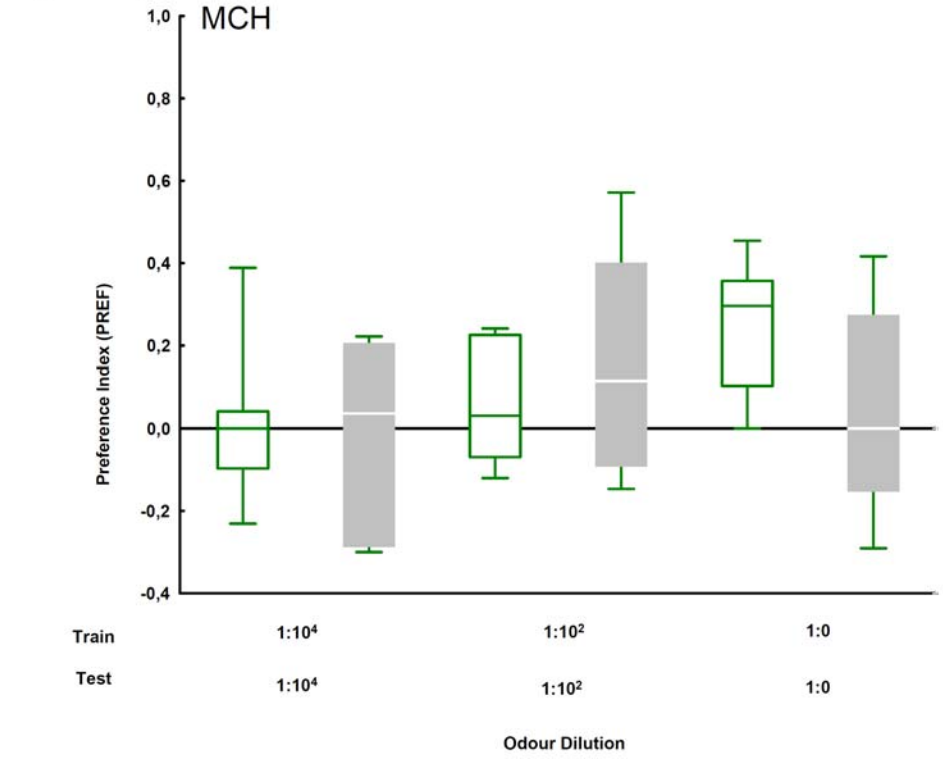
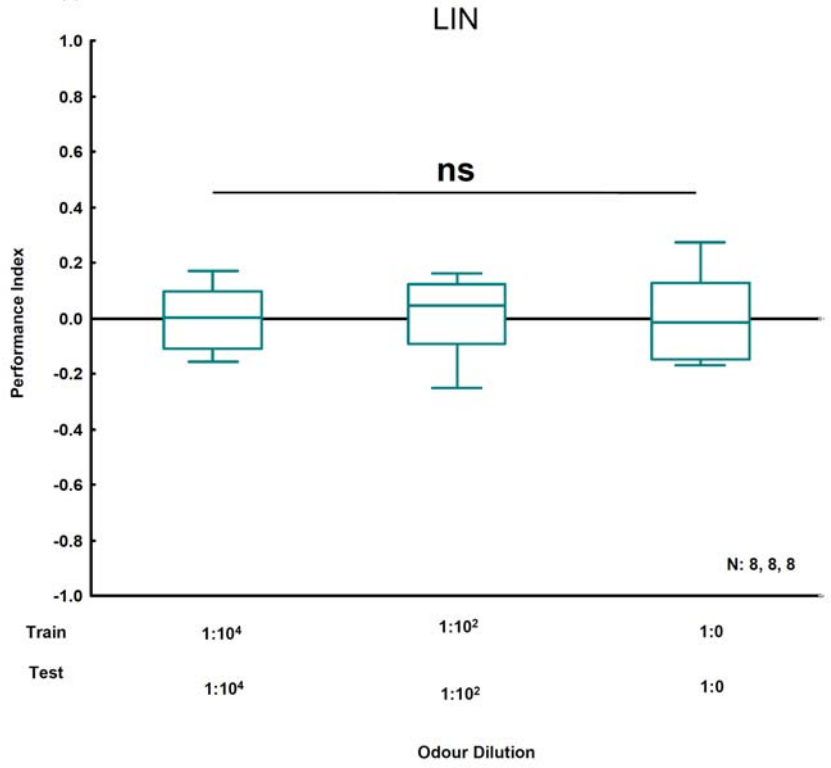


Fig: S2D (i)



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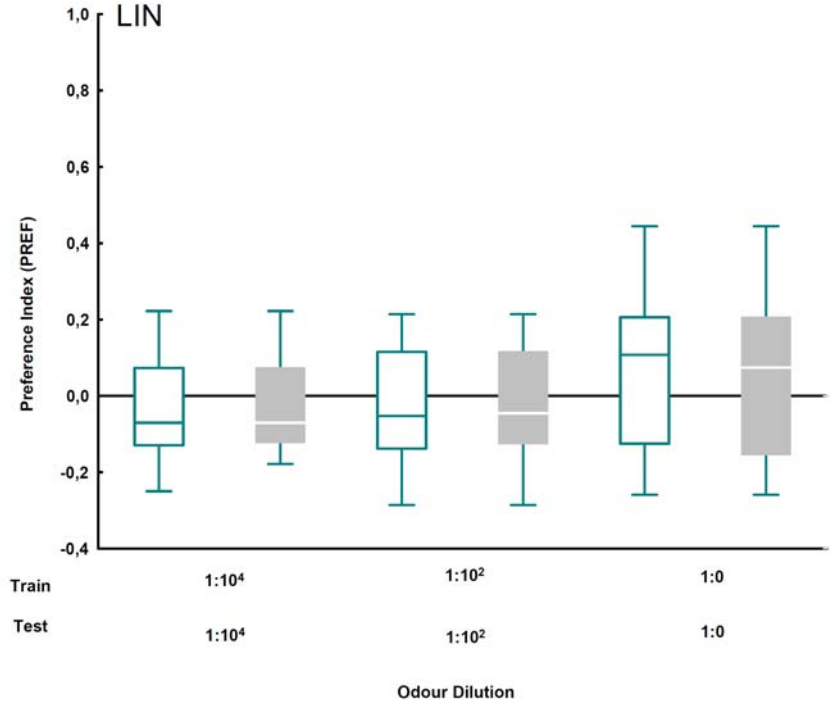

Figure S2

Associative scores and their respective preference values for 1-OCT, HA, MCH, and LIN

(Ai) For 1-OCT we find an optimum-function for associative performance scores across odour intensities. Performance scores at low ($1:10^3$, $1:10^4$) and very at high (1:0) odour intensities are not significantly different from zero, whereas all other groups do show significant learning scores, indicated by filling of the boxes (OSS tests at P<> 0.05/6). The KW test across groups yields H= 18, df= 5, P< 0.05. For this odour, intensity learning has not been probed for.

(Aii) Preference scores of the reciprocally trained groups (1-OCT+/EM; open boxes and 1-OCT/EM+; grey boxes) corresponding to the associative performance scores displayed in (Ai).

(Bi) At very low intensities of HA, performance indices are not significantly different from zero, whereas the other groups do show significant learning scores, indicated by filling of the boxes (OSS tests at P<> 0.05/7). The groups are significantly different from each other (KW: H= 60.1, df= 6, P< 0.05). For this odour, intensity learning has not been probed for.

(Bii) Preference scores of the reciprocally trained groups (HA+/EM; open boxes and HA/EM+; grey boxes) corresponding to the associative performance scores displayed in (Bi).

(Ci) For MCH, we do not find any appreciable associative performance scores across odour intensities, indicated by lack of filling of the boxes (OSS tests at P> 0.05/3); ns refers to lack of between-group significance (KW: H= 4.1, df= 2, P> 0.05). For this odour, intensity learning has not been probed for.

(Cii) Preference scores of the reciprocally trained groups (MCH+/EM; open boxes and MCH/EM+; grey boxes) corresponding to the associative performance scores displayed in (Ci).

(Di) For LIN, we do not find any appreciable performance scores across odour intensities, indicated by lack of filling of the boxes (OSS tests at P> 0.05/3); ns refers to lack of between-group significance (KW: H= 0.06, df= 2, P> 0.05). For this odour, intensity learning has not been probed for.

(Dii) Preference scores of the reciprocally trained groups (LIN+/EM; open boxes and LIN/EM+; grey boxes) corresponding to the associative performance scores displayed in (Di).

Fig: S3 (A)

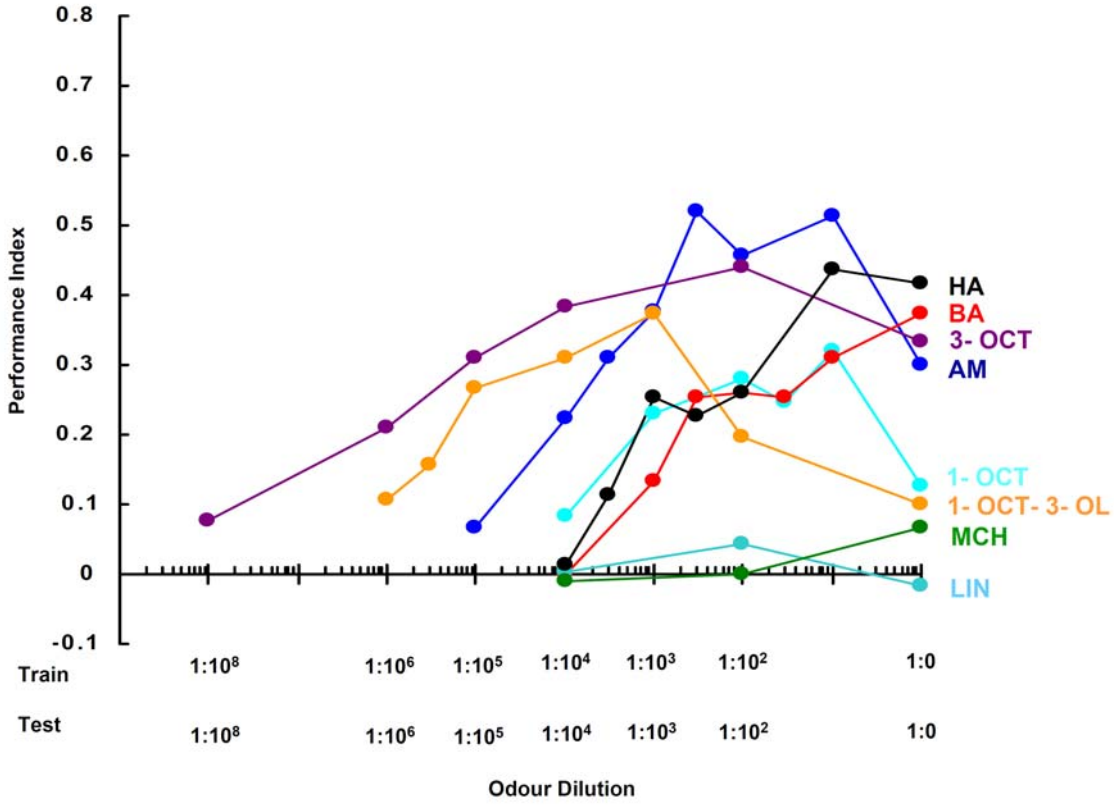
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Performance Index
HA
BA
3- OCT
AM
1- OCT
1- OCT- 3- OL
MCH
LIN
Train
Test
1:10^8
1:10^6
1:10^5
1:10^4
1:10^3
1:10^2
1:0
Odour Dilution

Fig: S3 (B)

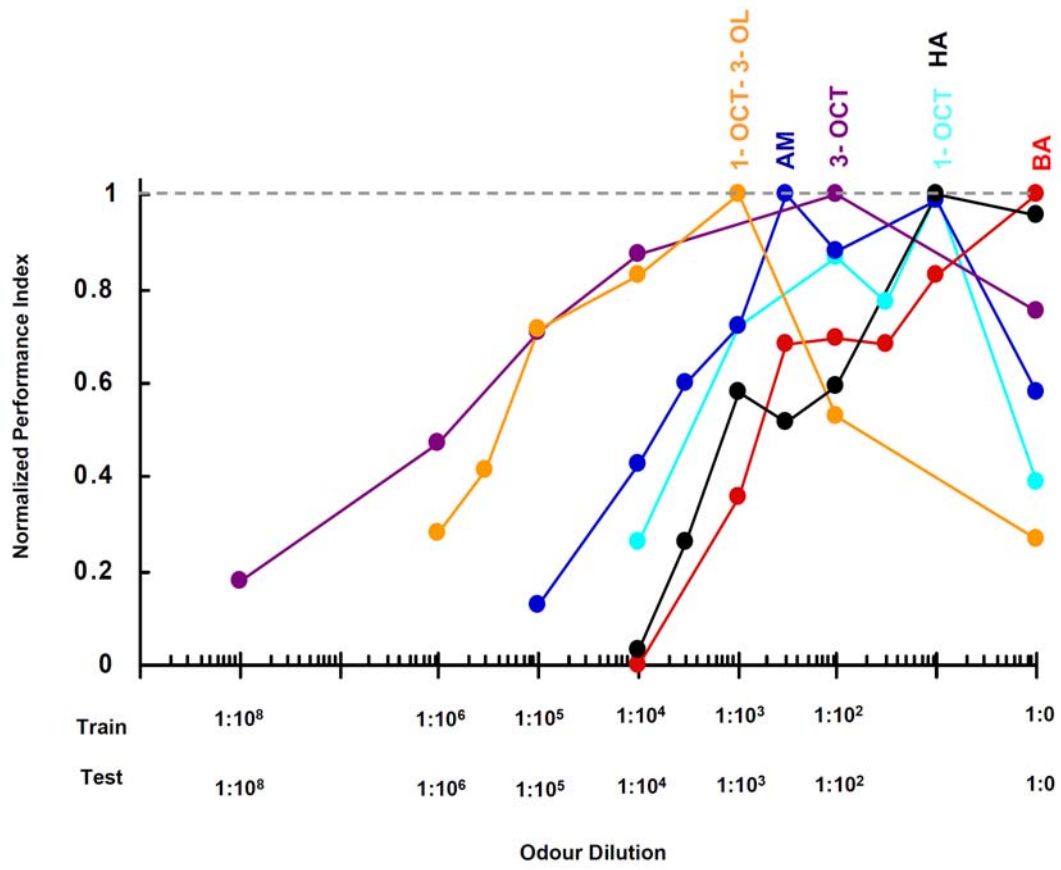

Figure S3

Semi-schematic summary of the dose response functions

(A) For eight different odours (*n*-amyl acetate [AM], 3-octanol [3-OCT], 1-octene-3-ol [1-OCT-3-OL], benzaldehyde [BA], 1-octanol [1-OCT], linalool [LIN], 4-methylcyclohexanol [MCH] and hexyl acetate [HA]), we plot the dose-effect curves of learnability, displaying odour intensity along the X-axis and the median values of associative performance indices along the Y-axis.

(B) With odour intensity along the X-axis, we plot the same data as in (A), normalized according to the respectively highest median associative performance index obtained for the respective odour.

Chapter I.2

A behavioural odour similarity ‘space’

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Bertram Gerber

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A Behavioral Odor Similarity ‘‘Space’’ in Larval *Drosophila*

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A behavioural odour-similarity 'space' in larval *Drosophila*

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Running title

Odour similarity

Key words

Olfaction, learning, sensory physiology, similarity, perception

Abstract

To provide a behaviour-based estimate of odour similarity in larval *Drosophila*, we use four recognition-type experiments: (i) We train larvae to associate an odour with food, and then test whether they would regard another odour as the same as the trained one. (ii) We train larvae to associate an odour with food, and test whether they prefer the trained odour against a novel, non-trained one. (iii) We train larvae differentially to associate one odour with food, but not the other one, and test whether they prefer the rewarded against the non-rewarded odour. (iv) In an experiment like (iii), we test the larvae after a 30min-break. This yields a combined, task-independent estimate of perceived difference between odour-pairs. Comparing these perceived differences to published measures of physico-chemical difference reveals a weak correlation. A notable exception are 3-octanol and benzaldehyde, which are distinct in published accounts of chemical similarity, and in terms of their published sensory representation, but nevertheless are consistently regarded as the most similar of the ten odour pairs employed. It thus appears as if at least some aspects of olfactory perception are 'computed' in post-receptor circuits *on the basis of* sensory signals, rather than being immediately *given* by them.

Keywords: discrimination, generalization, gustation, learning, olfaction, perception, sensory physiology, similarity

Introduction

The discoveries of the gene families coding for olfactory receptors in rodents (OR receptors: Buck and Axel, 1991; V1R receptors: Dulac and Axel, 1995; V2R receptors: Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; TAAR receptors: Liberles and Buck, 2006) and later also in *Drosophila* (*Or*-gene family: Clyne et al., 1999; Vosshall et al., 1999; *Ir*-gene family: Benton et al., 2009), have led to a reasonably satisfying working hypothesis of how different odour substances evoke different patterns of activity along the olfactory pathways (concerning *Drosophila* see Benton 2009; Gerber et al., 2009; Hallem and Carlson, 2006; Masse et al., 2009; Vosshall and Stocker, 2007). Still, olfactory coding is far from being understood: It remains challenging to understand how the temporal characteristics of neuronal activity contribute to olfactory coding (Laurent, 2002), whether and at which detail information about the physico-chemical properties of odour substances is available to the olfactory subject in these patterns of activity (Haddad et al., 2008; Schmuker et al., 2007), and, even more embarrassingly we believe, it remains largely unclear which aspects of these different patterns of activity, and at which sites along the sensory-motor loop, underlie olfactory perceptions. Obviously, addressing such questions in animals requires developing an operational handle on perception in terms of well-defined behavioural tasks. Here, we take a step in this direction, using olfactory recognition experiments after odour-food associative learning in larval *Drosophila*.

In principle, the architecture of the olfactory pathways in larval *Drosophila* is the same as in adult flies and in mammals- but at a numerically much reduced level (reviewed in Gerber and Stocker, 2007; Gerber et al., 2009; Stocker, 2008): The larva has only 21 olfactory sensory neurons, organized in the so-called dorsal organ, each expressing but one member of the *Or*-gene family (plus the co-receptor *Or83b*) with its respective ligand profile. The olfactory sensory neurons then innervate the antennal lobe (the functional analogue of the olfactory bulb), where they synapse onto both local interneurons (regarding adults: Wilson, 2008) and projection neurons (the functional analogue of the mitral cells). These connections are organized into glomeruli, such that one anatomically identifiable antennal lobe glomerulus contains input from but one genetically defined olfactory sensory neuron, gives rise to output of but one projection neuron, and harbours the lateral connections towards and from the local interneurons (Ramaekers et al., 2005). Notably, the projection neurons have two target areas: First, they innervate the so-called

lateral horn in an amazingly stereotyped way (Marin et al., 2002; Wong et al., 2002). The lateral horn in turn has access to pre-motor circuitry. It is arguably via this direct route that reflexive, innate olfactory behaviour is organized (regarding adult *Drosophila*: Heimbeck et al., 2001). Second, the projection neurons target the calyx of the so-called mushroom bodies (Ramaekers et al., 2005; Masuda-Nakagawa et al., 2005, 2009). In the larva, this structure is organized into approximately 40 anatomically identifiable glomeruli, such that one projection neuron provides input to typically but one of these calyx glomeruli; consequently, most of the projection neurons can be individually identified, based on the stereotyped combination of antennal input-glomerulus and calycal output-glomerulus (Ramaekers et al., 2005; Masuda-Nakagawa et al., 2009). In any event, the postsynaptic partners of the projection neurons in the mushroom bodies are the Kenyon cells. Each of the approximately 600 mature Kenyon cells receives input from an apparently random selection of 1- 6 glomeruli (Masuda-Nakagawa et al., 2005, 2009). This entails a dense network of divergence-convergence connections in the calyx, reminiscent of olfactory cortex (Davis, 2004; Tomer et al., 2010), and suitable for combinatorial coding. The mushroom bodies further receive input from aminergic reinforcement neurons, such that within the mushroom bodies the association of odour-evoked activity with salient rewarding or punishing events can take place (regarding adult *Drosophila*: Busch et al., 2009, Busch and Tanimoto, 2010; Gervasi et al., 2010; Riemensperger et al., 2005; Schwaerzel et al., 2003; Tomchik and Davis, 2009; regarding larval *Drosophila* Schroll et al., 2006; Selcho et al., 2009). The Kenyon cells in turn synapse onto remarkably few (based on findings in adult flies; Aso et al., 2009; Ito et al., 1998) output neurons that entertain connections towards pre-motor centres. It is arguably by this detour via the mushroom body that learned olfactory behaviour is organized (see discussions in Gerber et al., 2004, 2009; Heisenberg and Gerber, 2008). Given that, in addition to this fairly detailed account of the connectivity of the circuit, the ligand profiles of all larval-expressed *Or* gene products are at least partially described (using a panel of 26 odours: Kreher et al., 2005; 2008) (ligand profiles of the larval-expressed *Ir*-gene family receptors [Benton et al., 2009] are not yet known), it has been attempted to predict the combinatorial, yet not temporal, patterns of odour-evoked activity along the olfactory pathways of the larva (Masuda-Nakagawa et al., 2009). Still, how larvae actually perceive odours remains unknown. Here we make an attempt in this direction. Paramount to our approach is to *not* directly ask how the larvae perceive a given odour (because we did not

expect an answer), but rather to ask whether the larvae perceive two given odours as different *from each other*.

Figure 1

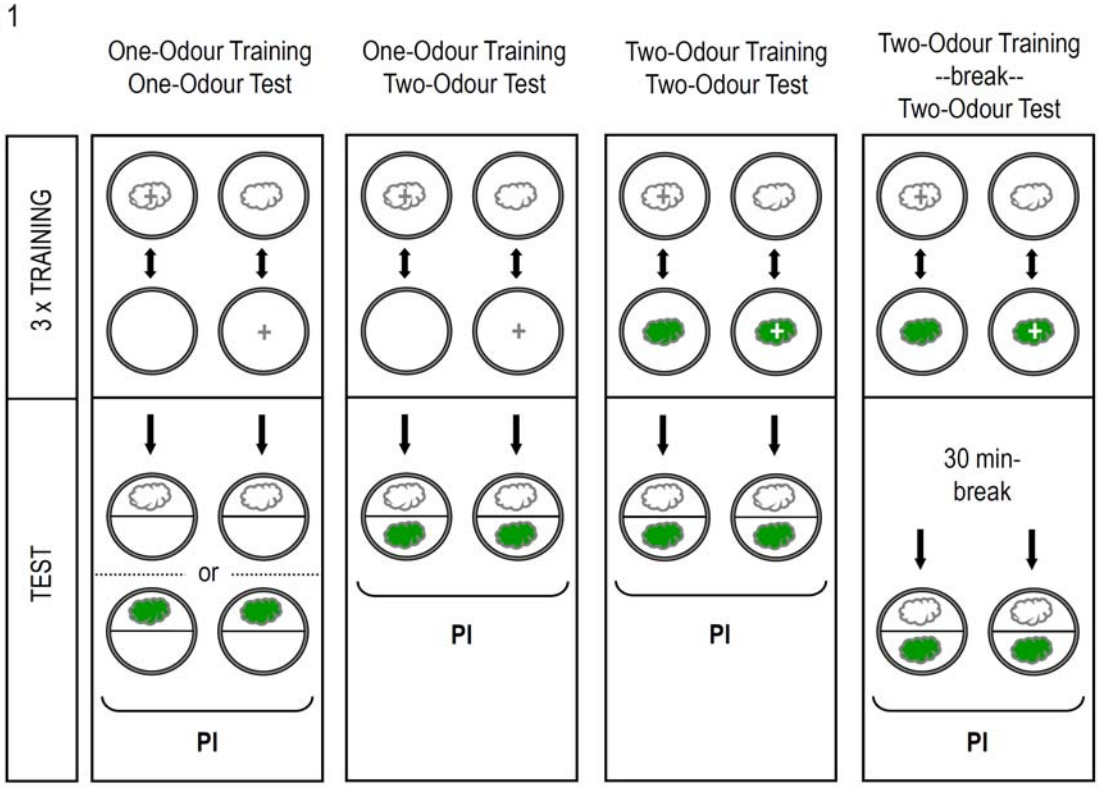

Figure 1 The rationale of the learning tasks

In Task (i), larvae are trained to associate an odour with a sugar reward, and are tested for their approach to either that trained odour or to a novel, not previously trained odour afterwards. In Task (ii), animals are trained to associate an odour with a sugar reward and are tested for their choice between that trained odour *versus* a novel odour. In Task (iii), larvae are trained differentially and tested for their choice between the previously rewarded *versus* the previously non-rewarded odour; the same procedure is employed in Task (iv), except that an additional retention period of 30 min is introduced.

Using four kinds of recognition task (Fig. 1), we seek to come up with one, task-independent estimate of perceived difference between ten odour-pairs. The tasks are: (i) we train larvae to associate an odour with a food reward, and then test whether, in a subsequent test, they would regard another odour as the same as the trained one; (ii) we train larvae to associate an odour with a food reward, and then test in a choice situation whether they can tell the trained odour from a novel, non-trained odour; (iii) we train larvae differentially to associate one, but not another odour with a food reward, and then test in a choice situation whether they can tell the previously rewarded from the

previously non-rewarded odour; (iv) in an experiment alike (iii), we test the larvae after an additional 30min-break.

Figure 2 A B

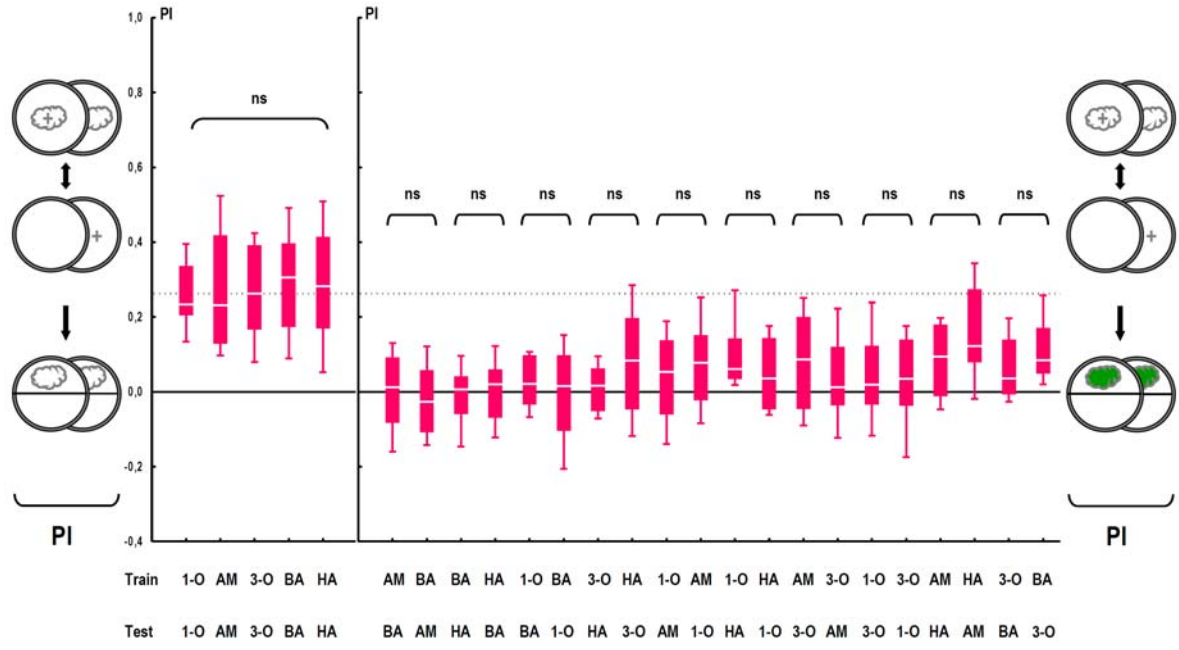

Figure 2 Symmetry of perceived distances

Associative Performance Indices (PIs) are presented depending on the combination of TRAINing *versus* TESTing odour (1-octanol: 1-O, *n*-amyl acetate: AM, 3-octanol: 3-O, benzaldehyde: BA, hexyl acetate: HA).

(A) Larvae are tested with the trained odour, yielding the same level of Performance Indices across the five odours used. Sample sizes are from left to right: 28, 28, 28, 44, 48. ns: KW-test, $P > 0.05$.

(B) Larvae are tested with a novel, not previously trained odour, yielding Performance Indices generally below the stippled line, i.e. below the median of the pooled data from (A). Note that Performance Indices are symmetrical in all cases: Scores are equal when e.g. AM is trained and BA is tested as in the case when BA is trained, and AM is tested. Sample sizes are from left to right: 12, 12, 12, 12, 12, 12, 12, 12, 28, 28, 12, 16, 28, 28, 28, 28, 12, 12, 12, 12. ns: MW-tests, $P > 0.05/10$ (Bonferroni correction).

For the underlying preference data, see Fig. S1.

Data are presented as box plots with the middle line as the median, box boundaries and whiskers as 25 / 75 and 10 / 90 % quantiles, respectively.

A distinguishing feature of our approach (as compared to Boyle and Cobb, 2005; Cobb and Domain, 2000; Guerrieri et al., 2005; Kreher et al., 2008) is that we choose odour dilutions on a behavioural, rather than physical, basis. That is, we were adjusting odour

dilutions for equal learnability (Fig. 2A), rather than using the same dilution for all odours. Why would this be important? Suppose we would use odour dilutions in task (i) such that a given odour A would be learnt well, whereas odour B would be less well learnable if the same dilution is used. Thus, after training with A, we may find strong learnt attraction to B, because A and B are to some extent similar, and because the memory for A is strong. In turn, after training with B, learnt attraction to A may be low, simply because the memory for B is weak- and *although* A and B actually are regarded as similar by the larvae. This would entail an apparent asymmetry of similarity judgments, which as we argue here complicates interpretation of previous approaches towards odour similarity (Boyle and Cobb, 2005; Cobb and Domain, 2000; Guerrieri et al., 2005; Kreher et al., 2008). Symmetry is an essential property for a metric in the mathematical sense (the distance between X and Y must be equal to the distance between Y and X). Odor similarity metrics based on physicochemical properties of the odorant molecules, or on odour-evoked physiological activity patterns, fulfil this criterion. Thus, in order to be comparable with such metrics, symmetric measures of perceptual similarity are indispensable.

In any event, using a recognition-based approach obviously relies on the faculty of the larvae to learn and remember odours and their association with food reward. Given that odour-food memory traces are arguably established in the mushroom bodies (Gerber et al., 2004, 2009; Heisenberg and Gerber, 2008), our approach therefore probes for behaviourally-relevant, central-brain aspects of olfactory perception (this approach had been pioneered by Pavlov [1927; loc. cit. chapter VII], who had attempted to describe the discrimination powers of the ‚cortical analyzers' by means of discrimination-generalization experiments in the dog). We will then discuss whether these aspects of olfactory perception are correlated to physico-chemical properties of the odours.

Materials and methods

We use feeding-stage third-instar larvae of the wild-type Canton-S strain (stock collection, Universität Würzburg), aged 5 days after egg laying. Larvae are maintained in mass culture on standard medium at 25 °C, 60 - 70 % relative humidity and a 14-h:10-h light:dark cycle. All experiments are performed under a fume hood in a regularly lit room at 21 – 26 °C room temperature.

Prior to the learning experiments, the odour stimuli are prepared by adding 10 µl of odour substance into custom-made Teflon containers of 5 mm diameter which are closed by a perforated lid (7 holes, 0.5 mm diameter). As stimuli we use 1-octanol (1-O, Sigma-Aldrich, CAS: 111-87-5), *n*-amyl acetate (synonymous for *n*-pentyl acetate) (AM, Merck, CAS: 628-63-7), 3-octanol (3-O, Merck, CAS: 589-98-0), benzaldehyde (BA, Fluka, CAS: 100-52-7), hexyl acetate (HA, Sigma-Aldrich, CAS: 142-92-7), or an odour container without any odour applied (empty: EM). Odorants are used diluted in paraffin oil (1-O: 1:100; AM: 1:3333; 3-O: $1:10^5$; BA: 1:100; HA: 1:100, unless mentioned otherwise; paraffin oil: CAS: 8012-95-1; Merck, Darmstadt, Germany). The choice of these dilutions is based on a comprehensive description of the dose-dependent learnability of these odours (Mishra et al., in prep): We chose dilutions such that learnability is equal for all odours, and as near as possible to the lowest intensity that supports asymptotic associative performance. Paraffin oil is behaviourally ineffective (Saumweber et al., 2010).

Petri dishes (Sarstedt, Nümbrecht, Germany) of 85-mm diameter are filled either with only 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany), or with agarose containing the sugar reward in addition (+; 2 mol/l fructose, purity 99 %, Roth, Karlsruhe, Germany) which is added to agarose 10 min after boiling. After solidification, Petri dishes are covered with their lids, and left untreated at room temperature until the following day. Before starting experiments, we replace the regular lids of the Petri dishes with lids perforated in the center by fifteen 1-mm holes to improve aeration.

General procedure of the learning experiments

A spoonful of food medium containing larvae is taken from the food vial, 30 animals are collected, briefly rinsed in distilled water and taken as a group used for the experiment. In

all experiments, we train larvae using either of two reciprocal training regimen: for one regimen, animals receive stimulus X with a positive reinforcer (+) and stimulus Y without a reinforcer (Train: X+ // Y; the chemical identity of X and Y as 1-O, AM, 3-O, BA, HA, or EM is mentioned along the Results); for the second regimen, animals are trained reciprocally (Train: X // Y+). Afterwards, animals are tested for their choice between stimulus X *versus* stimulus Y (please note that in half of the cases we start with stimulus X [i.e. X+ // Y and X // Y+], whereas in the other half of the cases we start with stimulus Y [Y // X+ and Y+ // X]). Associative learning is indicated by systematic differences in test performance between the reciprocally trained conditions; these differences are quantified by the Performance Index (PI; see below). This conclusion is compelling as during training animals from both training regimen have identical exposure to both odours and the reward - what differs between them is solely the contingency between these stimuli.

Immediately before a trial, two containers loaded with the same stimulus are placed on opposite sites of the Petri dish, which may or may not contain the sugar reward; animals are transferred to the Petri dish and the lid is closed. After 5 min, animals are transferred to a fresh Petri dish with the alternative stimulus–substrate combination. This training cycle is repeated three times. Fresh Petri dishes are used for each trial. After such training, animals are tested for their choice between two testing stimuli. They are placed in a 7-mm middle stripe of a testing Petri dish; this testing Petri dish does not contain the sugar reward. On either side of the Petri dish we place one odour container, 7 mm from the edge, each loaded with a different stimulus to create a choice situation. For example, in the simplest case (deviations are mentioned in the tasks below and along the Results section), the containers are loaded with stimulus X on one side and stimulus Y on the other side (Test: X -- Y). After 3 min, the number of animals on the X-side, the Y-side and the middle stripe is determined. We then calculate a preference score (PREF) by subtracting the number of animals observed on the Y-side from the number of animals observed on the X-side, divided by the total number of animals (larvae which remain in the 7 mm middle stripe are included in that total) (PREF scores for all experiments are documented in the Supplementary Material):

$$\text{(i)} \qquad PREF_{X+\,//\,Y} = (\#_{\text{Stimulus X}} - \#_{\text{Stimulus Y}}) / \#_{\text{Total}}$$

Then, another group of 30 animals is trained in a reciprocal manner, and the PREF score is determined as:

$$\text{(ii)} \qquad PREF_{X\ //\ Y+} = (\#_{Stimulus\ X} - \#_{Stimulus\ Y}) / \#_{Total}$$

To determine whether preferences are different depending on training regimen, we calculate a performance index (PI) from these two reciprocally trained groups ranging from –1 to 1 as:

$$\text{(iii)} \qquad PI = (PREF_{X+\ //\ Y} - PREF_{X\ //\ Y+}) / 2$$

Positive PIs thus indicate conditioned approach, negative PIs represent conditioned avoidance. Data from experimental conditions to be compared statistically are obtained in parallel. Larvae are trained and tested only once.

Features of the learning tasks

According to this general principle, a series of generalization-discrimination types of learning task is performed:

(i) In a 5 x 5 generalization-type of task, larvae are trained with any one of the five odour stimuli against EM. Afterwards, they are tested either for their choice between the trained odour *versus* EM, or for any one of the four remaining non-trained odours *versus* EM. An abbreviated form for this task may thus read as:

Train: X // EM
Test: X -- EM (or Y -- EM)

Thus, the larger the perceptual distance between X and Y is, the less conditioned behaviour towards Y we should observe (i.e. the smaller PI scores for Y should be). Note that this logic is valid only if odour intensities are adjusted for equal learnability (the same caveat also applies to the tasks ii- iv below).

(ii) Larvae are trained as in the previous task, but are tested in a two-odour choice situation between the trained *versus* any of the four non-trained odours:

Train: X // EM
Test: X -- Y

Thus, the larger perceptual distance between X and Y is, the more conditioned behaviour towards X we should observe (i.e. the larger PI scores should be).

(iii) In a discrimination-type of task, larvae are trained differentially between two odours and then are tested for their choice between them in a two-odour choice situation:

Train: X // Y
Test: X -- Y

Thus, the larger perceptual distance between X and Y is, the more conditioned behaviour we should obtain (i.e., the larger PI scores should be).

(iv) Larvae are trained and tested as in (iii) but testing takes place only after an additional 30-min break during which larvae are kept with few drops of water in an otherwise blank Petri dish:

Train: X // Y
30 min-break
Test: X -- Y

Again, the larger perceptual distance between X and Y is, the more conditioned behaviour we should obtain (i.e., the larger PI scores should be).

Data acquisition and statistics

Data are presented as box plots with the bold line showing the median, the 25 / 75 % and the 10 / 90 % quantiles as box boundaries and whiskers, respectively. Sample sizes are represented within the figure legends.

In a conservative approach, non-parametric analyses are performed; for multiple-group comparisons, we use Kruskal-Wallis (KW) tests, and for two-group comparisons Mann-Whitney U (MW) tests are performed. Significance of differences is assigned if $P < 0.05$. When multiple tests are performed within one experiment, we correct the significance level by dividing the P value of 0.05 according to the number of comparisons made (Bonferroni-correction) to maintain an experiment-wide error rate at 5 %; if e.g. three such comparisons are made, $P < 0.05/ 3$ is applied.

Spearman's rank correlation provides a distribution test of dependence between behavioural and chemical odour similarities.

All statistical analyses are performed with Statistica 7.0 (Statsoft).

Experimenters are blind with respect to treatment condition (reward status of the Petri dishes).

Results

The rationale of the experiments is to ask whether larvae perceive a test odour *as the same* as a previously trained odour. For this purpose, we first present the results of four independent recognition tasks, and then combine these results into one comprehensive, task-independent score of perceived odour distance.

Task (i)

Larvae are trained to associate an odour with a sugar reward and are tested for their approach either to that trained odour or to a novel, previously non-trained odour (see sketches in Fig.s 1, 2). Importantly, all five employed odours are equally learnable, yielding associative performance indices of about 0.3 (stippled line in Fig. 2A; KW-test: H= 1.07, P= 0.90; N= 28, 28, 28, 44, 48). When non-trained odours are used for testing,

performance indices are generally lower (Fig. 2B); for example, if AM is trained and BA is tested, performance indices are indistinguishable from zero, arguing that AM and BA are perceptually distinct to the larvae. Notably, these measures of perceptual distance are in all cases symmetrical: for instance, the performance indices of larvae trained with AM and tested with BA is as low as when BA is trained and AM is tested (Fig. 2B; MW-test: U= 63, *P*= 0.60; N= 12, 12); the same result we find for all other odour pairs as well (Fig. 2B). Therefore, we pool these respective subgroups (Fig. 3A). It turns out that performance indices differ among odour pairs, meaning that perceived distances (black arrows in Fig. 3A) are different among odour pairs (Fig. 3A; KW-test: H= 20.68, *P*< 0.05; N= 24, 24, 24, 24, 56, 28, 56, 56, 24, 24). In a conservative approach, we assign ranks to the perceived distances thus obtained (see Table 1); we note that odour pair AM-BA yields the highest perceptual distance, and odour pair AM-HA the lowest perceptual distance for the larvae- with respect of this kind of learning task.

Figure 3A

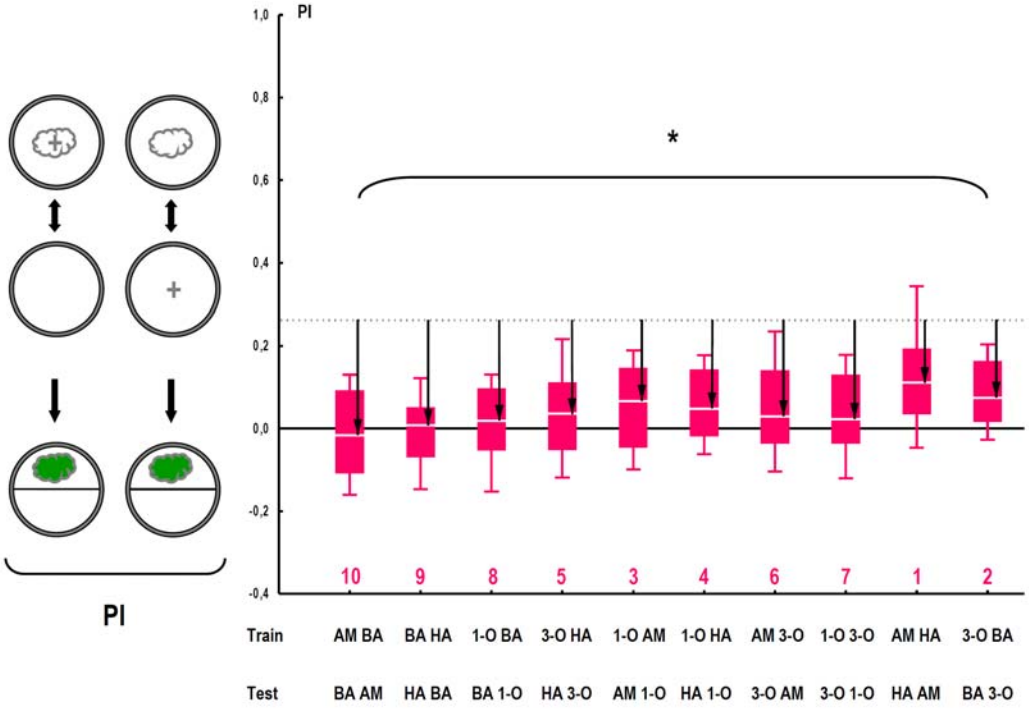

Figure 3B

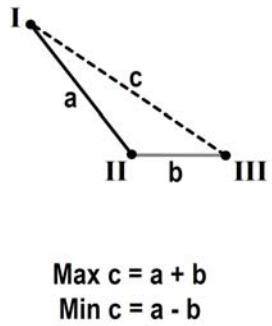

Figure 3C

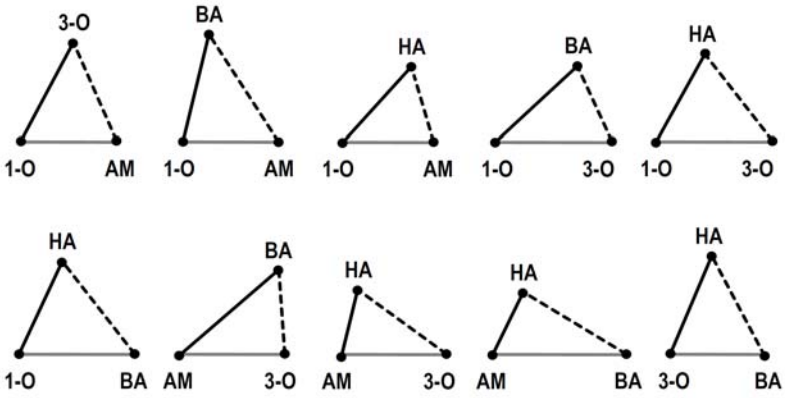

Figure 3 Odour pairs differ in perceived distances in an one-odour training, one-odour test task

(A) Re-presenting the pooled data from Figure 2. The stippled grey line shows the level of Performance Indices when TRAINing and TESTing odour are actually the same (pooled from Fig. 2A). The more different larvae regard the TESTing odour from the TRAINing odour, the smaller Performance Indices should be observed; this is quantified by the 'distance' arrows. Note that Performance Indices differ among odour pairs, indicating that perceived distances are different among odour pairs. Sample sizes are from left to right: 24, 24, 24, 24, 56, 28, 56, 56, 24, 24. *: KW-test: $P < 0.05$.

The numbers below the plots refer to the distance-rank of the respective odour pair (see Table 1). Other details are described in Fig. 2.

(B) Sketch to describe the minimal-maximal range of distances between odours I and III in relation to known distances between odours

I-II, as well as odours II-III.

(C) For all 10 sets of 3 odours, the distances form (A) can be represented as triangles, arguing that the consistency-criterion in (B) is met. The same holds true for 29 out of the 30 additional cases using the data from Figures 4, 5, and 6 (exception being triplet AM-3O-BA in task ii).

Considering perceived distances among three, rather than two odours, our results allow us to consistently describe distances for odour triplets (Fig. 3B). That is, for cases of known distance between odours I-II and odours II-III, the maximal distance between odour I and odour III is given by the sum of the I-II plus the II-III distance, whereas the minimal distance between I and III is given by the difference between the I-II distance minus the II-III distance. This is indeed the case for all 10 triplets (Fig. 3C), arguing for the internal consistency of the obtained perceptual distances. Similar analyses of the data from tasks ii, iii, and iv yield the same conclusion for 29/30 cases (not shown; the exception is the AM-3O-BA triplet in task ii).

Task (ii)

We train larvae to associate an odour with a sugar reward and test their choice between that trained odour *versus* a novel odour (see sketches in Fig.s 1, 4). If larvae regard these two odours as similar, i.e. if perceived distance is low, they should distribute equally between both odours in the test situation, resulting in low performance indices. We note that also for this experiment, performance indices are symmetrical, such that for example the performance index in the case when choice between AM-BA is tested after AM training is as high as in the case when the same choice is offered after BA training (Fig. S2A; MW-test: U= 57, *P*= 0.39; N= 12, 12); the same is found for all other odour pairs as well (Fig. S2A). Therefore, we can pool these respective subgroups; we find that performance indices are different among odour pairs (Fig. 4; KW-test: H= 17.19, *P*< 0.05; N= 24 in all cases), arguing that perceived distances also differ between odour pairs in this task. For example, the odour pair AM-BA yields the highest performance indices, and hence the largest perceived distance, whereas for the odour pair 3-O and BA we find the smallest perceptual distance (black arrows in Fig. 4). Again, we assign ranks to the odour pairs according to these perceived distances (Table 1).

Table 1

Odour pair	Task i PD	Rank	Task ii PD	Rank	Task iii PD	Rank	Task iv PD	Rank	Median Rank
AM vs. BA	0.279	10	0.171	10	0.389	8	0.225	4	9
BA vs. HA	0.254	9	0.148	9	0.545	10	0.261	7	9
1-OCT vs. BA	0.244	8	0.132*	6	0.417	9	0.240	5	7
3-OCT vs. HA	0.227	5	0.144	8	0.327	5	0.263	8	6.5
1-OCT vs. AM	0.196	3	0.132#	5	0.359	7	0.280	10	6
1-OCT vs. HA	0.215	4	0.143	7	0.355	6	0.222	3	5
AM vs. 3-OCT	0.233	6	0.102	2	0.263	3	0.264	9	4.5
1-OCT vs. 3-OCT	0.240	7	0.120	4	0.272	4	0.221	2	4
AM vs. HA	0.151	1	0.116	3	0.215	2	0.246	6	2.5
3-OCT vs. BA	0.188	2	0.027	1	0.146	1	0.139	1	1

* 0.1327
# 0.1326

Table 1 Ranks of perceived distance

For each of the four tasks, we assign the indicated odour pair a rank based on perceived distance (arrows in Fig. 3- 6); the right-most column presents the median of the obtained ranks for the respective odour pair.

Figure 4

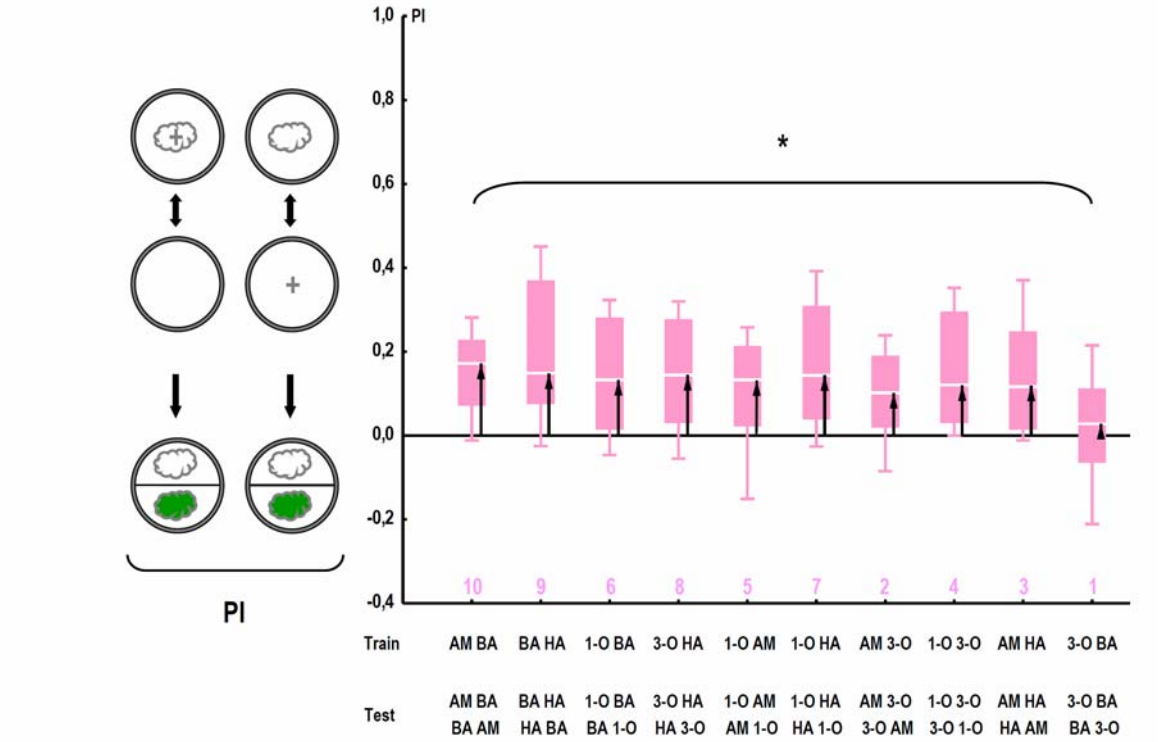

Figure 4 Odour pairs differ in perceived distances in an one-odour training, two-odour test task

Larvae are trained to associate one given odour, and then are offered a choice between this trained odour *versus* a novel odour. The more different larvae regard both odours, the larger Performance Indices would be observed; perceived distances can thus be estimated as indicated by the arrows. *: KW-test: $P< 0.05$; N= 24 in all cases.

For the underlying preference data, see Fig. S2.

The numbers below the plots refer to the distance-rank of the respective odour pair (see Table 1). Other details are described in Fig. 2.

Task (iii)

Larvae are trained to discriminate two odours such that one odour is paired with a sugar reward, whereas the other odour is presented without reward; at test, larvae are given the choice between these two odours (see sketches in Fig.s 1, 5). If the two odours are similar to the larvae, we expect low performance indices. We find that performance indices differ among odour pairs (Fig. 5; KW-test: H= 55.71, $P< 0.05$; N= 16 in all cases), once more arguing that perceived distances differ among odour pairs. For example, in this task, BA and HA appear as the most distinct pair to the larvae, whereas 3-O and BA appear to be

similar to them. In Table 1, we present the ranks of perceived distances (black arrows in Fig. 5) thus obtained.

Figure 5

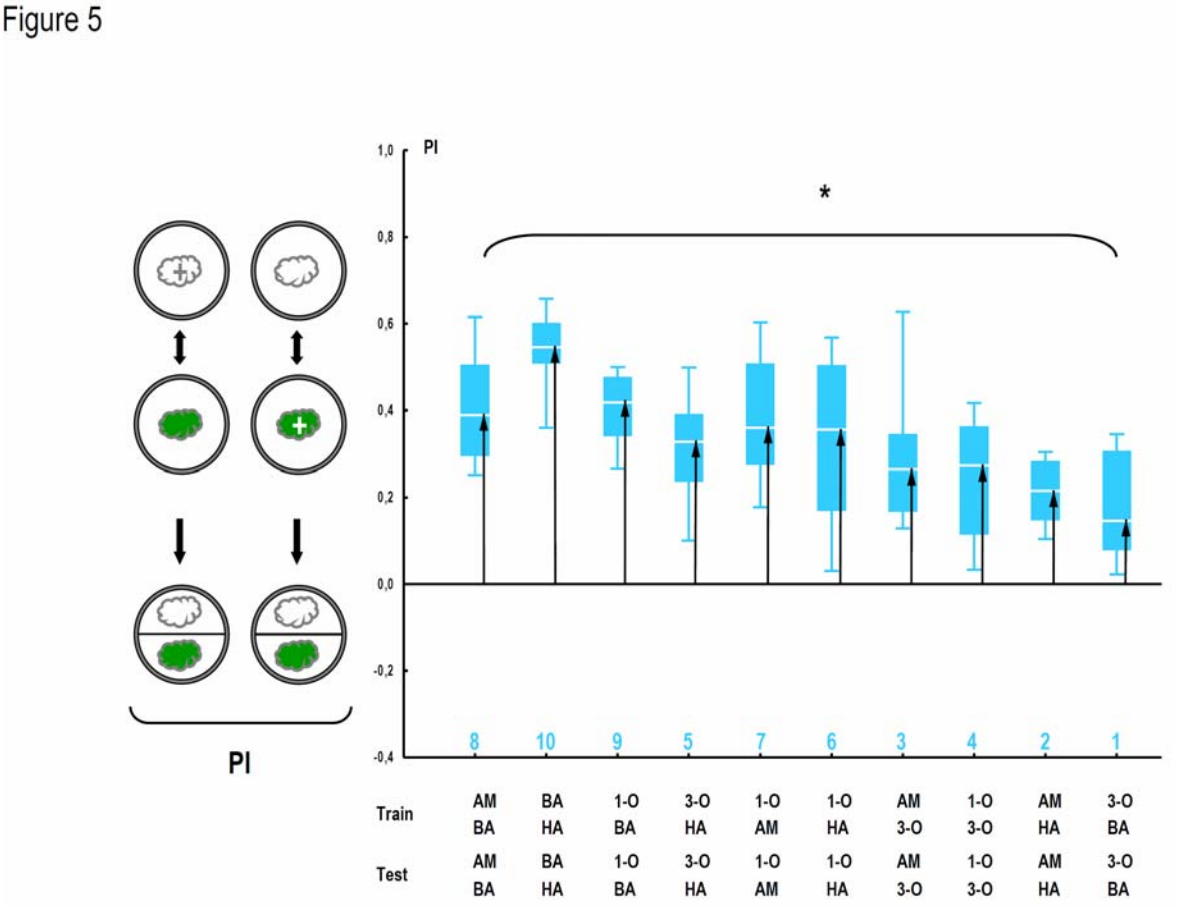

Figure 5 Odour pairs differ in perceived distances in a two-odour training, two-odour test task

Larvae are trained differentially by rewarding one but not the respective odour, and are then offered a choice between the previously rewarded *versus* the previously non-rewarded odour. The more distinct both odours are, the higher Performance Indices we should observe; perceived distance can thus be approximated as indicated by the arrows. *: KW-test: $P< 0.05$; N= 16 in all cases.

For the underlying preference data, see Fig. S3.

The numbers below the plots refer to the distance-rank of the respective odour pair (see Table 1). Other details are described in Fig. 2.

Task (iv)

The procedure of this task is exactly the same as in Task (iii), only that between training and test, an additional retention period of 30 min is introduced (see sketches in Fig.s 1, 6). Notably, in this case, performance indices do not formally differ among groups (Fig. 6; KW-test: H= 6.03, P= 0.74; N= 12 in all cases); in other words, perceived distances

(black arrows in Fig. 6) in this task do not differ between odour pairs. We note that performance indices for some odour pairs apparently decrease from immediate testing to testing after a 30-min retention period; for the odour pair BA and HA as an example, performance indices at 30 min are only about half as compared to immediate testing. For other odour pairs, such as AM and HA, in contrast, levels of performance indices are stable over time (see Smith, 1991 for similar dynamics in bees). In any event, regarding Task (iv) as well, we present the ranks of the obtained perceived distances (black arrows in Fig. 6) in Table 1.

Figure 6

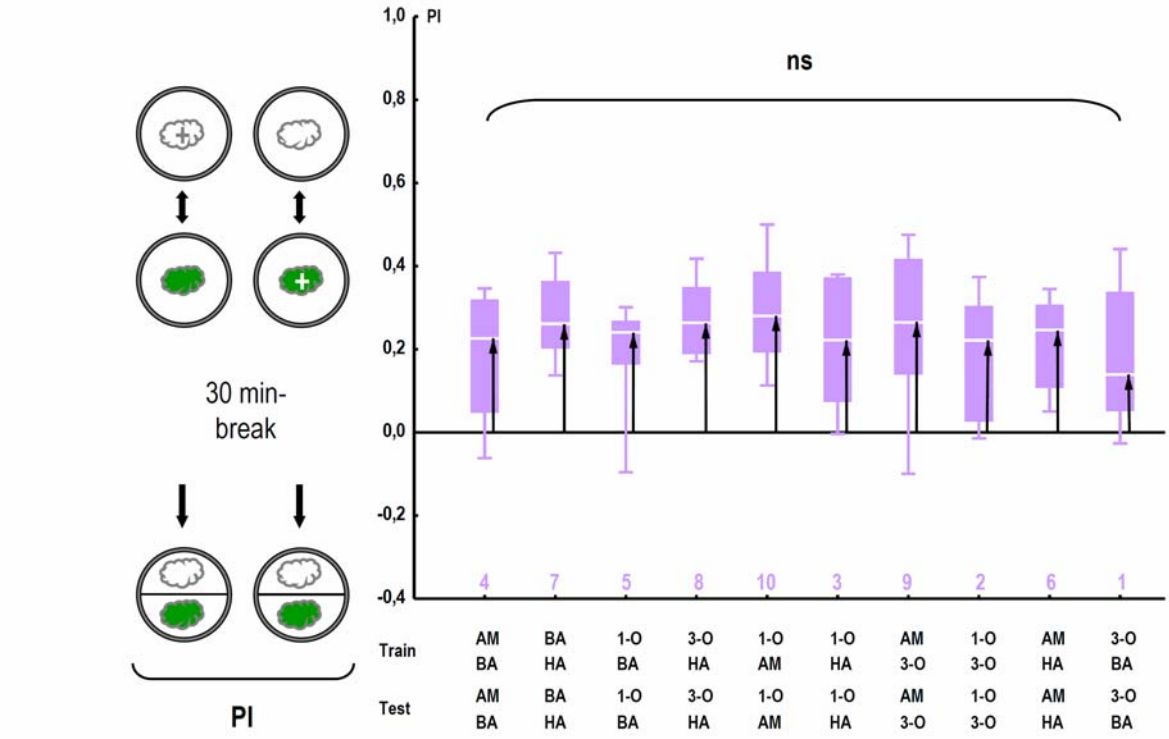

Figure 6 Perceived distances after a 30-min retention period

Larvae are trained and tested differentially in the same way as in the experiment displayed in Fig. 5; however, testing is performed only after an additional 30-min retention period. The arrows indicate perceptual distances; apparently, after this retention period, there are no significant differences among odour pairs in terms of their perceived distances. ns: KW-test: $P > 0.05$; N=12 in all cases.

For the underlying preference data, see Fig. S4.

The numbers below the plots refer to the distance-rank of the respective odour pair (see Table 1). Other details are described in Fig. 2.

Ranking perceived distances

Given that the pattern of perceptual distances we find appears fairly concordant across these four tasks, we combine all the data to come up with one, task-independent estimate of perceived distance. For this purpose, we take a conservative approach and use the ranked perceived distance scores from all tasks (Table 1), and present a summary of these ranks as a box plot in Figure 7. It turns out that these ranks differ among odour pairs (Fig. 7; KW-test: H= 22.22, $P< 0.05$; N= 4 in all cases), arguing that, irrespective of the task used, odour pairs are reliably different in their pair-wise perceived distances.

Figure 7

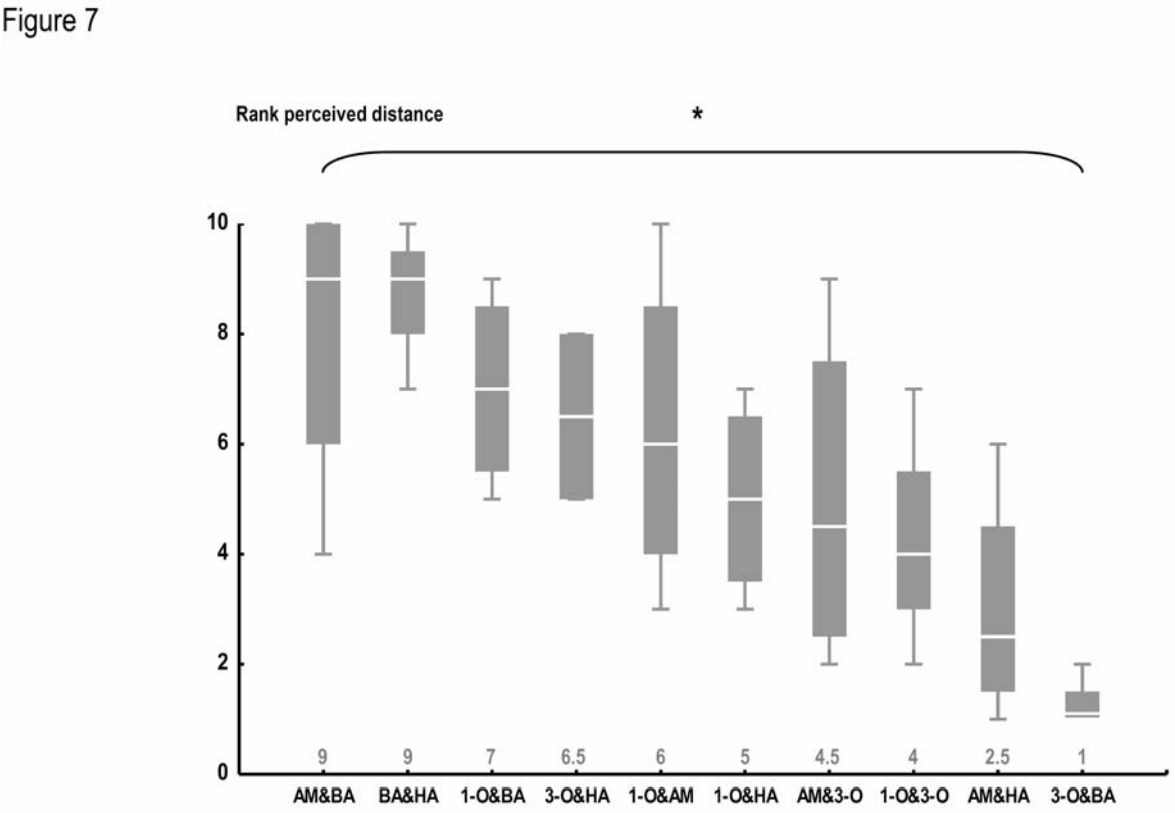

Figure 7 Estimating task-independent perceptual distances among odours

On the basis of the ranks in perceptual distances (Table 1), each box plot represents four combined perceptual distance ranks for each odour pair. *: KW-test: $P< 0.05$. N= 4 in all cases.

Discussion

Task-independence of odour distance

This study used four independent associative recognition tasks (Fig. 1) (Fig.s 3-6) in an attempt to provide a task-independent measure of perceived distance for ten odour pairs. We find that e.g. 3-O and BA consistently turn out as least distinct (i.e. most similar) in behaviour: Considering the ten odour pairs and all four tasks, there is a significant difference in perceived distances between odour-pairs (Fig. 7), meaning that our approach indeed could reveal consistent perceived distances between the ten odour pairs across all four tasks. This conclusion is in line with data from Niewalda et al. (in prep.) using recognition experiments after odour-shock training in adult flies.

As a drastic exception to this rule of task-independence, we have recently found (Mishra et al., 2010) that 3-O can be discriminated well from 1-octen-3-ol if larvae had been trained discriminatively, i.e. by rewarding one but not the other odour (task iii). On the other hand, no odour-specificity is observed after non-discriminative training (task i). That is, for this odour pair there is both strong discrimination and full generalization. If the test involves a choice between these two odours, larvae show conditioned preference for the rewarded odour if training had been performed discriminatively (task iii), but not if training had not been performed discriminatively (task ii). In other words, for 3-O and 1-octen-3-ol only discrimination training confers an odour-specific memory trace, whereas one-odour training does not. This means that, at least for 3-O and 1-octen-3-ol which have strongly overlapping electrophysiological activation profiles (Kreher et al., 2008), there is a degree of freedom in the olfactory system that allows enhancing or ignoring differences between odours flexibly, depending on the task.

Obviously, however, there is no perfect concordance among tasks. When we probe for correlations between tasks in ranks of perceived distance, we find a significant correlation only between task ii and task iii (Spearman's rank correlation: R= 0.82, $P< 0.05/ 6$; N= 10); this suggests that both the nature of the test situation (one-odour test / two-odour test), and the training-to-test interval (immediate / 30-min break) can modify the larvae´s odour distance 'landscape' to some extent. Regarding the training-to-test interval, we note that statistically speaking 30 min after training all odour pairs appear equally distant to the larvae; thus, in addition to an over-all decrease in associative scores between immediate

testing and testing after a 30-min break, it seems that memory is losing specificity over time. Interestingly, the data of Niewalda et al. (in prep.) suggest similar effects of the training-to-test interval for odour-shock associations in adult *Drosophila*. While in particular this loss of specificity is an interesting phenomenon from a mnemonic perspective, this practically means that longer-term memory assays should rather be avoided in future attempts to characterize the odour space in *Drosophila*.

Taken together, as a rule, associative odour recognition seems to draw upon a given, stable representation of the odours such that the features of the behavioural regimen are of little influence. Still, given that there are obvious and drastic exceptions to this rule, as mentioned above for 3-O and 1-octen-3-ol, and given some variance between the results obtained by different tasks, we do not believe there is any one best solution to estimate perceived distance from behavioural experiments. Rather, we believe it is wise to use more than one behavioural task to 'distil' the stable perceptual distances between odour pairs. Clearly, the labour invested in using multiple behavioural tasks then has to be traded off with the number of odour pairs one can include in the analysis.

Physico-chemical distances

Given the fair concordance of perceived distances across tasks, we wonder whether the physico-chemical properties of the odours might be a determinant for these perceived distances. To this end, we follow the approaches by Schmuker et al. (2007) and Haddad et al. (2008) (Table 2). In the Schmuker et al. (2007) approach, a set of 184 physico-chemical descriptors is calculated using the MOE software (Chemical Computing Group, Montreal, Canada). Descriptors are normalized to zero mean and unit variance. Distances are calculated using the sum of absolute coordinate differences (Manhattan or city-block metric) and are reported in Table 2. In the Haddad et al. (2008) approach, each odour structure is obtained from PubChem (http://pubchem.ncbi.nlm.nih.goc) and entered into the Dragon software (http://www.talete.mi.it/products/dragon_description.htm). Then, each odour is represented as a vector of 1664 molecular descriptor values. For the respective odour pairs we obtain the distance values as displayed in Table 2.

We then assign ranks to the odour pairs according to the respective physico-chemical distance values obtained (Table 2). In Figure 8, we can thus plot the ranks of perceived

distance *versus* the ranks of physico-chemical distance. When considering the combined dataset, i.e. when treating the results of the Schmuker et al. (2007) and the Haddad et al. (2008) approaches as independent approaches, we find a just-significant correlation between physico-chemical and perceived distance (Fig. 8C; Spearman's rank correlation: R= 0.45, *P*= 0.04; N= 20) (within each of these two approaches, only trends for such correlations are observed [Fig. 8A; Spearman's rank correlation: R= 0.41, *P*= 0.24; N= 10; Fig. 8B; Spearman's rank correlation: R= 0.48, *P*= 0.16; N= 10]). This suggests that, as a rule, small differences in the physico-chemical properties of odours entail small differences in perception, and that associative memory trace formation and associative recognition draw upon these task-invariant percepts. Still, we should note that both Schmuker et al. (2007) and Haddad et al. (2008) implicitly assume odour intensity, which can be a profound determinant of olfactory perception, to be equal. However, meeting this assumption in behavioural experiments is not trivial and requires experimental scrutiny to adjust odour dilutions for equal effectiveness in the respective behavioural task.

Table 2

Odour pair	**Distance** Schmuker et al.	**Rank**	Distance Haddad et al.	Rank
AM vs. BA	**169.66**	**7**	34.17	7
BA vs. HA	**189.01**	**8**	38.80	9
1-OCT vs. BA	**202.40**	**10**	41.71	10
3-OCT vs. HA	**88.76**	**4**	18.31	3
1-OCT vs. AM	**94.33**	**5**	22.02	6
1-OCT vs. HA	**80.16**	**3**	19.35	4
AM vs. 3-OCT	**94.61**	**6**	16.50	2
1-OCT vs. 3-OCT	**28.57**	**2**	19.65	5
AM vs. HA	**25.74**	**1**	11.99	1
3-OCT vs. BA	**197.45**	**9**	37.08	8

Table 2 Physico-chemical distances between odours

Physico-chemical distance values for odour pairs are determined according to Schmuker et al. (2007) and Haddad et al. (2008), respectively. Within each approach, odour pairs are assigned ranks according to the respective values obtained.

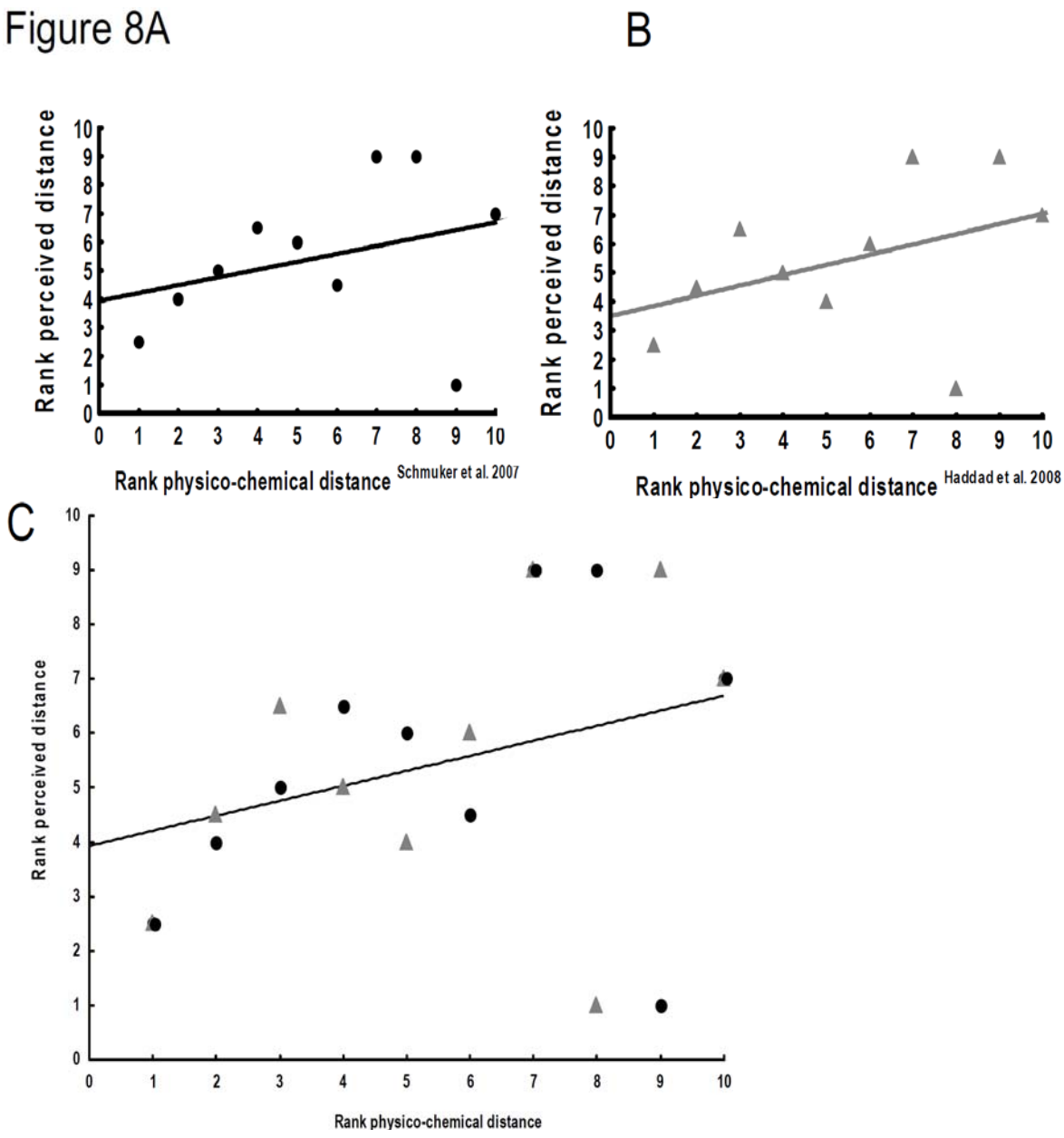

Figure 8 Comparing perceived distances to physico-chemical distances

(A) (B) According to two independent odour-distance metrics (based on Schmuker et al. [2007] and Haddad et al. [2008], respectively) (Table 2), we rank odour pairs according to physico-chemical distance. The plot presents perceived distance ranks on the y-axis and physico-chemical distance ranks on the x-axis. Spearman's rank correlation: R= 0.41, 0.48, *P*= 0.24, 0.16; N= 10, 10 for (A) and (B) respectively.

(C) The combined data from (A) and (B) suggest a just-significant correlation between physio-chemical and perceived odour distances. Spearman's rank correlation: R= 0.45, *P*= 0.048; N= 20.

Physiology

Again, it seems important to draw attention to the exception (see also the discussion in Sell [2006]): The pair 3-O and BA is regarded as most similar by the larvae, across all tasks (Fig.s 3-6; Fig. 7); however, both the Schmuker et al. (2007) and the Haddad et al. (2008) approach agree that these odours are relatively different in their physico-chemical features (Fig. 8C). Interestingly, from an electrophysiological perspective, 3-O and BA appear clearly distinct, too: 3-O activates *Or85c*-expressing olfactory sensory neurons, whereas BA activates *Or45b*-expressing cells (Kreher et al. 2008), a distinctiveness that is maintained even at relatively higher odour concentrations (3-O: *Or13a, Or35a, Or45a, Or47a, Or85c*; BA: *Or7a, Or24a, Or30a, Or45b, Or67b*) and also with regard to inhibition (relatively high concentration, 3-O: *Or22c, Or24a, Or33b;* BA: *Or13a, Or42b, Or82a;* relatively low concentration, 3-O: *Or33b;* BA: *Or33b, Or85c*). Unfortunately, a comprehensive comparison of our behavioural data to the physiology of *Or*-expressing neurons is not possible, because the odour set used by Kreher et al. (2008) does not include data for all odour pairs employed here. In any event, although 3-O and BA are distinct chemically as well as in terms of their sensory representation, the larvae still regard them as the most similar of all the ten odour pairs employed in our study. This suggests a step of 'merging' of both odours at a point between the first-order sensory layer and behavioural control (see Niewalda et al. [in prep.] for a similar suggestion on the basis of a combined behavioural and optical-imaging approach in adult flies). It therefore appears as if, similar to the case of colour vision, for example, relevant aspects of the olfactory percept need to be 'computed' in post-receptor circuits *on the basis of* the sensory signals, rather than being immediately *given* by the sensory signals.

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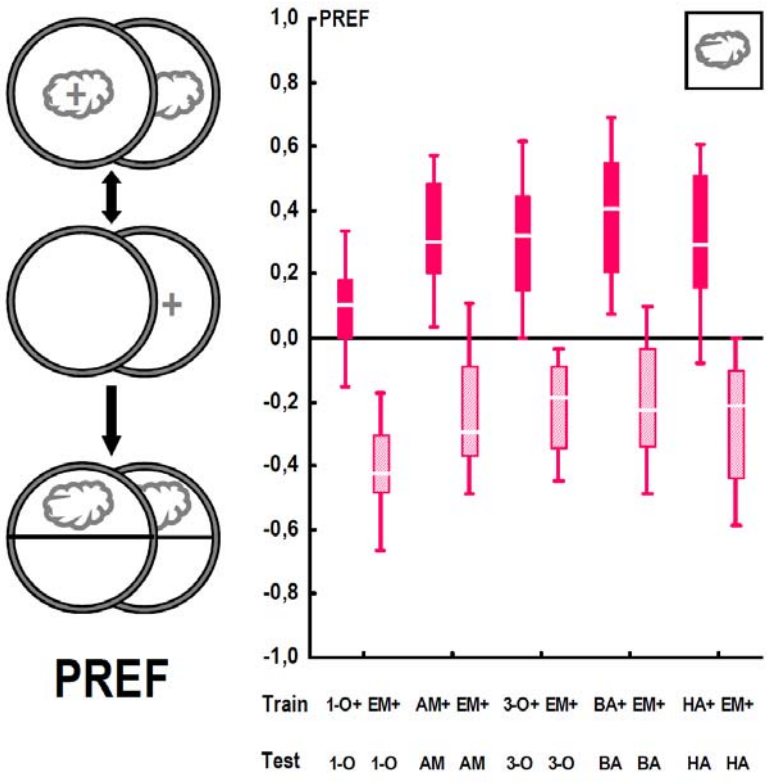
Supplimentary Material

Figure S1A



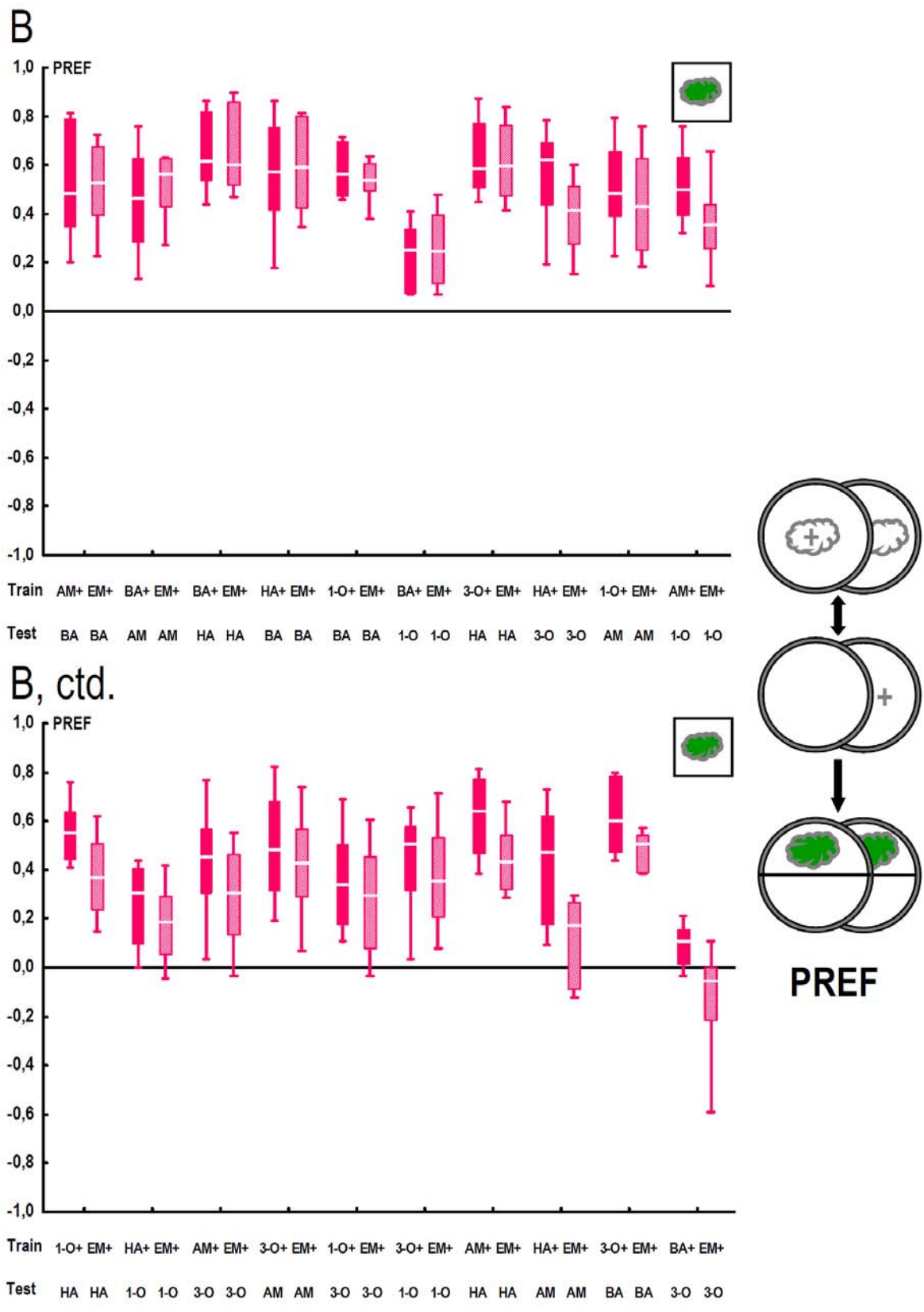
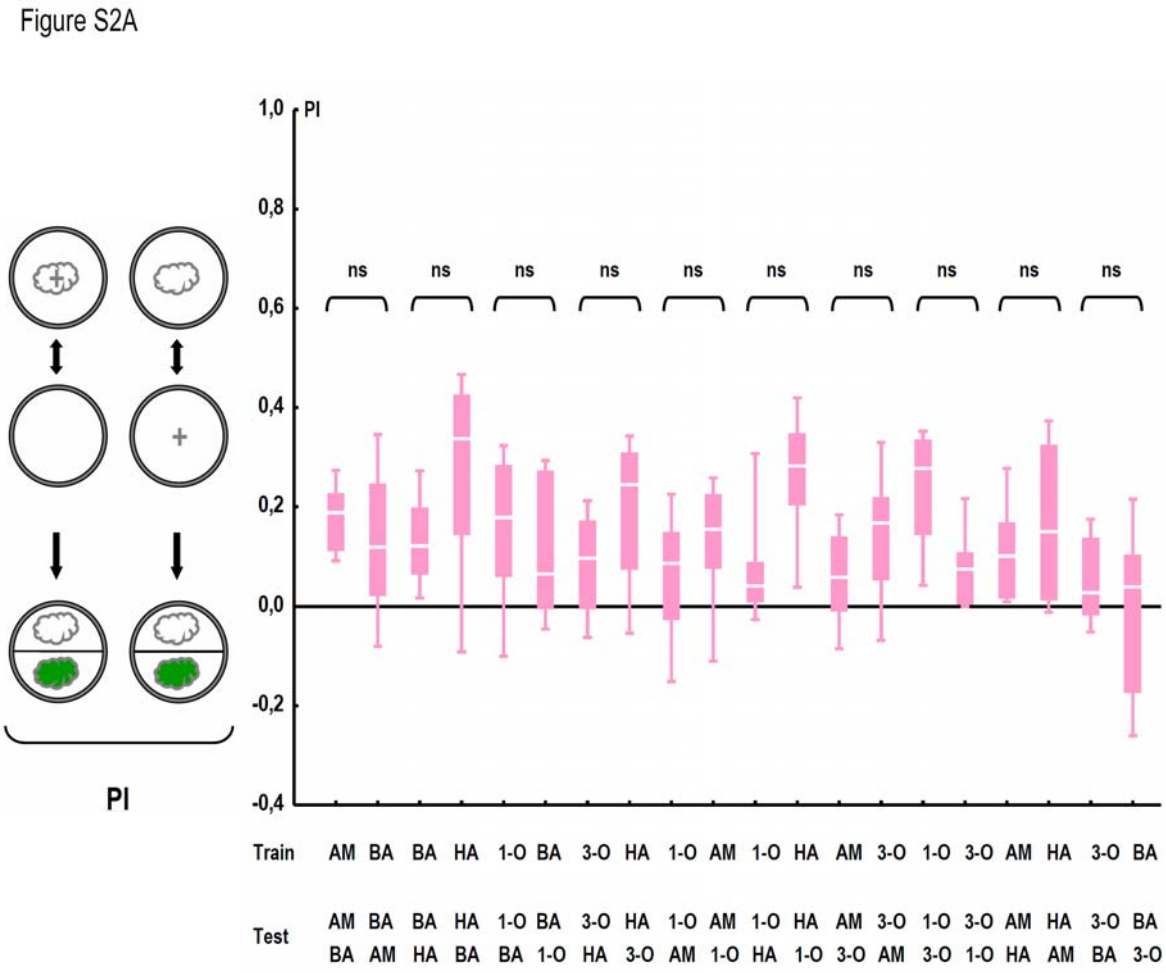

B
PREF
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
-0,6
-0,8
-1,0
Train AM+ EM+ BA+ EM+ BA+ EM+ HA+ EM+ 1-O+ EM+ BA+ EM+ 3-O+ EM+ HA+ EM+ 1-O+ EM+ AM+ EM+
Test BA BA AM AM HA HA BA BA BA BA 1-O 1-O HA HA 3-O 3-O AM AM 1-O 1-O
B, ctd.
PREF
1,0
0,8
0,6
0,4
0,2
0,0
-0,2
-0,4
-0,6
-0,8
-1,0
Train 1-O+ EM+ HA+ EM+ AM+ EM+ 3-O+ EM+ 1-O+ EM+ 3-O+ EM+ AM+ EM+ HA+ EM+ 3-O+ EM+ BA+ EM+
Test HA HA 1-O 1-O 3-O 3-O AM AM 3-O 3-O 1-O 1-O HA HA AM AM BA BA 3-O 3-O
+
+
PREF

Figure S1 Preference data of Task (i)

Preference scores of the reciprocally trained groups of larvae underlying the associative Performance Indices presented in Fig.s 2A, B.

(A) Larvae are tested with the trained odour. Sample sizes are from left to right: 14, 14, 14, 14, 14, 14, 22, 22, 24, 24.

(B) Larvae are tested with a novel, not previously trained odour. Sample sizes are from left to right: 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 6, 14, 14, 14, 14 (for the upper part), and 6, 6, 8, 8, 14, 14, 14, 14, 14, 14, 14, 14, 6, 6, 6, 6, 6, 6, 6, 6 (for the lower part).



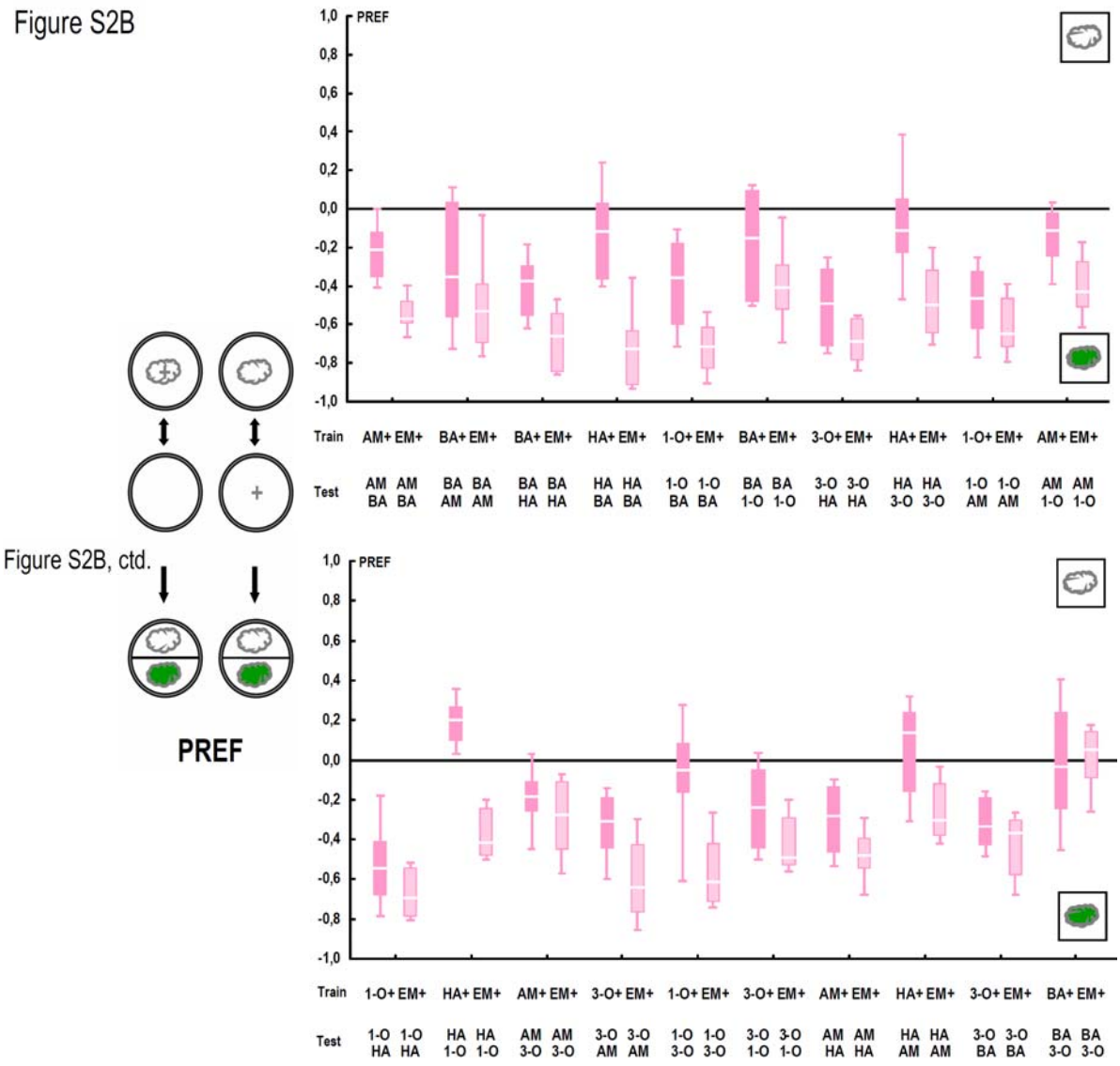


Figure S2 Symmetry of perceived distances (A) and Preference data for Task (ii)

(A) Data from Fig. 4, separated by the combination of TRAINing odour and TESTing odour pair. In all cases, Performance Indices are symmetrical: For example, scores are equal when AM is trained and a choice between AM *versus* BA is tested, as when BA is trained and an AM *versus* BA choice is tested. ns: MW-test, *P*> 0.05/ 10 (Bonferroni correction); N= 12 in all cases. Other details as in Fig. 2.

(B) Preference scores of the reciprocally trained groups of larvae underlying the associative Performance Indices presented in (A). N= 6 in all cases. Please note the strong over-all preference for that odour which had not been presented during the training phase (the 'green odour').

Figure S3

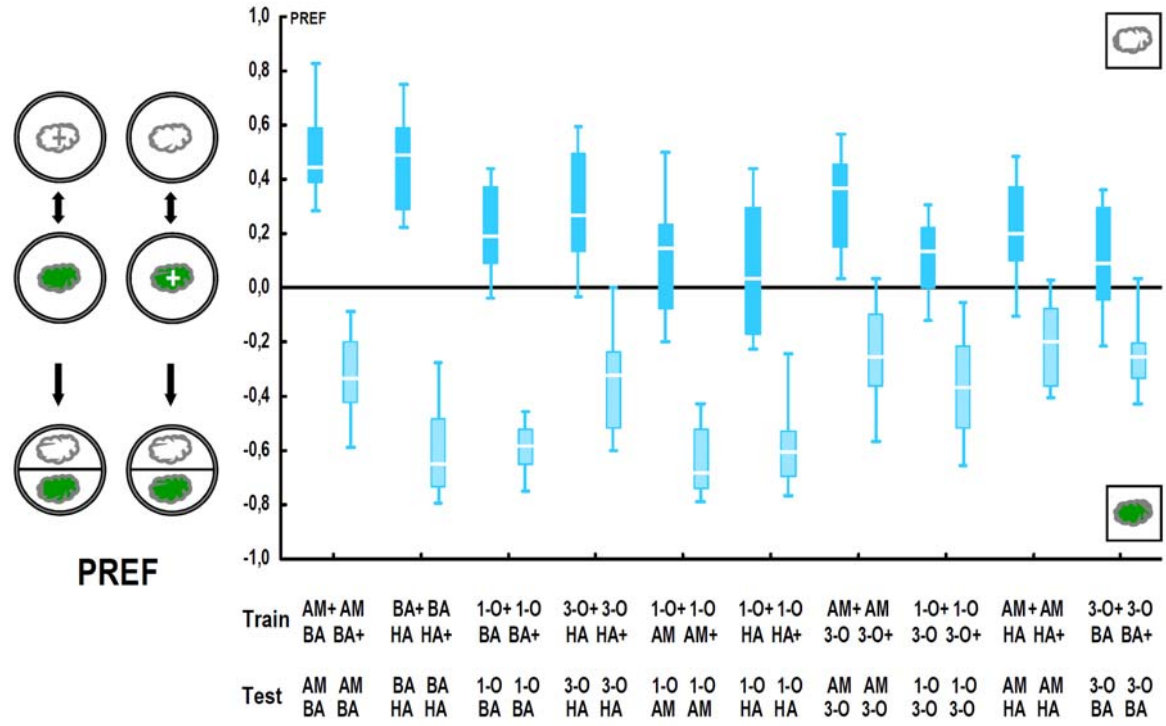


Figure S3 Preference data of Task (iii)

Preference scores of the reciprocally trained groups of larvae as they underlie the associative Performance Indices presented in Fig. 5. N= 8 in all cases.

Figure S4

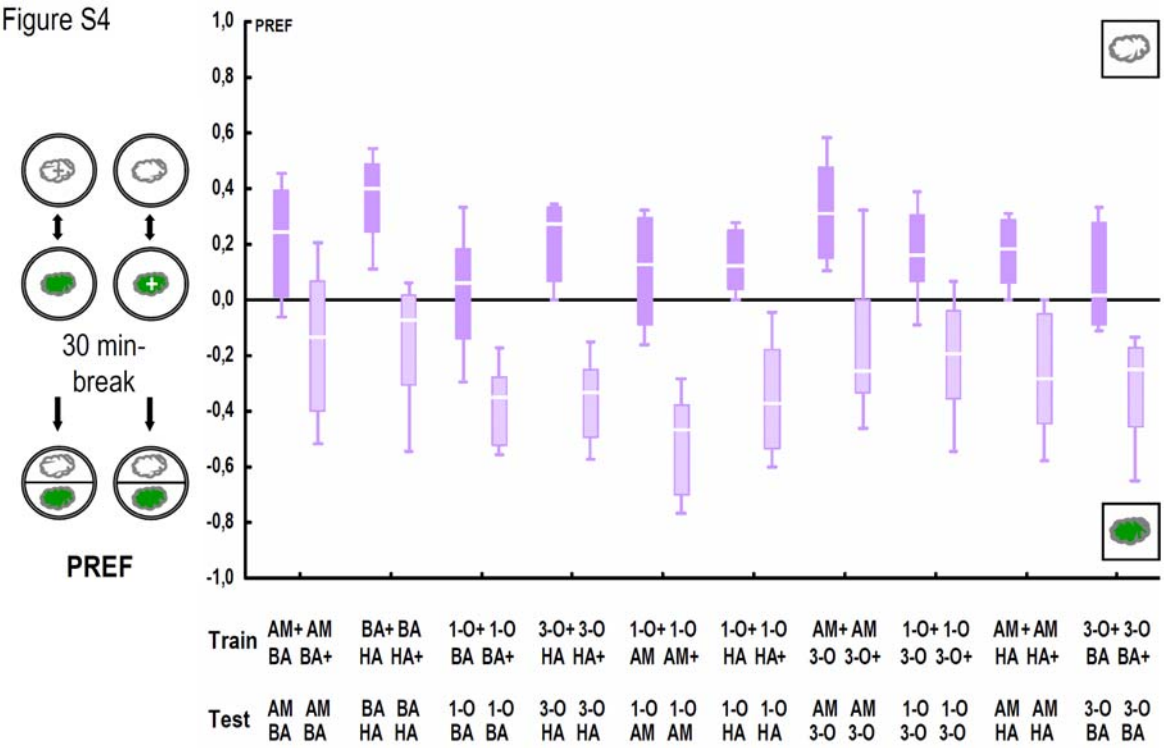


Figure S4 Preference data of Task (iv)

Preference scores of the reciprocally trained groups of larvae as they underlie the associative Performance Indices presented in Fig. 6. N= 6 in all cases.

Figure S5

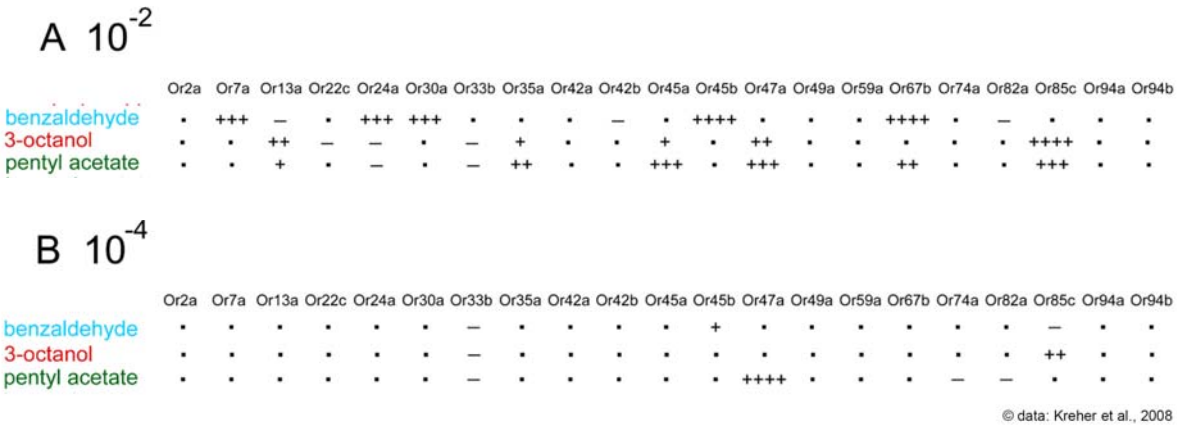


Figure S5 Electrophysiological activity from Kreher et al., 2008

(A) (B) Electrophysiological responses to odorants at 10^{-2} and 10^{-4} dilution respectively, as measured upon expression of the indicated *Or* genes. "•", < 50 spikes/s; "+", 50 - 100 spikes/s; "++", 100 - 150 spikes/s; "+++", 150 - 200 spikes/s; "++++", n ≥ 200 spikes/s; "-" denotes inhibition to ≤ 50% of the spontaneous firing rate. data from Kreher et al., 2008.

Chapter I.3

Odour percepts can be adaptively adjusted

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Adaptive Adjustment of the Generalization-Discrimination Balance in Larval *Drosophila*

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Adaptive adjustment of the generalization-discrimination balance in larval *Drosophila*

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Running head: Generalization-discrimination balance in the larva

Key Words

Generalization, Discrimination, Learning, Olfaction, *Drosophila melanogaster*

ABSTRACT

Learnt predictive behaviour faces a dilemma: predictive stimuli will never 'replay' exactly as during the learning event, requiring generalization. In turn, minute differences can become meaningful, prompting discrimination. To provide a study case for an adaptive adjustment of this generalization-discrimination balance, we ask whether *Drosophila melanogaster* larva are able to either generalize or discriminate between two odours (1-octen-3-ol and 3-octanol), depending on the task. We find that after discriminatively rewarding one but not the other odour, larvae show conditioned preference for the rewarded odour. On the other hand, no odour-specificity is observed after non-discriminative training, even if the test involves a choice between both odours. Thus, for this odour pair at least, discrimination training is required to confer an odour-specific memory trace. This requires that there is at least some difference in processing between the two odours *already at the beginning* of the training. Therefore, as a default, there is a small yet salient difference in processing between 1-octen-3-ol and 3-octanol; this difference is ignored after non-discriminative training (generalization), while it is accentuated by odour-specific reinforcement (discrimination). Given that, as we show, both faculties are lost in anosmic *Or83b*^{1} mutants, this indicates an adaptive adjustment of the generalization-discrimination balance in larval *Drosophila* taking place downstream of *Or83b*-expressing sensory neurons.

INTRODUCTION

The ability to learn and remember is of obvious advantage as it allows predictive behaviour. However, predictive behaviour is haunted by a dilemma (for an early discussion see Pavlov, 1927, loc. cit. chapter VII): no predictive stimulus will ever 'replay' exactly as during the learning phase, so animals have to behave according to likeness, lumping together different inputs to behave equally towards them, despite actual and recognizable differences (generalization). In turn, however, minute differences can be or can become meaningful, such that animals have to behave differently towards them, despite their actual and recognizeable commonalities (discrimination). To get this generalization-discrimination balance right in all cases is a mission impossible: in logical terms, this would amount to minimizing both false-positive and false-negative errors, i.e. to both avoid regarding a stimulus as different from the learnt one although they have the same consequence, *and* to put an effective curb on regarding it as the same although it does not have the same consequences as the learnt one.

To complicate matters further, a reasonable compromise suitable for any one task may not be suitable for another task, dependent on what is at stake: animals should be very little tolerable of not timely escaping a predator when actually there is one, whereas dismissing a food-predicting hint and missing out on a meal may not have dramatic consequences. Obviously, however, even within each of these tasks the most reasonable point of compromise may shift: when starving, any stimulus vaguely similar to a food-predicting hint will be tracked down, because missing out on *that opportunity* for a meal may mean death. Thus, one may expect animals to be able to shift their generalization-discrimination balance in an adaptive way.

To provide a study case for such a readjustment of the generalization-discrimination balance, we chose chemosensory learning of the *Drosophila melanogaster* larva (Neuser et al., 2005; Scherer et al., 2003) because it offers a fortunate combination of genetic tractability, cellular simplicity in terms of cell number, and versatility for behavioural analysis (Gerber & Stocker 2007; Gerber et al., 2009). In particular, we focus on whether the larval olfactory system is flexible enough to either generalize or discriminate between two odours, depending on the task.

MATERIALS AND METHODS

Third instar *Drosophila* larvae (5 days after egg laying) from the Canton Special wild type strain or, as indicated, of the *Or83b*^{1} mutant (Bloomington *Drosophila* stock centre, code: 23129) (Larsson et al., 2004) are used. The *Or83b*^{1} mutation is a protein-null mutation due to replacement gene-targeting (Gong & Golic, 2003) deleting the putative transcription start site and large portions of the coding regions of the gene. The flies are kept in mass culture under a 14/ 10 h light/ dark cycle at 25 ^{o}C and 60-70 % relative humidity. For experiments, a spoon-full of medium containing larvae is taken into an empty Petri dish and 30 larvae are collected and briefly washed in distilled water.

One day prior to the experiment, Petri dishes of 85 mm inner diameter (Sarstedt, Nümbrecht, Germany) are filled either with a solution of 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany) or with 1 % agarose added with 2 M fructose (Roth, Karlsruhe, Germany). Once the agarose solidified, dishes are covered with their lids and left until the following day.

As odours, we use 1-octen-3-ol (1-OCT-3-OL, CAS: 3391-86-4) and 3-octanol (3-OL, CAS: 589-98-0) (Merck, Hohenbrunn, Germany, purity 99 %). These two odours are selected as they induce a similar pattern of activity in the peripheral olfactory system of the larva (Kreher et al., 2008); also, 1-OCT-3-OL is of interest because a 1-OCT-3-OL sensitive olfactory receptor arguably is a target of the insect repellent DEET (Ditzen et al., 2008). Odours are diluted in paraffin oil (Merck, Darmstadt, Germany) at a ratio of 1: 10,000 in the case of 1-octen-3-ol and 1: 100,000 in the case of 3-octanol (these dilutions were chosen as preliminary experiments [Mishra, Chen, Yarali & Gerber, in preparation] had revealed that at these dilutions both odours support equal levels of learning; specifically, the dose-dependencies of learning follow an inverted U-shape for both odours; dilutions were picked at the lowest concentration that supports a performance index [see below] of 0.3, which is below asymptotic levels of the performance index). On the day of the experiment, 10 µl of odour-solution is applied into custom-made Teflon containers with an inner diameter of 5 mm, and a perforated cap with 7 holes of 0.5 mm diameter, each. Containers without any odour added are denoted as empty (EM).

In addition, for the experiment displayed in Figure 4A we use amyl acetate (AM, CAS: 628-63-7, Merck, Darmstadt, Germany, purity 98 %; diluted at a ratio of 1: 10,000 in paraffin oil), benzaldehyde (BA, CAS: 100-52-7, Sigma-Aldrich, Steinheim, Germany, purity 99.5 %; diluted at a ratio of 1: 100 in paraffin oil), as well as a 1: 1000,000 dilution of 3-OL and a 5.6: 100,000 dilution of 1-OCT-3-OL. For this four-odour set, these dilutions yield odour intensities low enough for performance indices remaining below asymptote for all four odours, but high enough to support performance indices of at least 0.2 (Mishra, Chen, Yarali & Gerber, in preparation).

One-odour training, one-odour test

Learning assays are performed under a fume hood at 21- 26 ^{o}C, under the light from a fluorescent lamp. Larvae are trained and tested in groups of 30, using either of two reciprocal training regimen. For example, at the beginning of training, two odour-filled Teflon containers filled with 1-octen-3-ol are placed at opposite sides of a Petri dish containing agarose, added with fructose (+). Larvae are placed in the middle of this dish and left crawling for 5 min. They are then removed to another dish containing two empty Teflon containers (EM) and filled with only agarose, where they also spent 5 min (1-OCT-3-OL+ // EM). This cycle of training is repeated three times, using fresh dishes each time. At the end of this training, larvae are placed in the middle of a dish filled with only agarose; on opposing sides, Teflon containers are placed, one container filled with the trained odour and one empty container (1-OCT-3-OL -- EM) (note that the sidedness of placing these containers is balanced across repetitions of the experiment). After 3 min, larvae on each half of the dish are counted to calculate a preference score (PREF) as:

(i) $$PREF = (\#_{Odour} - \#_{Empty}) / \#_{Total}$$

In this formula, # designates the number of larvae on the corresponding side of the dish. PREF values thus range from -1 to 1; positive values indicate approach to the odour, negative ones reflect avoidance.

Alternately, we train larvae reciprocally (1-OCT-3-OL // EM+) (note that for either reciprocal regimen, the sequence of training trials is balanced across repetitions of the experiment; that is, in half of the cases training is 1-OCT-3-OL+ // EM and in the reciprocal case 1-OCT-3-OL // EM+ as in the example above, and in the other half it is EM // 1-OCT-3-OL+ and EM+ // 1-OCT-3-OL; the same measures are taken for the subsequently described experiments in an analogous manner). An associative performance index (PI) can then be calculated based on the difference in odour preference between the reciprocally trained groups (Hendel et al., 2005):

(ii) $$PI = (PREF_{Odour+ // EM} - PREF_{Odour // EM+}) / 2$$

The subscripts of PREF indicate the respective training regimen. Performance indices thus range from -1 to 1, positive values indicating conditioned approach, whereas negative values indicate conditioned aversion. This reciprocal training procedure is designated in the sketches below Fig. 1A as:

TRAINING 1-OCT-3-OL // EM

TEST 1-OCT-3OL -- EM

The same types of experiment are run for 3-octanol as well:

TRAINING 3-OL // EM

TEST 3-OL -- EM

Thus, the test odour is always the trained odour.

Generalization

To test for generalization between 1-octen-3-ol and 3-octanol, experiments are run as above in that either of these two odours is trained; however, the test odour is always the respective other odour. That is, larvae are trained, *e.g.*, towards 1-octen-3-ol (using the above reciprocal training regimen) but are tested for their preference for 3-octanol:

TRAINING 1-OCT-3-OL // EM

TEST 3-OL -- EM

The same type of experiment is performed for 3-octanol training and 1-octen-3-ol testing:

TRAINING 3-OL // EM

TEST 1-OCT-3-OL -- EM

Thus, the test odour is always 'novel' to the larvae.

Discrimination

To test for discrimination between 1-octen-3-ol and 3-octanol, larvae are trained either such that 1-octen-3-ol is rewarded, but 3-octanol is not (1-OCT-3-OL+ // 3-OL), or such that in the reciprocal group 3-octanol is rewarded, but 1-octen-3-ol is not (1-OCT-3-OL // 3-OL+) (again, the sequence of training trials is balanced across repetitions of the experiment). Then, animals are tested for their relative preference between the two odours, one of them being the previously rewarded, the other one being the previously non-rewarded odour:

TRAINING 1-OCT-3-OL // 3-OL

TEST 1-OCT-3-OL -- 3-OL

One-odour training, two-odour test

To see whether the memory trace established by one-odour training would be sufficiently specific to allow discrimination at test, larvae are trained, *e.g.*, to 1-octen-3-ol (using the reciprocal training regimen detailed above) and then are tested for their relative preference between the two odours:

TRAINING 1-OCT-3-OL // EM

TEST 1-OCT-3-OL -- 3-OL

Or training is for 3-octanol, respectively:

TRAINING 3-OL // EM

TEST 1-OCT-3-OL -- 3-OL

In this regimen, therefore, one of the two tested odours is always the trained odour, the other one is a 'novel' odour.

Preference behaviour of experimentally naive larvae

To measure innate (in the sense of: experimentally naive) olfactory preference behaviour, we determine PREF scores according to equation (i), but of larvae without any prior training.

To measure innate gustatory preference behaviour, we also use experimentally naive larvae. We separate Petri dishes (85 mm inner diameter) into two halves with a piece of overhead transparency, fill one side with only 1 % agarose (PURE) and the other side with 1 % agarose plus 2M fructose. Once the agarose has solidified, we remove the overhead transparency, cover the dishes with their lids and leave them at room temperature until the following day. We place 30 larvae to the middle of the dish, close the lid and after 3 min record the number of larvae (#) on either side of the dish. This allows calculating a gustatory preference index ($PREF_{Gustatory}$) as:

(iii) $PREF_{Gustatory} = (\#_{Fructose} - \#_{Pure}) / \#_{Total}$

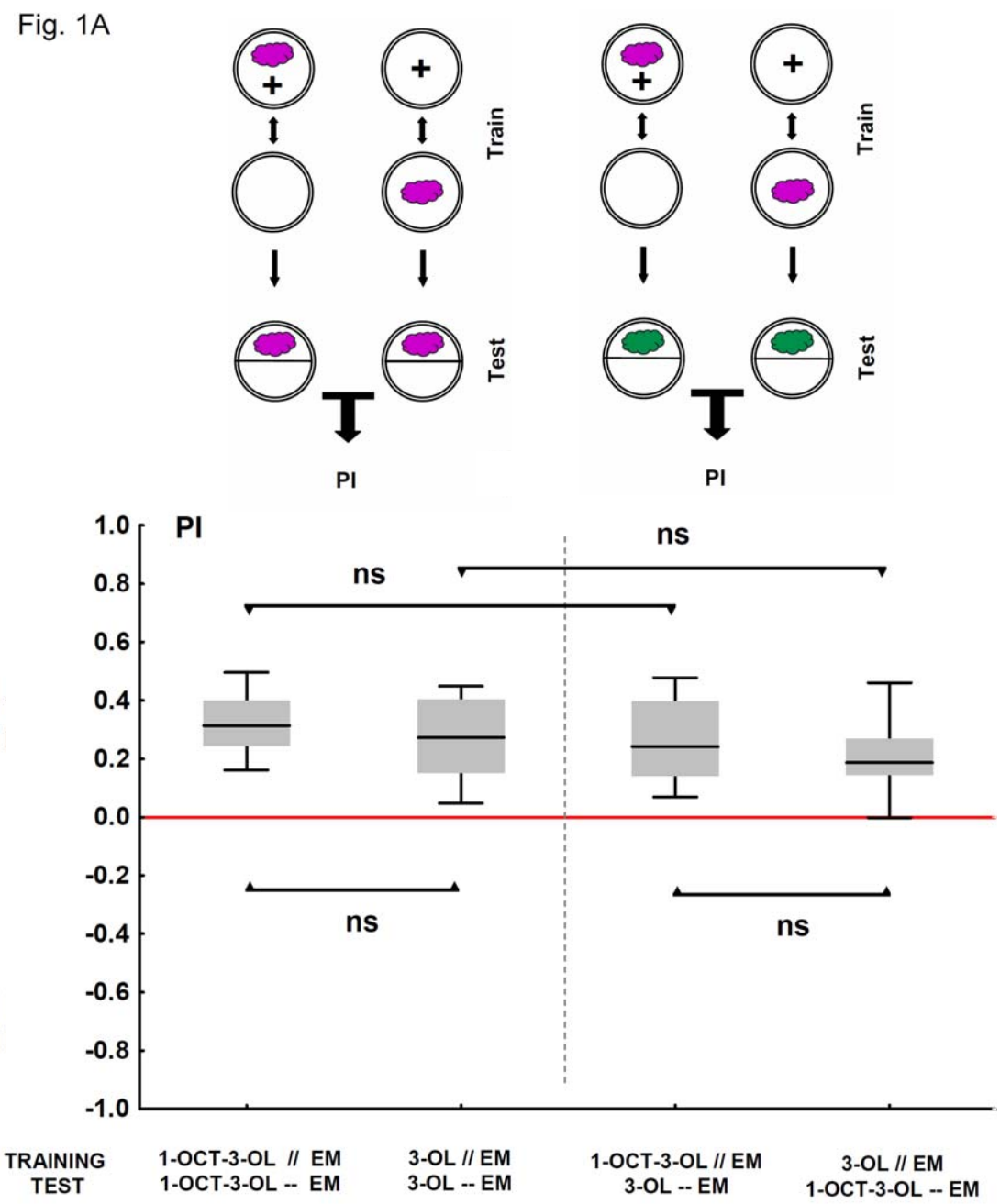
Thus, positive values indicate preference for fructose.

Statistics

Data is obtained in parallel for all the groups to be compared statistically, using non-parametrical analyses throughout. Kruskal-Wallis (KW) tests are used to compare between multiple groups. To test the scores of single groups against zero we use one-sample sign (OSS) tests. To test for pair-wise group differences we use a Mann-Whitney U (MWU) test. If applicable, the significance level of 0.05 is corrected to account for multiple comparisons such that an experiment-wide error rate of 5 % is maintained upon Bonferroni corrections. For instance, when the data of four groups are individually compared to zero, the corrected significance level is 0.05/4 = 0.0125.

All statistical analyses are performed with Statistica (StatSoft, Tulsa, OK, USA) on a PC. The data is presented as box plots with the median as bold line, box boundaries as the 25 / 75% quantiles and whiskers as the 10 / 90% quantiles.

Fig. 1A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Train
Test
PI
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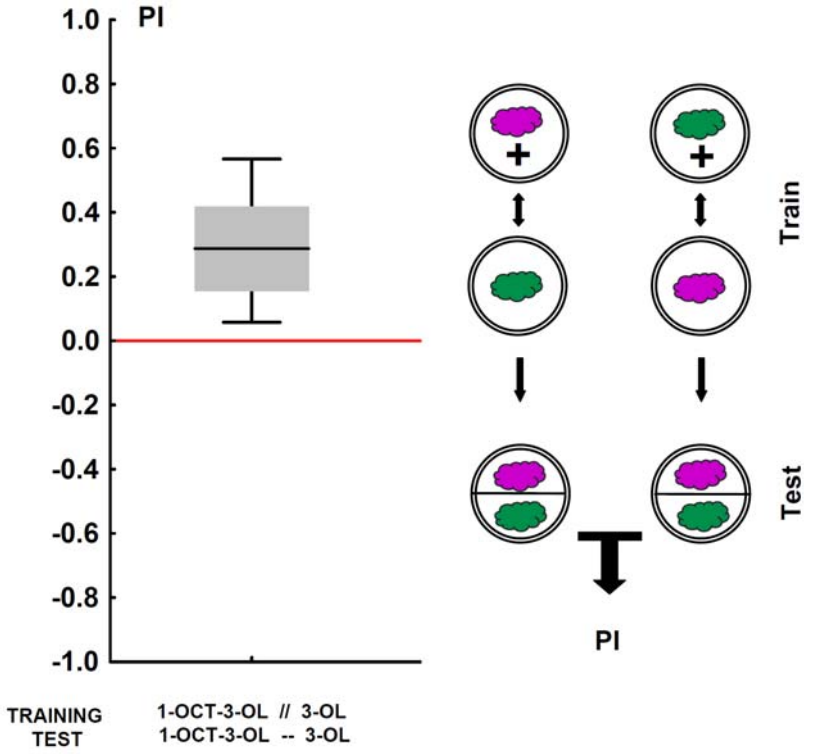
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
ns
ns
ns
ns
TRAINING
TEST
1-OCT-3-OL // EM
1-OCT-3-OL -- EM
3-OL // EM
3-OL -- EM
1-OCT-3-OL // EM
3-OL -- EM
3-OL // EM
1-OCT-3-OL -- EM

Fig. 1B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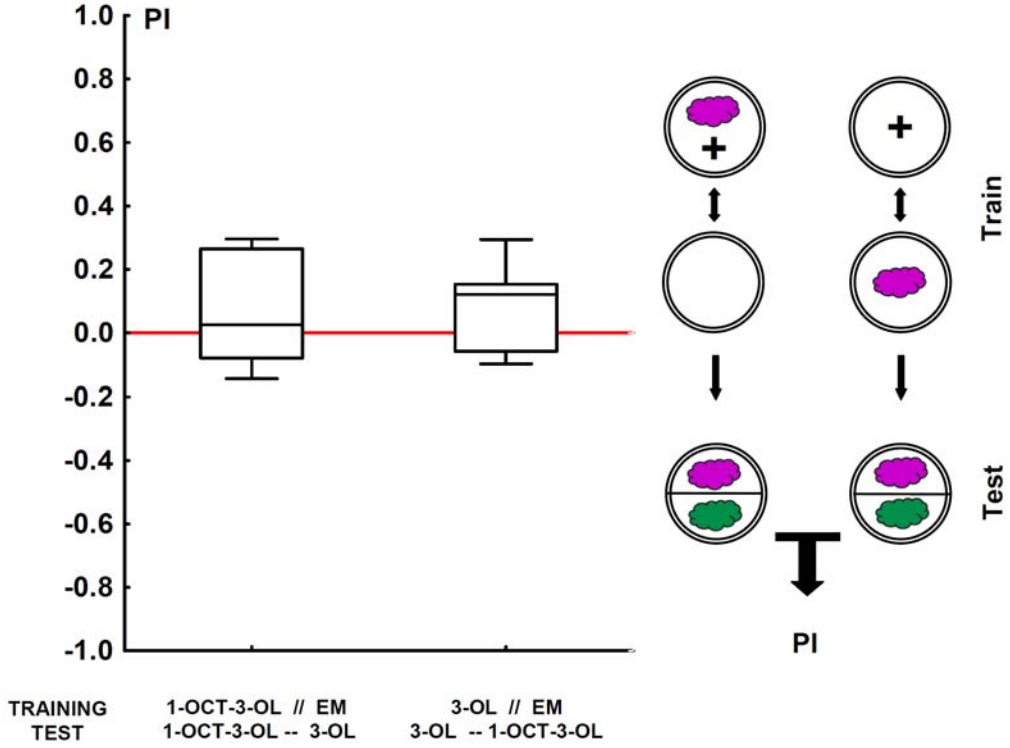
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Train
Test
PI
TRAINING
TEST
1-OCT-3-OL // 3-OL
1-OCT-3-OL -- 3-OL

Fig. 1C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-0.4
-0.6
-0.8
-1.0
Train
Test
PI
TRAINING
TEST
1-OCT-3-OL // EM
1-OCT-3-OL -- 3-OL
3-OL // EM
3-OL -- 1-OCT-3-OL

Figure 1: The generalization-discrimination balance

A) Learnability is equal for 1-OCT-3-OL and 3-OL (two left plots), and generalization is both symmetrical (two right plots) and practically complete (comparisons between left versus right plots). Shown are the associative performance indices after either the one-odour training, one-odour test task (left) or the generalization task (right); positive values indicate conditioned approach towards and negative values conditioned avoidance of the odour.

The sketches above indicate the nature of the task: The circles indicate the Petri dishes used, the clouds indicate odour, and a difference in colour of the clouds indicates a difference in odour identity; "+" indicates the presence of the sugar reward in the Petri dish. Note that in half of the cases the sequence of training trials is as indicated (in e.g. the left-most sketch: first the odour-reward trial, then the trial with neither odour nor reward), whereas in the other half of the cases the sequence of training trials is reversed (first the trial with neither odour nor reward, then the odour-reward trial). The lettering below the plots shows the chemical identity of the odours employed for training (top line) and test (bottom line), respectively. Box plots display the median as the middle line, and 25, 75 % quantiles as box boundaries, and 10, 90 % quantiles as whiskers, respectively. $P > 0.05/4$ in MWU tests is indicated by ns; shading of the boxes refers to $P < 0.05/4$ in OSS tests. Sample sizes from left to right: 17, 20, 22, 26.

B) The discrimination task reveals the larvae´s ability to discriminate between 1-OCT-3-OL and 3-OL. Positive scores indicate conditioned approach to the rewarded odour. Shading of box indicates $P < 0.05$ in an OSS test. Other details as in A. Sample size: 25.

C) One-odour training does not confer an odour-specific memory trace. Shown are the associative performance indices after the one-odour training, two-odour test task. Positive scores indicate conditioned approach to the rewarded odour. Lack of shading of boxes indicates $P > 0.05/2$ in OSS tests. Other details as in A. Sample sizes from left to right: 15, 12.

RESULTS

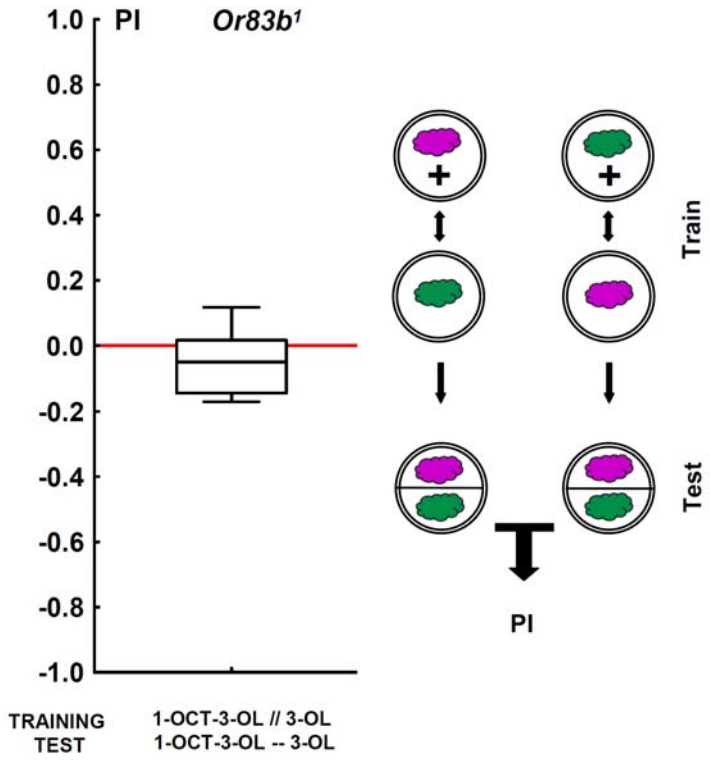
Using the one-odour training, one-odour test task, larvae show equal levels of conditioned approach towards 1-octen-3-ol and 3-octanol (Fig. 1A; MWU-test: $P = 0.42$; U= 143.0, sample sizes: 17, 20), indicating that associative learnability is equal between them. This suggests that differences in perceived intensity, if any, are not relevant to the obtained associative performance index.

In the generalization task, we find that performance indices are not smaller when the test odour is 'novel' to the larvae. Conditioned behaviour after 1-octen-3-ol training is equal when the trained odour 1-octen-3-ol is used for the test, as when the 'novel' odour 3-octanol is used for the test (Fig. 1A; MWU-test: $P = 0.19$; U= 141, sample sizes: 17, 22). The same holds true when conditioned behaviour upon 3-octanol training is compared between 3-octanol and the 'novel' odour 1-octen-3-ol as test odours (Fig. 1A; MWU-test: $P = 0.17$; U= 198, sample sizes: 20, 26). Also, conditioned performance towards the two 'novel' odours is not different (Fig. 1A; MWU-test: $P = 0.24$; U= 229, sample sizes: 22, 26). This means that there is symmetrical and practically complete generalization between 1-octen-3-ol and 3-octanol (Fig. 1A; KW-test: $P = 0.09$, H= 6.27, df= 3, sample sizes as above) (for the PREF scores relating to this experiment see Fig. S1A).

These results prompt the question of whether 1-octen-3-ol and 3-octanol are 'actively' generalized, such that, although in principle discriminable, they are *regarded* as the same by the larvae. Therefore, we run a discrimination task, such that one of the two odours is rewarded and the other odour is not; subsequently, we test whether the larvae show discrimination between the previously rewarded versus the previously non-rewarded odour. This is indeed the case, as shown by significant conditioned approach in this kind of discrimination experiment (Fig. 1B; OSS-test: $P< 0.05$, sample size: 25) (for the PREF scores relating to this experiment see Fig. S1B). As argued by the equal levels of learnability of these two odours (see above, Fig. 1A), it seems unlikely that differences in perceived intensity are the basis for this discrimination.

Clearly, the question remains whether successful discrimination between 1-octen-3-ol and 3-octanol is *conferred* by differential training, or whether discrimination is *revealed* by differential testing. That is, it may be that even one-odour training supports a memory trace specific enough to prefer the trained odour if presented in a choice situation against a 'novel' odour. We therefore use a one-odour training, two-odour test task: larvae are trained to either 1-octen-3-ol or 3-octanol, but then are tested for their choice between the two odours, one being the trained odour, and the other one being a 'novel' odour. We find no conditioned behaviour in this experiment (for 1-octen-3-ol training: Fig. 1C; OSS-test: $P= 0.30$, sample size: 15; for 3-octanol training: Fig. 1C; OSS-test: $P= 0.38$, sample size: 12) (for the PREF scores relating to this experiment see Fig. S1C), suggesting that the formation of a memory trace allowing for discrimination between 1-octen-3-ol and 3-octanol requires differential training.

Fig. 2A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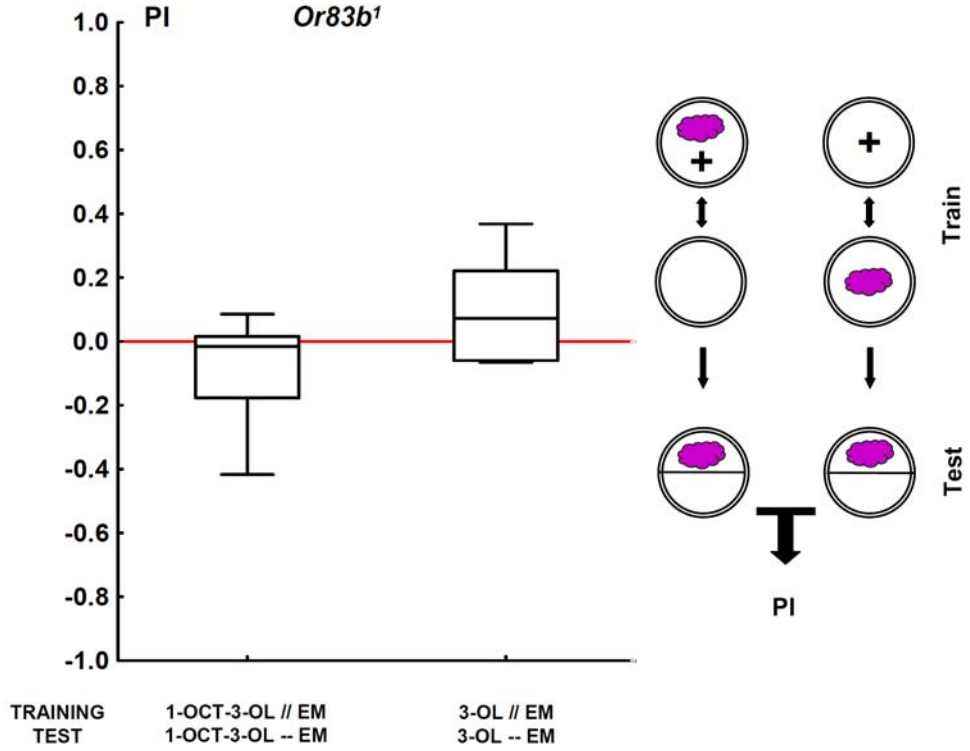
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Train
Test
PI
TRAINING
TEST
1-OCT-3-OL // 3-OL
1-OCT-3-OL -- 3-OL

Fig. 2B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Or83b1
1.0
0.8
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0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Train
Test
PI
TRAINING
TEST
1-OCT-3-OL // EM
1-OCT-3-OL -- EM
3-OL // EM
3-OL -- EM

Figure 2: Loss of discriminability and learnability of 1-OCT-3-OL and 3-OL in the *Or83b*^{1} mutant

In the *Or83b*^{1} mutant, discrimination ability is abolished (A), as is associative function in the one-odour training, one-odour test task (B). Lack of shading of the boxes indicates P> 0.05 (A) or P> 0.05/2 (B) in OSS tests. Other details as in the legend of Fig. 1. Sample sizes: 12 (A); from left to right: 10, 11 (B).

To open up subsequent neurogenetic analyses of how this discrimination ability comes about, we test the *Or83b*^{1} mutant in the discrimination task. *Or83b* encodes for a protein required for the function of ligand-binding odour receptors of the *Or* gene family (Larsson et al., 2004; Pellegrino & Nakagawa 2009), but not for the function of receptors of the *Ir* gene family (Benton et al., 2009). Obviously, *Or83b*^{1} mutants are not able to discriminate between 1-octen-3-ol and 3-octanol (Fig. 2A; OSS-test: *P*= 0.38, sample size: 12). Also, neither odour is learnable in the one-odour training, one-odour test task (Fig. 2B; for 1-octen-3-ol: OSS-test: *P*= 0.75, sample size: 10; for 3-octanol: OSS-test: *P*= 0.22, sample size: 11) and, when considering the preference scores rather than the associative performance index, there is no evidence for the *Or83b*^{1} mutant larvae showing any preference towards either odour (Fig. 3A; OSS-tests: *P*= 0.14, 0.38, sample sizes: 12, 12; Fig. 3B-1; OSS-tests: *P*= 0.34, 0.34, sample sizes: 10, 10; Fig. 3B-2; OSS-tests: *P*= 0.75, 0.34, sample sizes: 11, 11). This suggests that in the context of odour-reward learning olfactory larval behaviour towards either 1-octen-3-ol or towards 3-octanol requires *Or83b* function. In addition, innate behaviour towards these two odours, as well as behaviour towards AM and BA, requires *Or83b* function, too (Fig. 4A; OSS test: P= 0.80, 0.80, 0.26, 0.15, sample sizes: 16, 16, 20, 24). This is not a trivial finding, because innate preference and associative learnability can be dissociated (T. Saumweber, Universität Würzburg, pers. comm.).

Fig. 3A

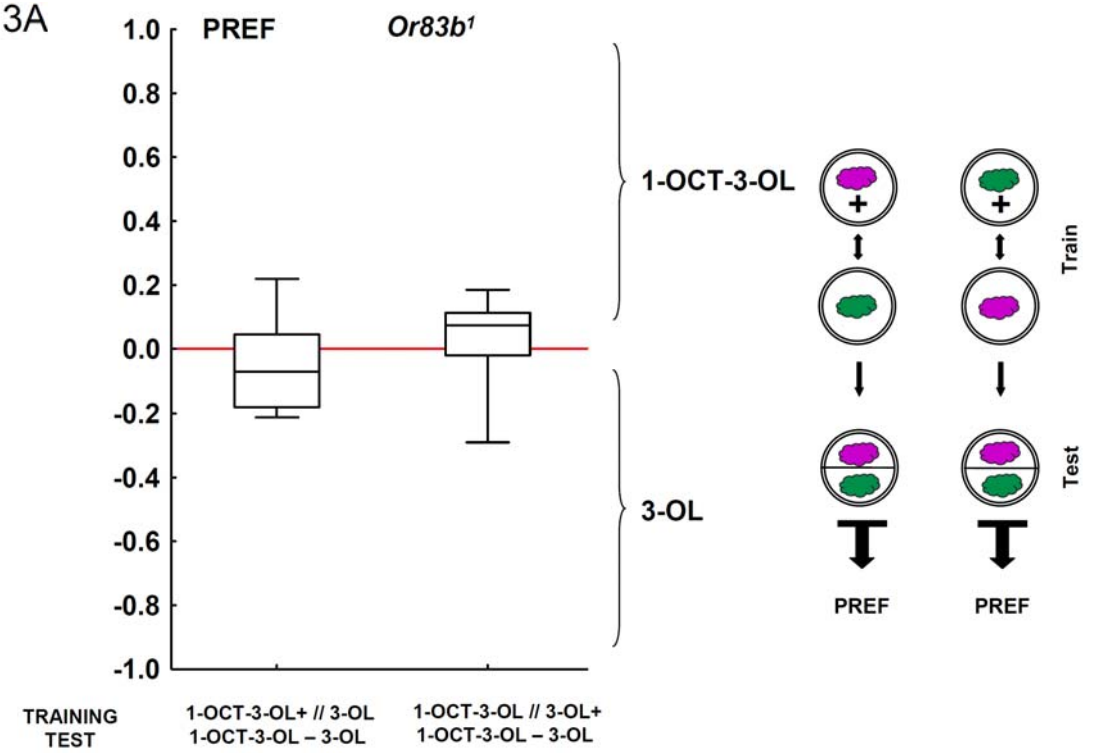
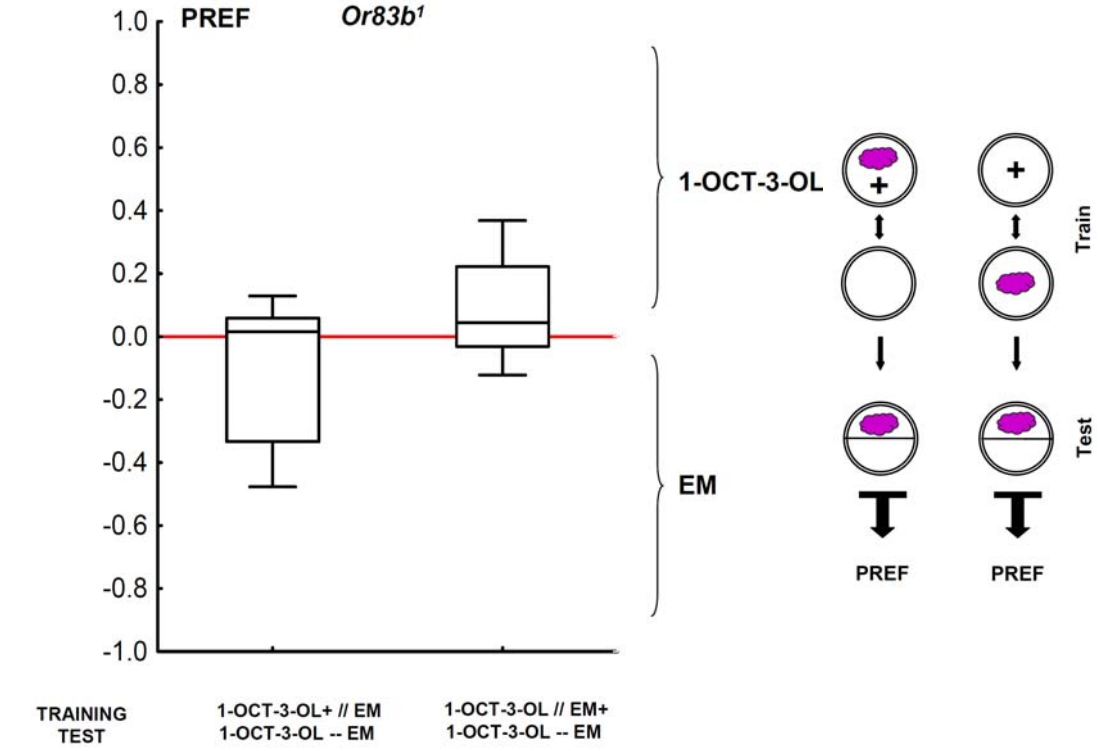


Fig. 3B-1



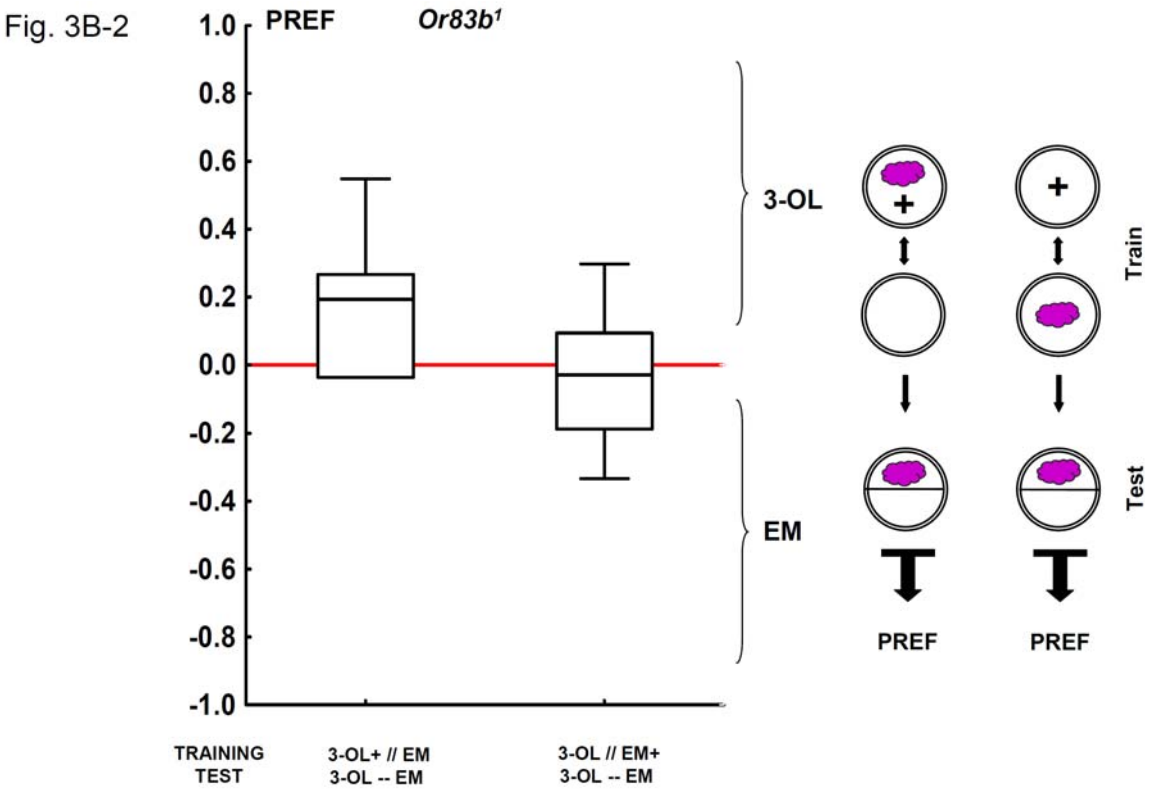


Figure 3: Loss of preference behaviour after either training regimen in the *Or83b*^{1} mutant

In the *Or83b*^{1} mutant, animals distribute equally between 1-OCT-3-OL and 3-OL after two-odour training (A). Also, preference behaviour towards 1-OCT-3-OL (B-1) as well as towards 3-OL (B-2) after one-odour training is abolished. Note that the PREF data displayed here are the basis for the associative PI scores in Fig. 2. Lack of shading of boxes indicates $P > 0.05/2$ in OSS tests. Other details as in the legend of Fig. 1. Sample sizes from left to right: 12, 12 in (A) and 10, 10 (B-1) and 11, 11 (B-2).

These fairly general defects of the *Or83b*^{1} mutant in olfactory behaviour prompted us to test whether behaviour in another chemosensory dimension would be impaired as well. We find no obvious defect of *Or83b*^{1} mutants to sense and behave towards the sugar reward employed in our learning experiments (Fig. 4B; OSS-test: $P < 0.05$, sample size: 22), relative to the strength of preference behaviour typically found in wild-type larvae (Schipanski et al., 2008). The same conclusion had, without an actual display of the data, been reached by Larsson et al. (2004) in the initial description of the *Or83b*^{1} mutant.

Fig. 4A

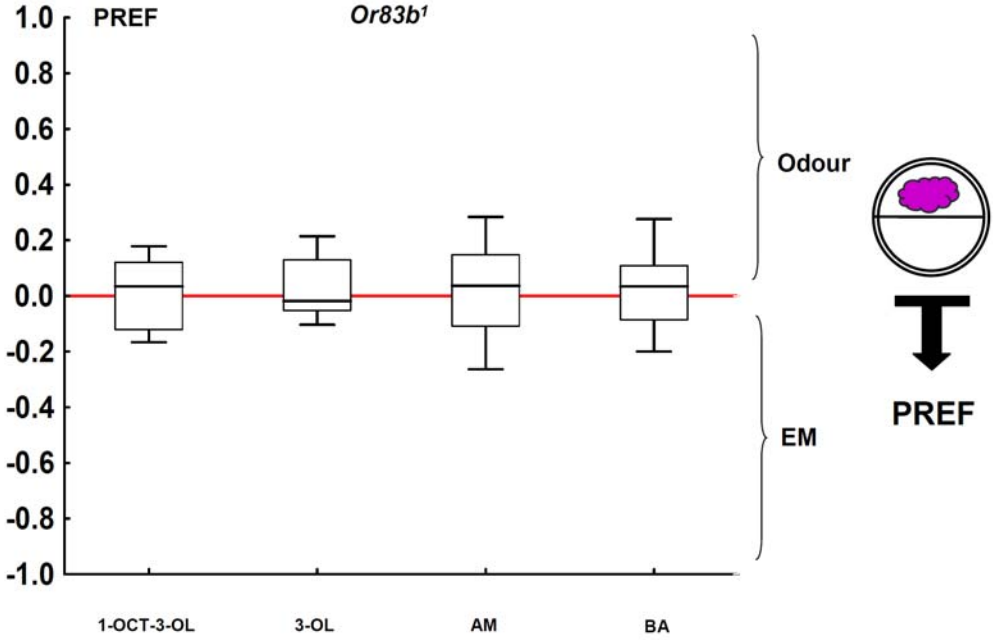


Fig. 4B

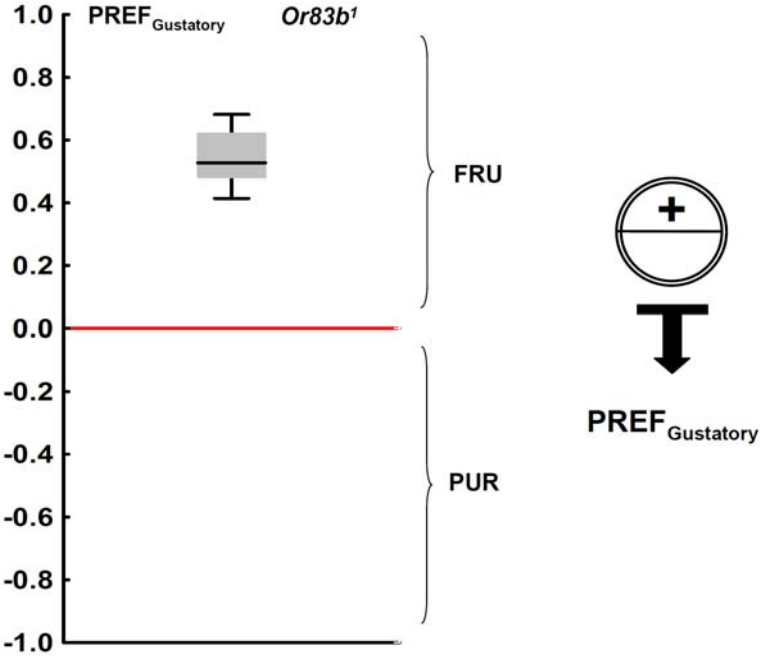


Figure 4: Loss of innate preference for odours, but not the sugar reward, in the *Or83b*[1] mutant

In the *Or83b*[1] mutant, innate olfactory preference behaviour (towards 1-OCT-3-OL, 3-OL, AM, and BA) is abolished (A), whereas innate gustatory preference behaviour towards fructose is apparently intact (B). Lack of shading of boxes indicates $P > 0.05/4$ (A) or $P > 0.05$ (B) in OSS tests. Sample sizes in (A) are from left to right: 16, 16, 20, 24; sample size in (B) is 22.

DISCUSSION

We have shown that, depending on the nature of the task, larval *Drosophila* do or do not make a difference between a pair of odours (1-octen-3-ol and 3-octanol). On the one hand, if 'explicitly' trained to make a difference between both odours, by discriminatively rewarding one but not the other odour, larvae show conditioned preference for the rewarded over the unrewarded odour (Fig. 1B). On the other hand, no odour-specificity of conditioned behaviour is observed after non-discriminative (i.e. one-odour) training (Figs 1A, C). In turn, if the test involves a choice between both odours, larvae prefer the rewarded odour only if training had been discriminative (Fig. 1B versus 1C). In other words, only discrimination training confers an odour-specific memory trace, whereas one-odour training does not.

Specifically, for discrimination training to lead to an odour-specific memory trace requires that there is at least some difference in processing between the two odours already at the *beginning* of training - if both odours would induce exactly the same kind of peripheral activity, discriminative reinforcement would not have any initial difference to work on. This means that, as a default, there is a small yet salient difference in processing between 1-octen-3-ol and 3-octanol, as is indeed observed (see below); this difference can either be ignored as is the case after one-odour training (generalization), or it can be accentuated by odour-specific reinforcement (discrimination). This reveals an adaptive adjustment of the generalization-discrimination balance in larval *Drosophila*. Given that all tested behaviours towards 1-octen-3-ol and 3-octanol require *Or83b* function (Figs 2, 3, 4A), these processes take place in the neuronal circuitry downstream of the *Or*-expressing sensory neurons, whereas the pathways downstream of *Ir*-gene expressing neurons are not sufficient in this regard (Benton et al., 2009).

Peripheral representation of 1-octen-3-ol and 3-octanol

The peripheral olfactory system of the larva comprises 21 olfactory sensory neurons. Each of them typically expresses one odorant receptor (OR) together with a co-receptor encoded by the *Or83b* gene (Fishilevich et al., 2005; Kreher et al., 2005; Larsson et al., 2004). The activation profile of a given sensory neuron is primarily determined by the activation profile of its cognate OR (Asahina et al., 2009; Kreher et al., 2008). The two stimuli considered here, 1-octen-3-ol and 3-octanol, activate a similar subset of larval ORs. At high odour concentrations, five broadly tuned ORs get activated by both stimuli

(the receptors encoded by the *Or13a*, *Or35a*, *Or45a*, *Or47a* and *Or85c* genes) (Kreher et al., 2008). Among this array of receptors, two display a particularly high affinity for both 1-octen-3-ol and 3-octanol, namely those encoded by the *Or13a* and *Or85c* genes (Kreher et al., 2008). Notably, the *Or13a*-encoded OR has a relatively higher affinity for 1-octen-3-ol than for 3-octanol, whereas the *Or85c*-encoded OR is more sensitive for 3-octanol than for 1-octen-3-ol (Kreher et al., 2008). In future experiments, it will be interesting to test whether and how these differences contribute to the adaptive adjustment of the generalization-discrimination balance between these two odours.

Symmetry and level of generalization

We found *symmetrical* generalization between 1-octen-3-ol and 3-octanol. This symmetry is also observed in a parallel study using a 5 x 5 matrix of odours (Chen, Mishra, Gerber, in prep.), all of which equated for equal learnability by adjusting odour dilution. Studies not adjusting odour dilution for generalization (Guerrieri et al., 2005), or cross adaptation experiments (Boyle & Cobb, 2005; Kreher et al., 2008), can yield asymmetric results.

In contrast, the observation that generalization between 1-octen-3-ol and 3-octanol is practically *complete* is unusual: in the mentioned 5 x 5 matrix (Chen, Mishra, Gerber, in prep.), response levels towards the 'novel' odour are typically much less than 50 % of the responses to the trained odour. Also, in that dataset measures of generalization between odour pairs on the one hand and measures of discrimination between them on the other hand are fairly well correlated, suggesting that the observed mismatch found here between full generalization and good discriminability is the exception, rather than the rule.

ACKNOWLEDGEMENTS

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Analyses of Olfaction). BG is a Heisenberg Fellow of the DFG. ML acknowledges funding from the Spanish Ministry of Science and Innovation (MICINN), and the CRG-EMBL Systems Biology Program.

This paper is dedicated to Erich Buchner in acknowledgement of his role as scientist, mentor and colleague, and in particular of his group's early discoveries regarding odour processing (Rodrigues & Buchner, 1984), which have initiated a long-standing German-Indian scientific collaboration.

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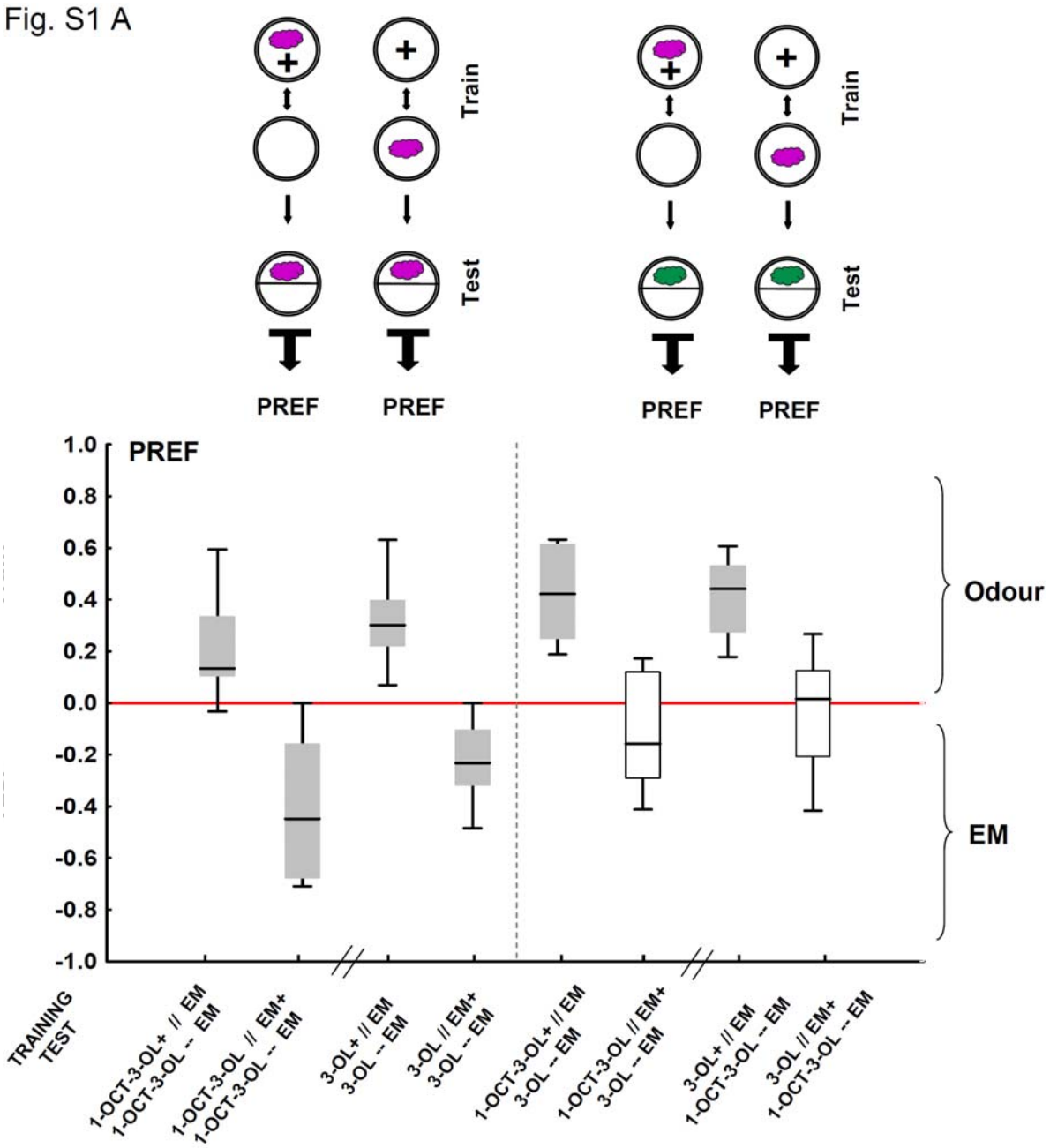
SUPPLEMENTARY MATERIAL

Fig. S1 A



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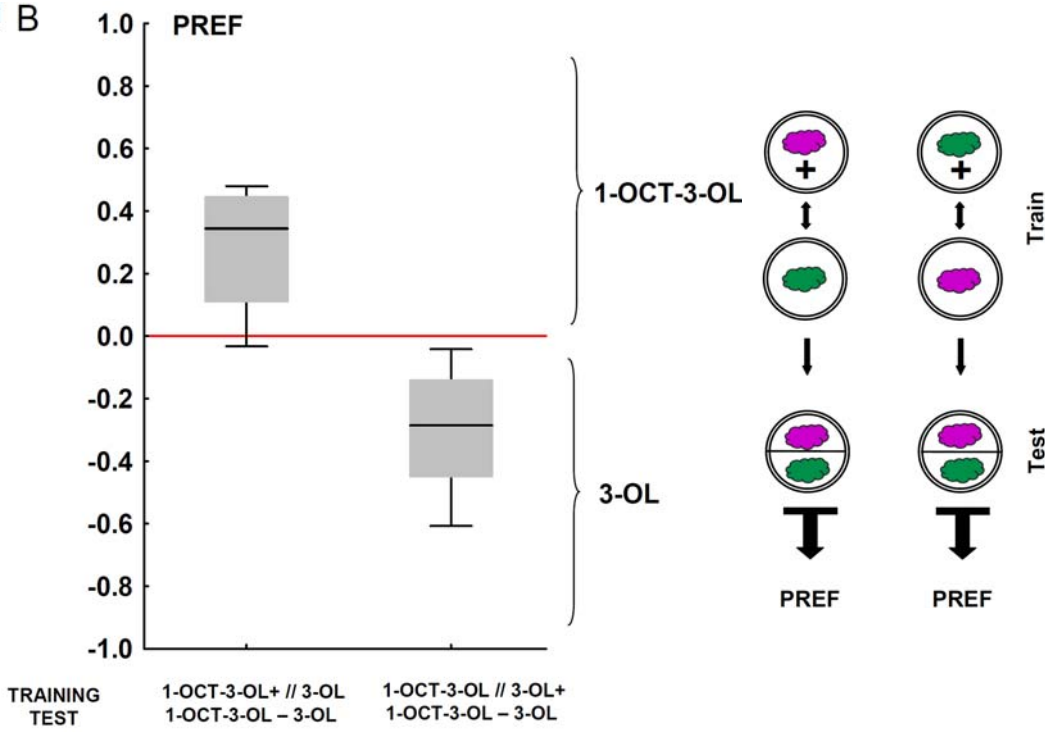

Fig. S1 C

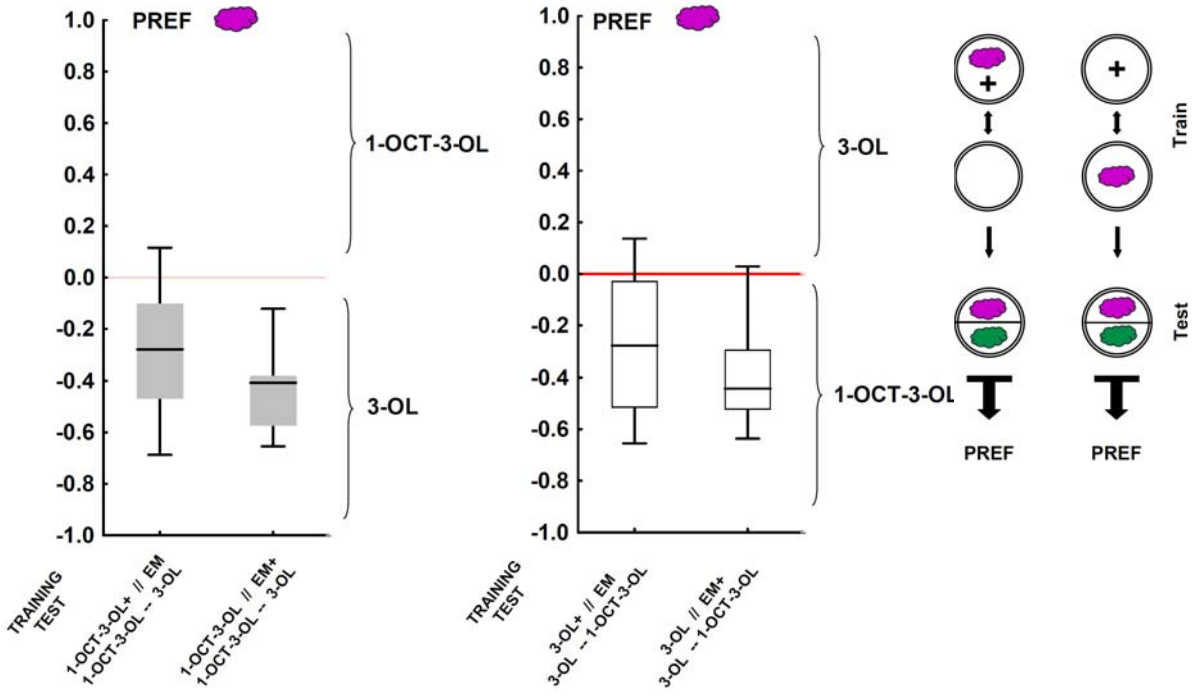

Figure S1: Preference scores for the generalization-discrimination balance

(A) Preferences scores for one-odour training and one-odour test task (left) and then for the generalization task (right). Positive values indicate approach towards the odour and negative values indicate odour avoidance. Data displayed here are the basis for the associative

PI scores in Fig. 1A. Shading of the boxes refers to $P< 0.05/2$ in OSS tests. Sample sizes from left to right: 17, 17, 20, 20, 22, 22, 26, 26.

(B) Preference scores for the odour discrimination task, which uses differential training and differential testing with 1-OCT-3-OL and 3-OL. Positive values indicate approach towards 1-OCT-3-OL and negative values indicate approach towards 3-OL. PREF data displayed here are the basis for the associative PI scores in Fig. 1B. Shading of the boxes refers to $P< 0.05/2$ in OSS tests. Sample sizes from left to right: 25, 25.

(C) Preference scores for one-odour training and two-odour test task. Data are displayed such that positive scores mean approach towards that odour which the larvae had experienced during training (i.e. the 'magenta odour'). We note that, after one-odour training, the larvae strongly prefer the novel ('green') odour when given the choice between the previously experienced versus the novel odour. PREF data displayed here are the basis for the associative PI scores in Fig. 1C. Shading of the boxes refers to $P< 0.05/2$ in OSS tests. Sample sizes from left to right: 15, 15, 12, 12.

Part II.

Mechanisms of learning and memory in larval *Drosophila*

Chapter II.1

Cognitive enhancement in larval *Drosophila*

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Cognitive enhancement' in *Drosophila*: Associative function increased by *Rhodiola rosea* food supplementation

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Running head

Cognitive enhancement by *Rhodiola rosea*

SUMMARY

Humans traditionally use extracts from *Rhodiola rosea* roots for their anti-stress and 'cognitive-enhancing' remedy. Here, we scrutinize this effect in larval *Drosophila melonogaster*. We show that food supplementation with freshly-grinded, dried roots of *Rhodiola rosea* improve odour-reward associative function; sensory and motor functions that are relevant for the employed task, as well as general locomotor parameters, however, remain unaltered. Notably, supplementing fly food with either commercially available, grinded tablets or extract containing *Rhodiola* root material do not have a 'cognitive enhancing' effect. *Drosophila* as a genetically tractable study case should now allow accelerated analyses of the molecular mechanism(s) that underlie the 'cognitive enhancement' conveyed by *Rhodiola rosea*. To the extent that the molecular determinants of 'cognition' are shared between animals and man, such research may have bearings for humans as well.

HIGHLIGHTS

- Humans traditionally use *Rhodiola* root preparations for 'cognitive enhancement'
- *Rhodiola* food supplementation doubles associative function in *Drosophila*
- Sensory-motor functions unaffectd by *Rhodiola* food supplementation
- *Drosophila* genetic tool-box can now be used to unravel mode of action of *Rhodiola*

RESULTS and DISCUSSION

Extracts from *Rhodiola rosea* roots, a perennial mountain plant of the family *Crassulaceae*, are used for their anti-stress and cognitive-enhancing remedy (Darbinyan et al., 2000; Spasov et al., 2000). In animal experiments, food supplementation with *Rhodiola rosea* preparations promotes resistance to stress and increases life span (see e.g. Wiegant et al., 2009 regarding *Caenorhabditis elegans*). Importantly for the current context, it was found that *Rhodiola rosea* root extracts dose-dependently improve mnemonic function in rats (Petkov et al., 1986). Here, we scrutinize this 'cognitive enhancing' effect in larval *Drosophila melonogaster*, an animal model system that appears suitable to investigate the cellular and molecular mechanisms behind such effects. The *Drosophila* larva is particularly suited for such an endeavor because of the availability of a robust odour-food associative learning paradigm (Gerber and Stocker, 2007; Gerber et al., 2009; Neuser et al., 2005; Scherer et al., 2003) combined with the cellular simplicity and genetic tractability of its brain.

Fig 1

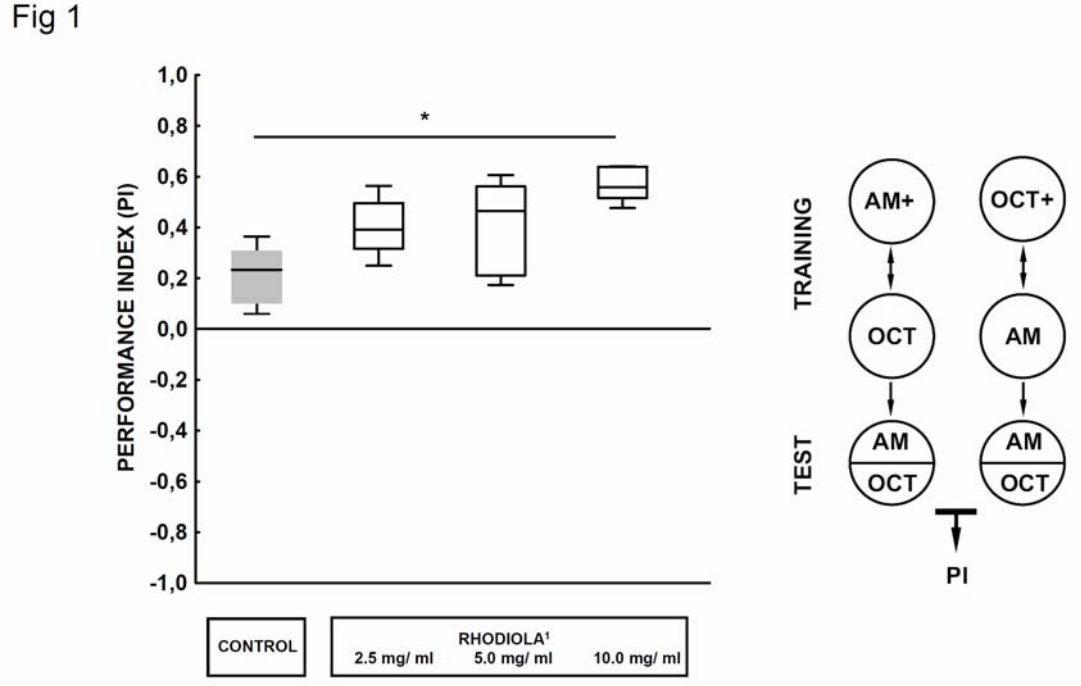


Figure 1 'Cognitive enhancement' by RHODIOLA[1] food supplementation

Shown are the Performance Indices (PI) of larvae reared on standard food (CONTROL) and of larvae reared with 2.5, 5.0, or 10.0 mg/ml RHODIOLA[1] added to their food. The performance index measures associative function by comparing the distribution of larvae between AM versus OCT after either AM was rewarded and OCT was not (AM+/ OCT), or after the reciprocal training regimen (AM/ OCT+); the inset figure illustrates this experimental procedure. Please note that in half of the cases we started training with AM+ or OCT+ as indicated; for the other half of the cases, we started training with OCT or AM, respectively.

Box plots represent the median as the middle line, 25 and 75 % quantiles as box boundaries, as well as 10 and 90 % quantiles as whiskers, respectively. Plots that share shading with the CONTROL are not significantly different from CONTROL in a Mann-Whithney U-test at $P < 0.05/3$.

RHODIOLA[1] treatment improves associative function

We raise *Drosophila* larvae on food medium that contains various concentrations of RHODIOLA[1] and test whether such food supplementation affects associative function in odour-sugar classical conditioning. This is indeed the case (Fig. 1; KW-test: $P < 0.05$, H= 20.6, df= 3, N= 15, 12, 12, 11): For the highest concentration of RHODIOLA[1] in the food, learning indices are almost tripled (MW U-test: 10.0 mg/ml versus CONTROL: U= 3.0, $P < 0.05/3$). Even the lowest concentration of RHODIOLA[1] doubles learning indices (MW U-test: 2.5 mg/ml versus CONTROL: U= 31.5, $P < 0.05/3$); the same is seen, as a trend, for the medium RHODIOLA[1] concentration (MW U-test: 5.0 mg/ml versus CONTROL: U= 46.0, $P = 0.03$). We note that this effect of RHODIOLA[1] is fairly acute as compared to the diet-effects on associative function as reported by Guo et al. (1996), which become apparent only with a lag period of several generations.

Fig 2A

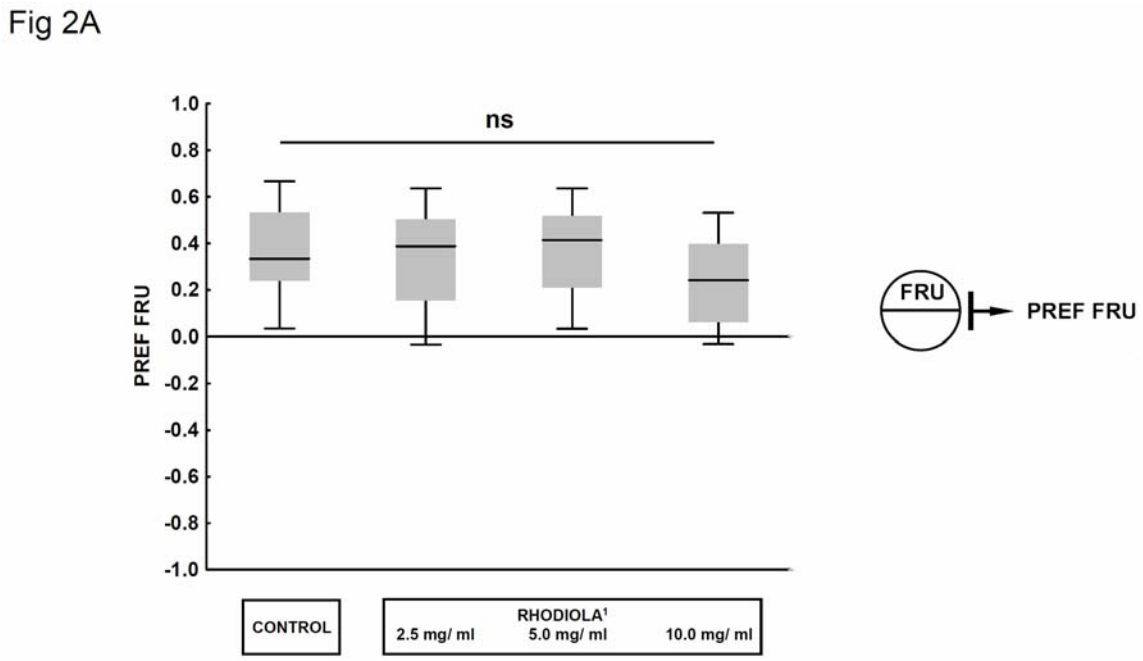
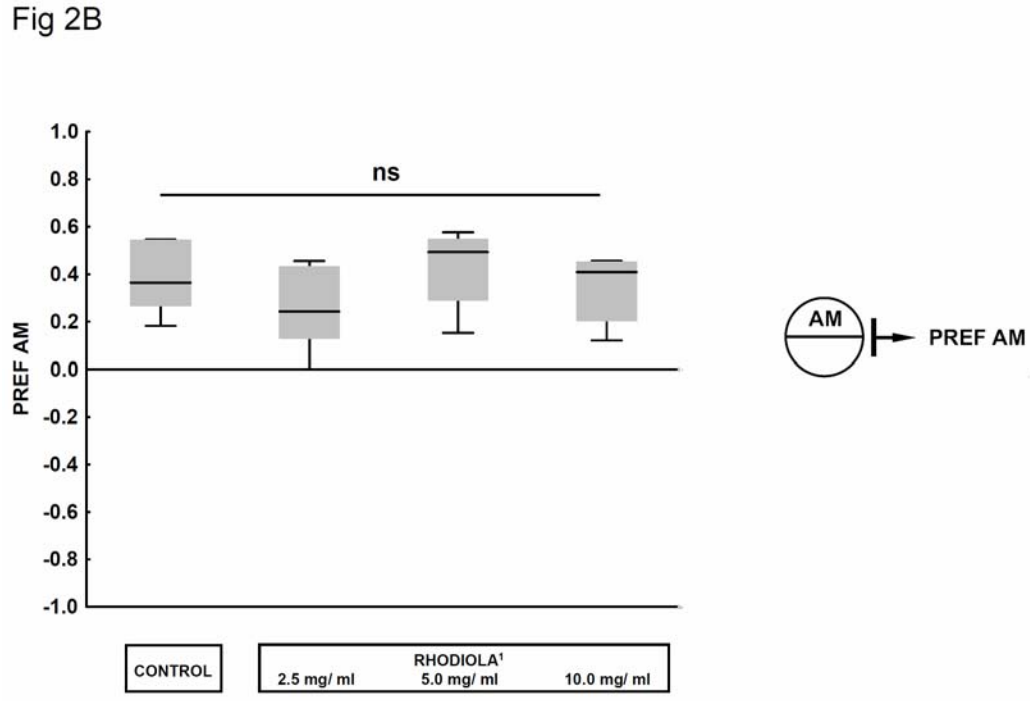

ns
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
PREF FRU
CONTROL
RHODIOLA[1]
2.5 mg/ ml
5.0 mg/ ml
10.0 mg/ ml
FRU
PREF FRU

Fig 2B


ns
1.0
0.8
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0.0
-0.2
-0.4
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PREF AM
CONTROL
RHODIOLA[1]
2.5 mg/ ml
5.0 mg/ ml
10.0 mg/ ml
AM
PREF AM

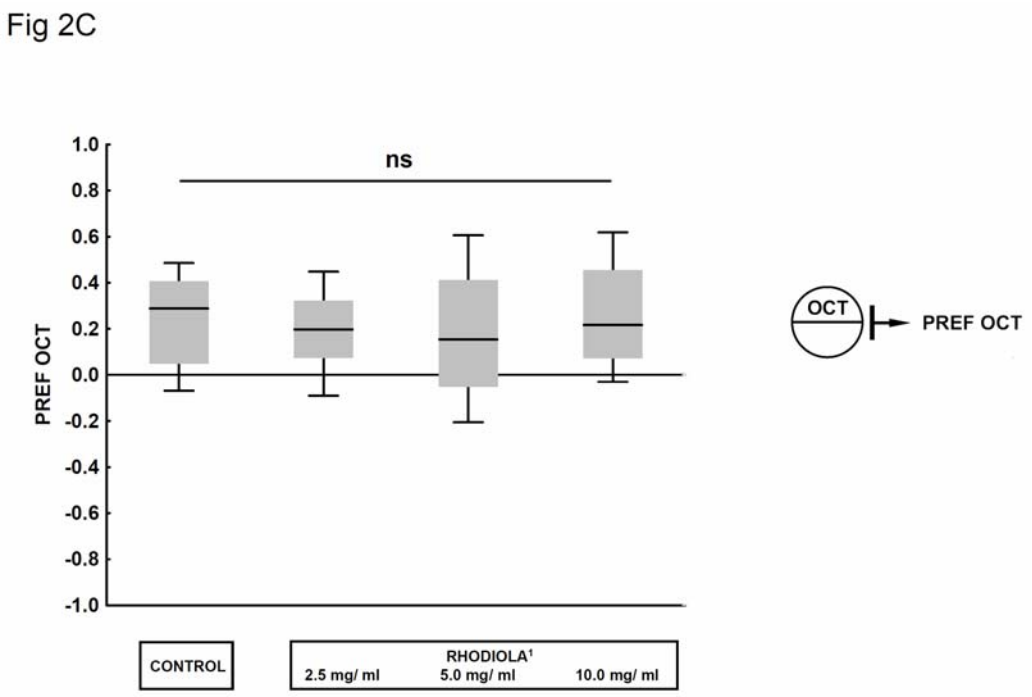

Figure 2 RHODIOLA[1] does not affect olfactory or gustatory behaviour in naïve larvae

Behaviour towards the reward (A: FRU) and detection of the used odours (B: AM; C: OCT) are not different between CONTROL and RHODIOLA[1] groups in KW-tests at $P < 0.05$. The inset figures show the respective behavioural procedures. For further details see legend of Fig. 1.

Importantly, behaviour of experimentally naïve larvae towards the sugar reward (i.e. fructose: FRU) as well as to the odours (i.e. *n*-amylacetate: AM and 1-octanol: OCT) is unaffected by RHODIOLA[1] (Fig. 2) (KW-tests: [A: FRU] $P = 0.31$, H= 3.5, df= 3, N= 17, 17, 17, 17; [B: AM] $P = 0.18$, H= 4.8, df= 3, N= 12, 12, 12, 12; [C: OCT] $P = 0.71$, H= 1.3, df= 3, N= 12, 12, 12, 12). Also, RHODIOLA[1] treatment does not affect the behaviour of the larvae towards the odours after training-like reward exposure (Fig. 3) (KW-tests: [A: AM] $P = 0.27$, H= 3.9, df= 3, N= 12, 12, 12, 12; [A': OCT] $P = 0.19$, H= 4.7, df= 3, N= 11, 12, 12, 12). Likewise, behaviour towards the odours after training-like odour-exposure is not influenced by RHODIOLA[1] (Fig. 3) (KW-tests: [B: AM] $P = 0.24$, H= 4.1, df= 3, N= 12, 12, 12, 12; [B': OCT] $P = 0.34$, H= 3.3, df= 3, N= 12, 12, 12, 12). Last but not least, when using a custom-written software for the analysis of locomotion, we find that RHODIOLA[1]-treated larvae (10.0 mg/ml) do not differ from CONTROL larvae in the two measured motor parameters: There are no differences in terms of SPEED (Fig 4B; MW U-test: $P = 0.49$, U= 403, N= 30, 30), or the frequency of TURNS (Fig 4C; Mann- Whitney U-test: $P = 0.17$, U= 357, N= 30, 30). Thus, food supplementation with

RHODIOLA[1] affects associative function in a dose-dependent way, but leaves sensory-motor processing unaltered.

Fig 3A

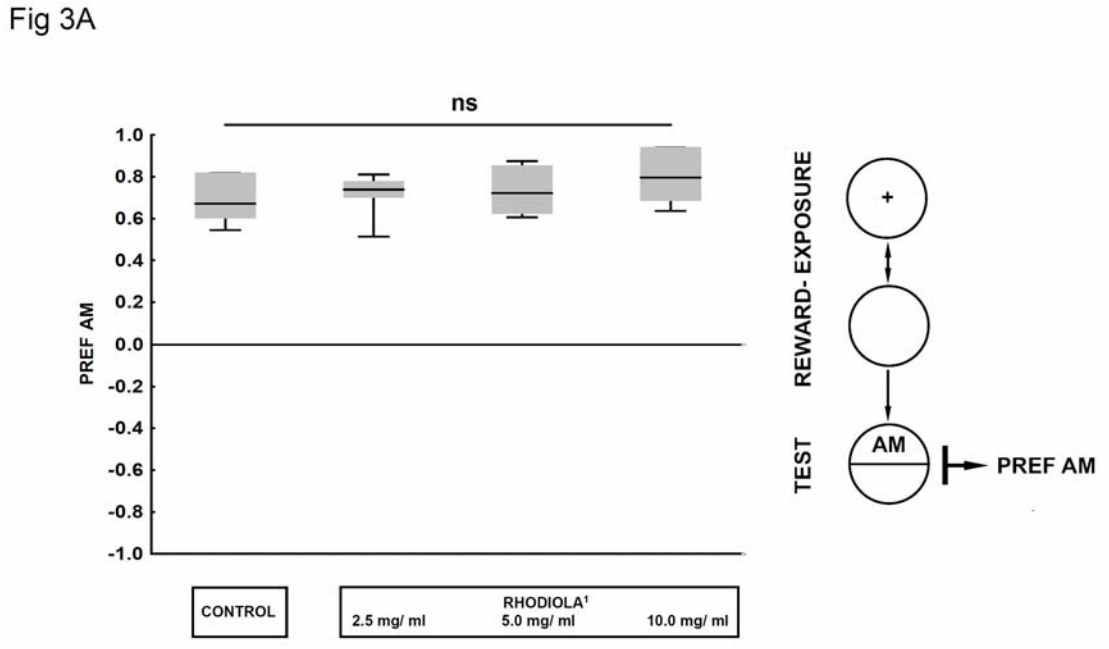

Fig 3A'

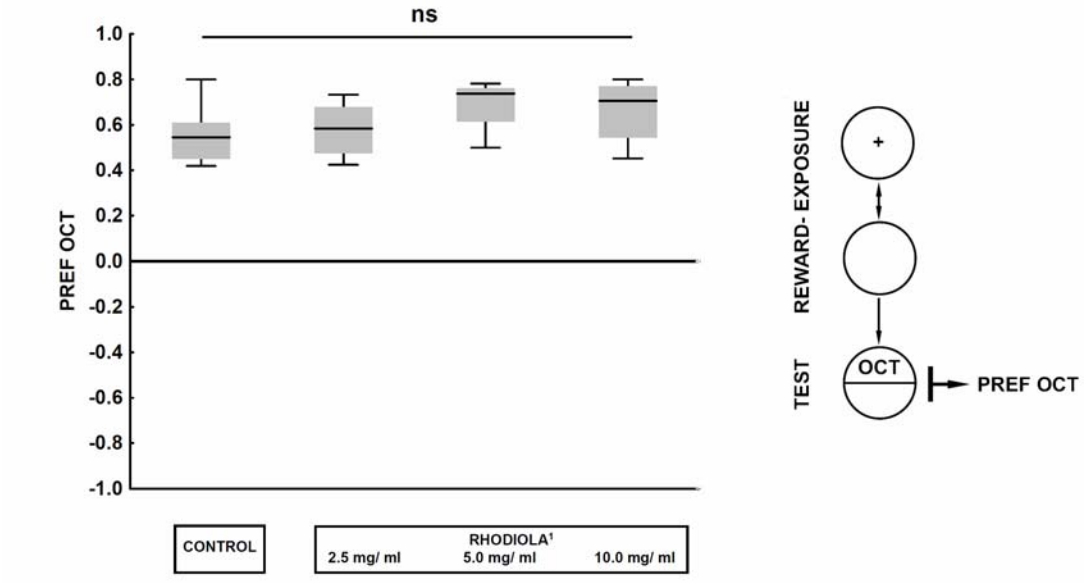

Fig 3B

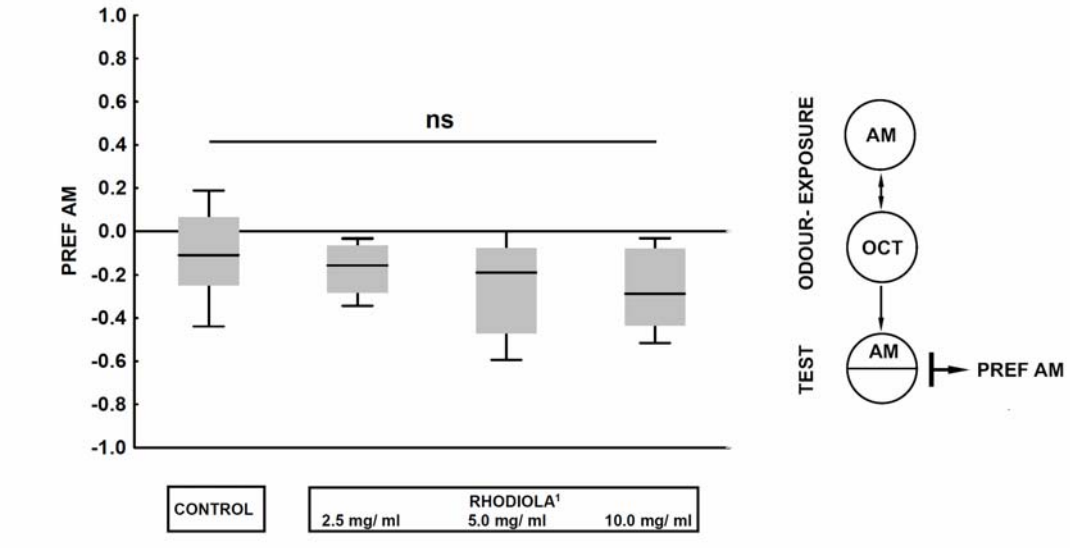

Fig 3B'

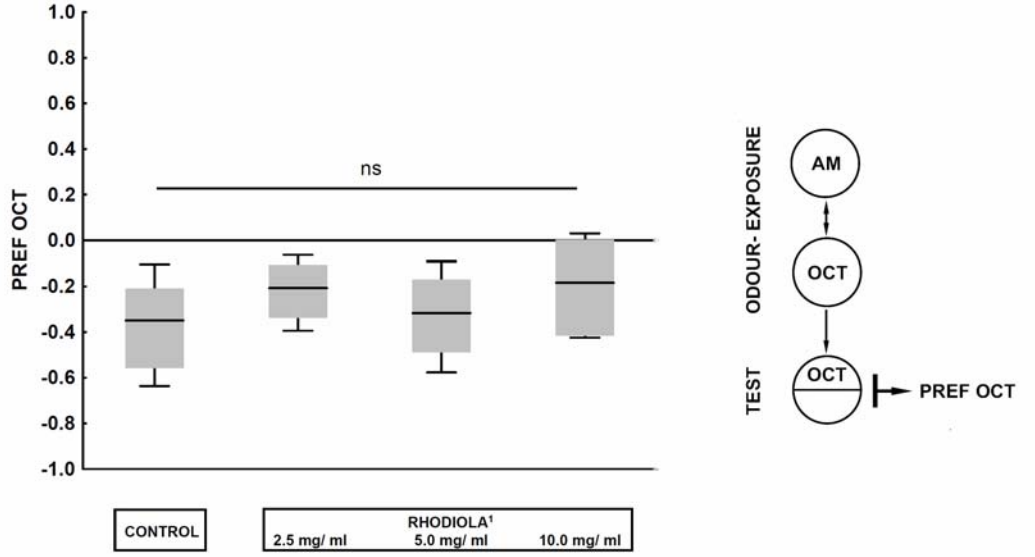

Figure 3 RHODIOLA[1] does not affect olfactory behaviour after training-like stimulus-exposure

Stimulus exposure involves the same handling as during normal training (inset of Fig. 1), except that we omitted either the odours (A, A´) or the reward (B, B´). After these kinds of exposure regimen, larvae are tested for their ability to detect AM (A, B) and OCT (A,´ B´), respectively. In neither case do we uncover any difference between CONTROL and RHODIOLA[1]. Note that for half of the cases the sequence is as indicated (i.e. the first trials in [A, A'] involve reward exposure; the first trials in [B, B'] involve AM exposure), whereas for the other half of the cases the sequence is reversed. Further details are as in the legend of Fig. 2.

Fig. 4A

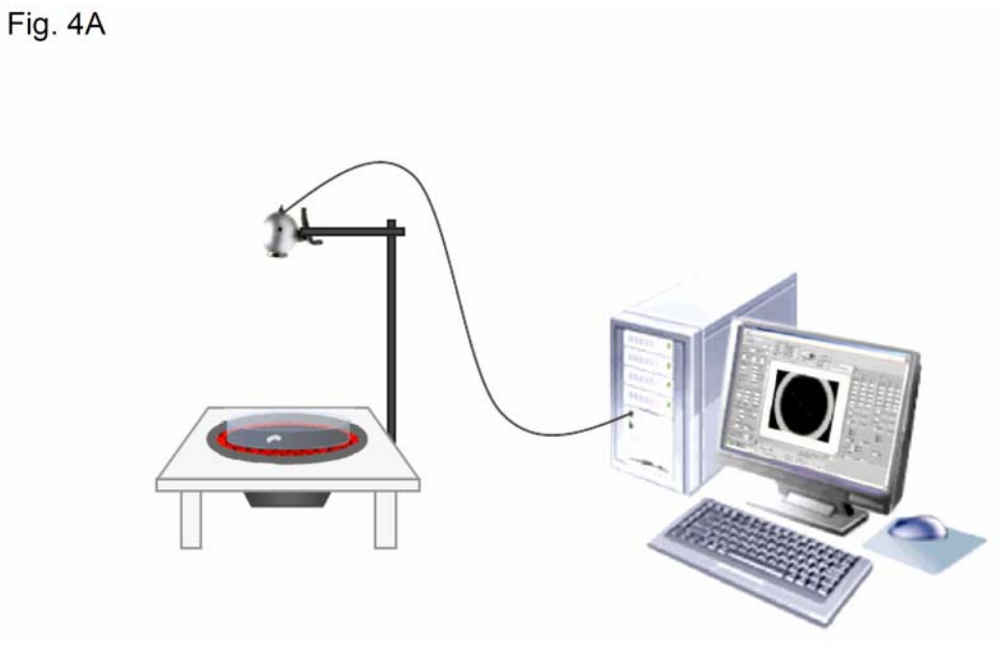

Fig. 4B

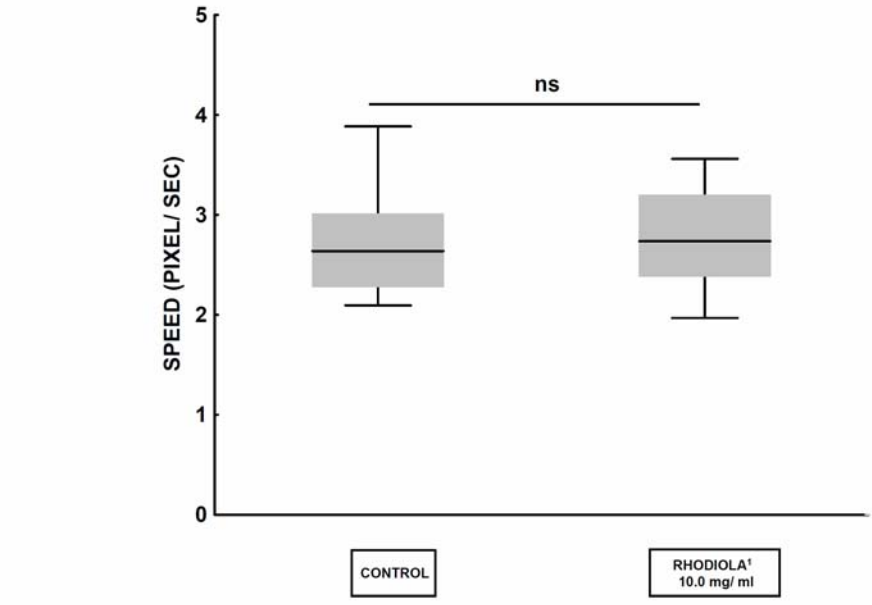

Fig. 4C

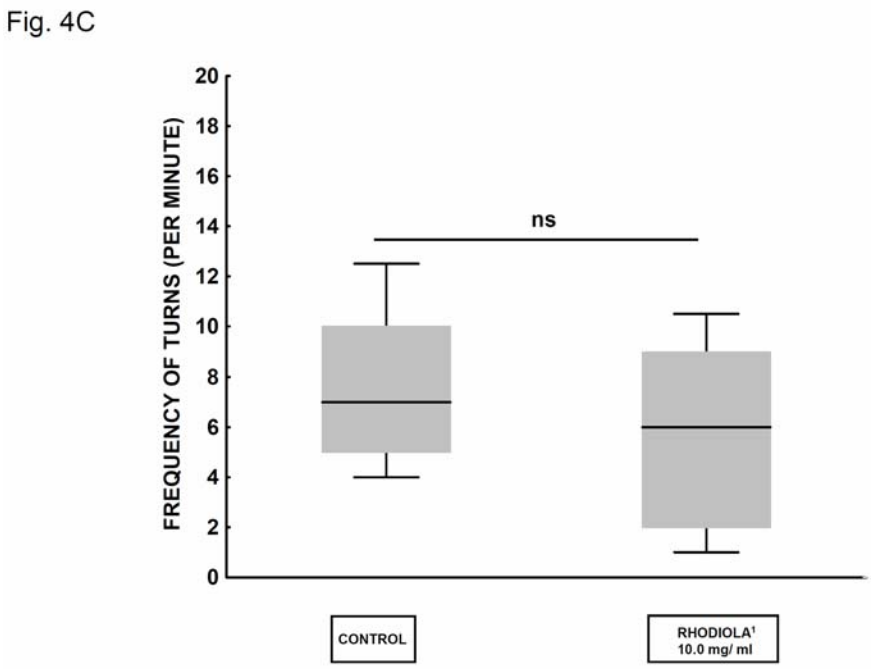

Figure 4 RHODIOLA[1] does not affect larval locomotion

(A) Schematic of larval tracking. Single larvae are observed for 1 min crawling on a Petri dish filled with agarose. This set-up is placed into a dark box (not shown) and through a light dispenser is illuminated by a red LED ring to allow for a webcam recording larval locomotion for off-line analysis.

There is no difference between CONTROL and RHODIOLA[1] larvae in the two locomotion parameter assayed (SPEED [B], frequency of TURNs [C]).

To repeat and extend this finding of enhanced associative function upon RHODIOLA[1] treatment, we compared learning ability of CONTROL and RHODIOLA[1] larvae in an one-odour paradigm (Saumweber et al. 2011), using only AM as odour. This paradigm also uncovered a massive influence of RHODIOLA[1] on associative function (Fig. 5) (KW-test: $P< 0.05$, H= 15.6, df= 3, N= 11, 11, 11, 11): For all concentrations of RHODIOLA[1] in the food, learning indices are increased as compared to CONTROL (MW U-tests: U= 10.0, U= 20, U= 8.0 for 2.5, 5.0, and 10.0 mg/ml, respectively; $P< 0.05/ 3$ in all cases).

Fig 5

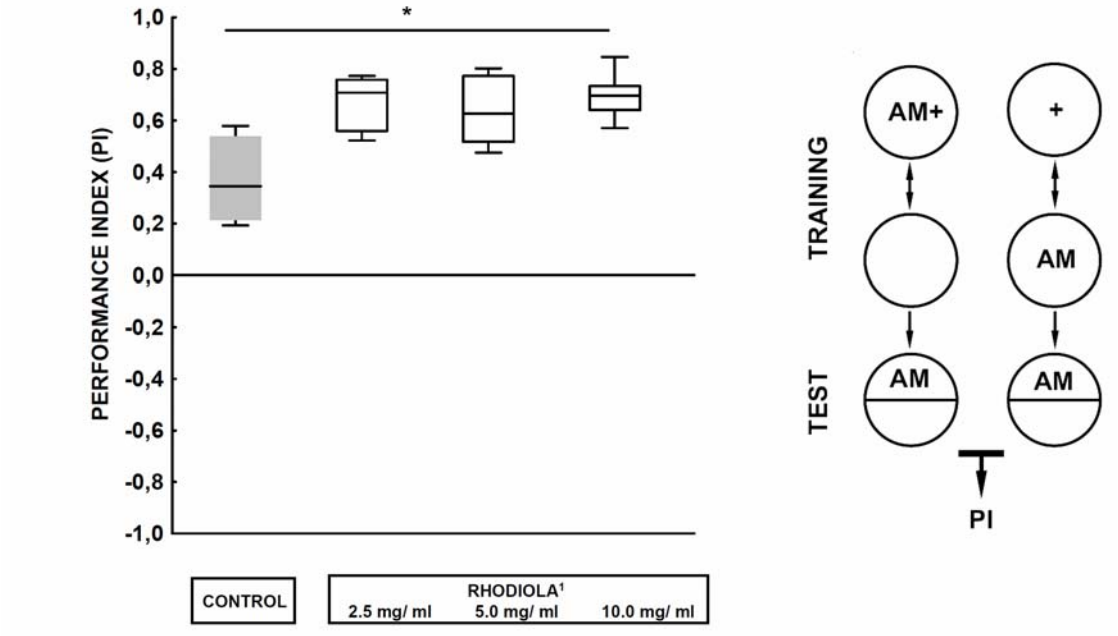

Figure 5 'Cognitive enhancement' by RHODIOLA[1] also in an one-odour paradigm

Shown are the Performance indices (PI) of larvae in a regimen identical to Figure 1, but omitting OCT from the experiment. Also in this case, learning indices are higher upon RHODIOLA[1] treatment. Please note that in half of the cases we started training with AM+ or + as indicated; for the other half of the cases, sequences were reversed. Further details are as in the legend of Fig. 1.

Fig 6

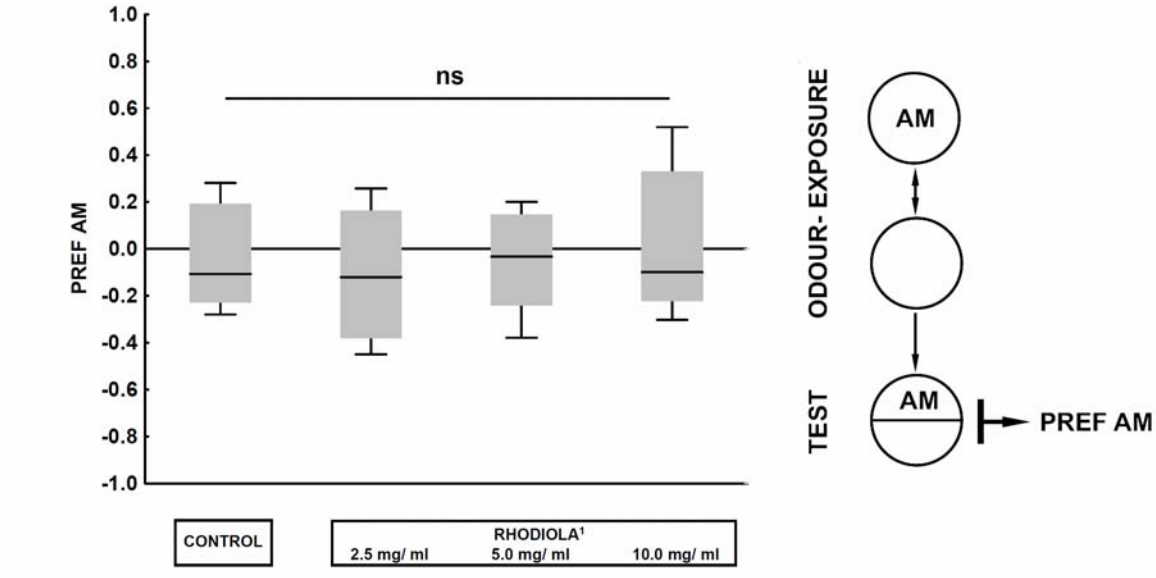

Figure 6 RHODIOLA[1] does not affect olfactory behaviour after one-odour exposure

To control for the effect of one-odour exposure, we tested the behaviour of the larvae towards AM after training-like exposure to AM. This does not uncover a difference between CONTROL and RHODIOLA[1] treated larvae. Note that for half of the cases the sequence is as indicated (i.e. the first trial includes AM exposure), whereas for the other half of the cases the sequence is reversed. For further details see legend of Fig. 3.

These effects are not due to an influence of RHODIOLA[1] on the behaviour towards AM after training-like exposure to AM (Fig. 6) (KW-test: P= 0.83, H= 0.9, df= 3, N= 12, 12, 12, 12) (for the other controls see above).

Fig 7

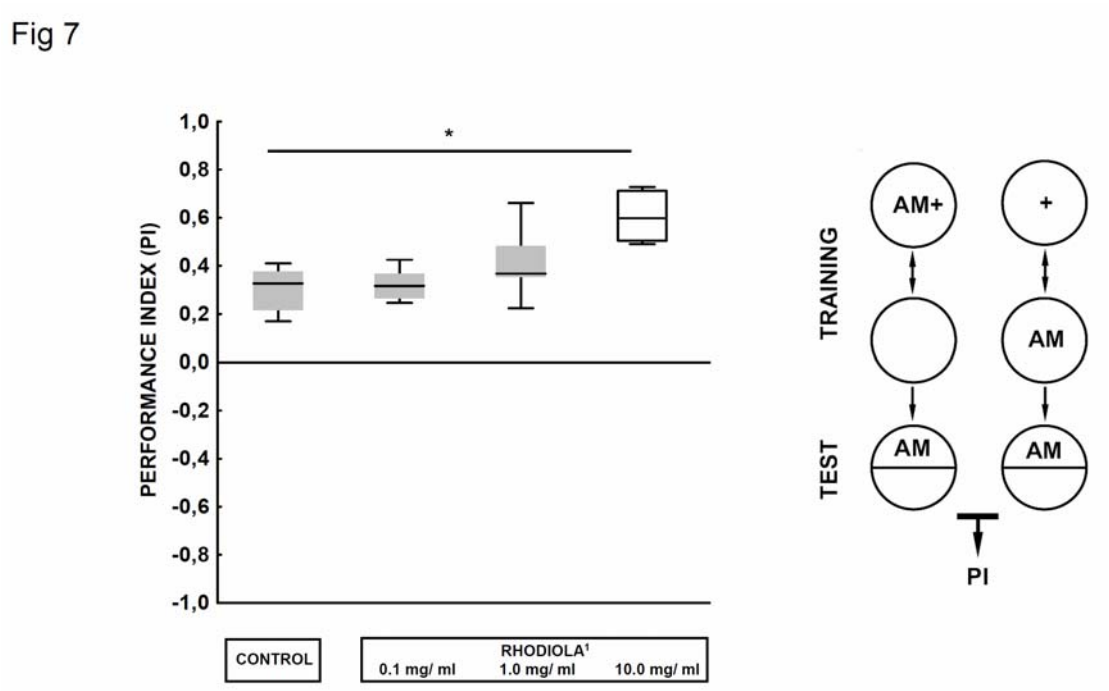

Figure 7 Probing for the lower limit of 'cognitive enhancement' by RHODIOLA[1]

This experiment repeats the one shown in Figure 4, but uses 0.1, 1.0, or 10.0 mg/ml RHODIOLA[1]. Both lower doses do not have an effect upon associative function. Please note that in half of the cases we started training with AM+ or + as indicated; for the other half of the cases, sequences were reversed. Further details are as in legend of Fig. 5.

To determine the lower range of the effective RHODIOLA[1] concentration, we extend this experiment, using concentrations of 0.1, 1.0 or 10.0 mg/ml RHODIOLA[1] (Fig. 7) (KW-test: P< 0.05, H= 21.7, df= 3, N= 15, 15, 13, 11). It turns out that 10.0 mg/ml RHODIOLA[1] increase learning indices (MW U-test: U= 6.0; P< 0.05/ 3); however, both 1.0 mg/ml (MW U-test: P= 0.04, U= 54.5) and 0.1 mg/ml (MW U-test: P= 0.86, U= 108.5) are without effect.

Fig 8

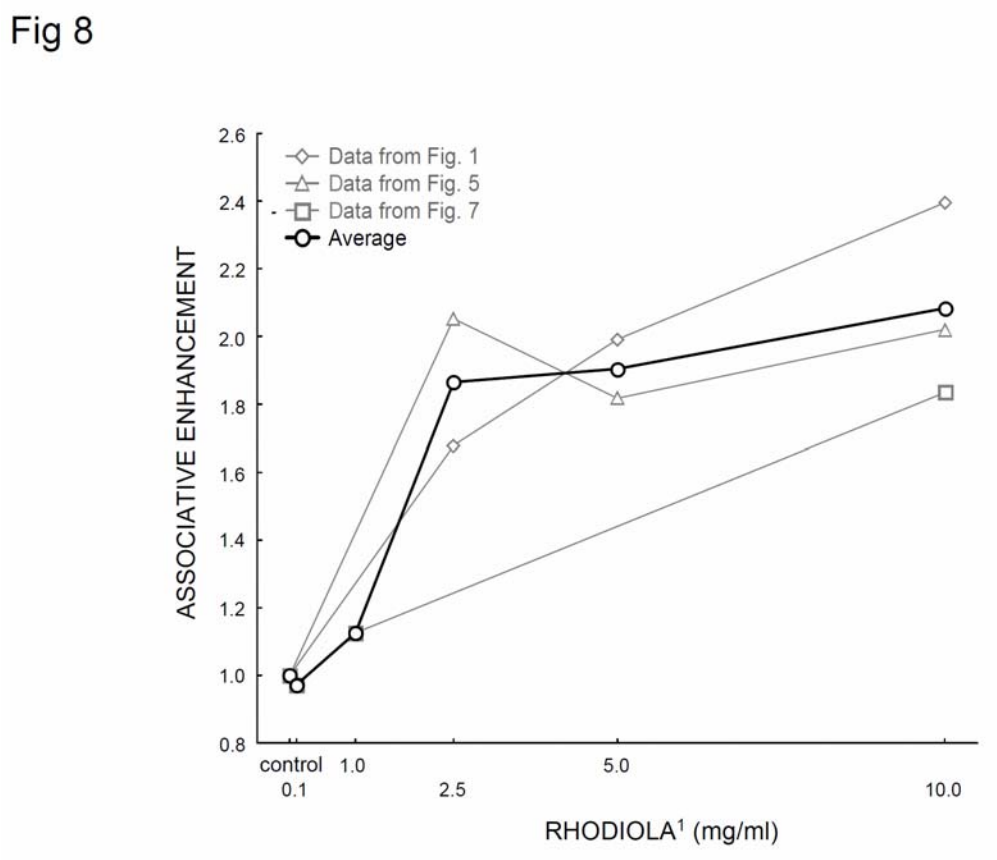

Figure 8 Semi-schematic dose-dependency of RHODIOLA[1] 'cognitive enhancement'

For the data displayed in Figures 1, 5, and 7, we normalize the median performance indices by the median of the respective CONTROL and plot them over RHODIOLA[1] concentration (grey lines). In those cases where more than one experiment used a given RHODIOLA[1] concentration, we present the mean of the respective normalized performance indices (solid line).

In Figure 8, we provide a semi-schematic summary of the dose-dependency of RHODIOLA[1]-mediated enhancement of associative function as combined from the data in Figures 1, 5, and 7.

Fig 9

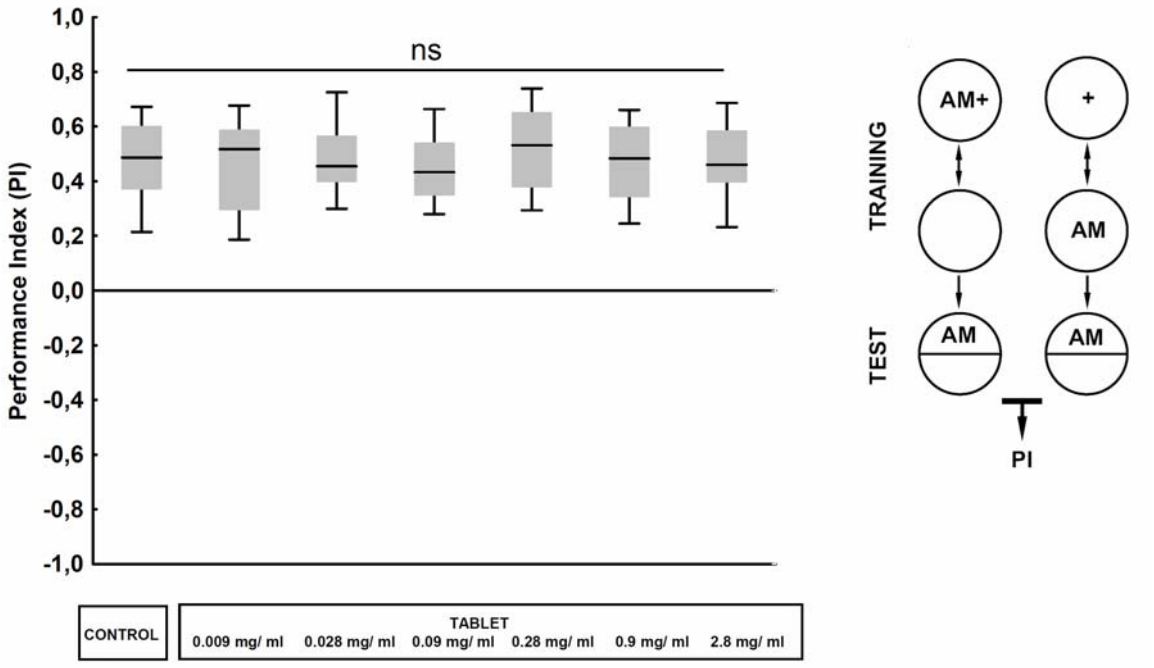

Figure 9 A *Rhodiola* TABLET preparation does not have a 'cognitive enhancement' remedy

Shown are the Performance Indices (PI) of larvae reared on standard food (CONTROL) and of larvae reared with the indicated amounts of a grinded *Rhodiola* TABLET preparation added to their food.

There is no difference in associative function between the treatment groups (KW-test at $P > 0.05$). Further details are as in legend of Fig. 6.

Rhodiola *TABLET or* Rhodiola *EXTRACT do not improve associative function*

Given that tablet preparations are commercially available that contain the patented SHR-5 extract from *Rhodiola rosea* ('Arctic root' tablets, Swedish Herbal Institute), and given that this extract has been reported to be behaviourally effective in humans (Darbinyan et al., 2000; Spasov et al., 2000) and *C. elegans* (Wiegant et al., 2009), we test whether food supplementation with grinded 'Arctic root' TABLET would affect associative function; this, however, is not the case (Fig. 9; KW-test: P=0.74, H= 3.5, df= 6, N= 69, 56, 54, 54 , 15, 14, 15). The same lack of effect is seen using a *Rhodiola rosea* EXTRACT (Fig. 10; KW-test: P=0.14, H= 5.41, df= 4, N= 46, 23, 48, 25).

Fig 10

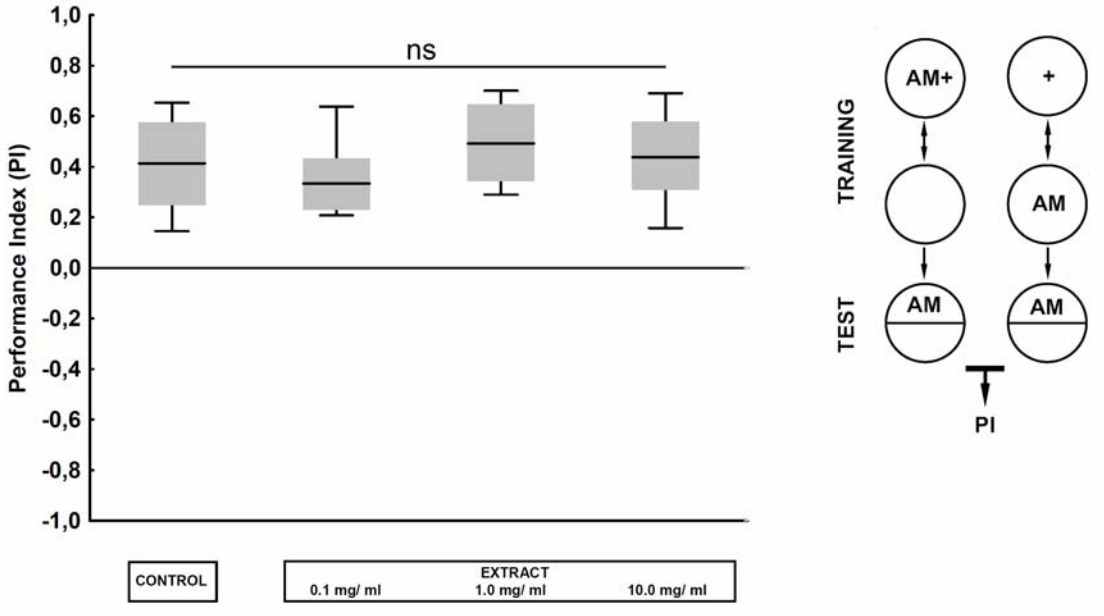

Figure 10 A *Rhodiola* Extract preparation also does not have a 'cognitive enhancement' remedy

Shown are the Performance Indices (PI) of larvae reared on standard food (CONTROL) and of larvae reared with the indicated amounts of a *Rhodiola* EXTRACT preparation added to their food.

There is no difference in associative function between the treatment groups (KW-test at $P > 0.05$). Further details are as in legend of Fig. 6.

Comparing Rhodiola rosea *roots of different origin*

We next test whether *Rhodiola rosea* roots provided by the supplier of the EXTRACT (RHODIOLA^2) would enhance associative function; this, however, is not the case (Fig. 11A; KW- test: $P=0.83$, H= 0.85, df= 3, N= 15, 14, 31, 32). Given this series of negative results, we return to the initially used *Rhodiola rosea* root (RHODIOLA^1), and find that the enhancement of associative function does replicate (Fig. 11B; MW-test: $P< 0.05$; U= 300.5; N= 35, 36). Indeed, in a subsequent experiment a direct comparison reveals a difference in associative function between CONTROL larvae and larvae raised on food containing the RHODIOLA^1 root, the root from the supplier of the EXTRACT (RHODIOLA^2), root from the same place as the initially used RHODIOLA^1 root but of a new crop (RHODIOLA^3), a root sample of Russian origin (RHODIOLA^4), or the commercially available TABLET (Fig. 11C; KW- test: $P< 0.05$, H= 36.6, df= 5, N= 40,

41, 28, 15, 13, 25): Food supplementation with RHODIOLA[1] , RHODIOLA[3], and RHODIOLA[4] (MW-test: $P< 0.05/5$, U= 309, U= 123, U= 117 respectively), but not with RHODIOLA[2] (MW-test: $P= 0.31$; U= 479) or the TABLET (MW-test: $P= 0.81$; U= 483) increases associative function above CONTROL levels.

Fig 11A

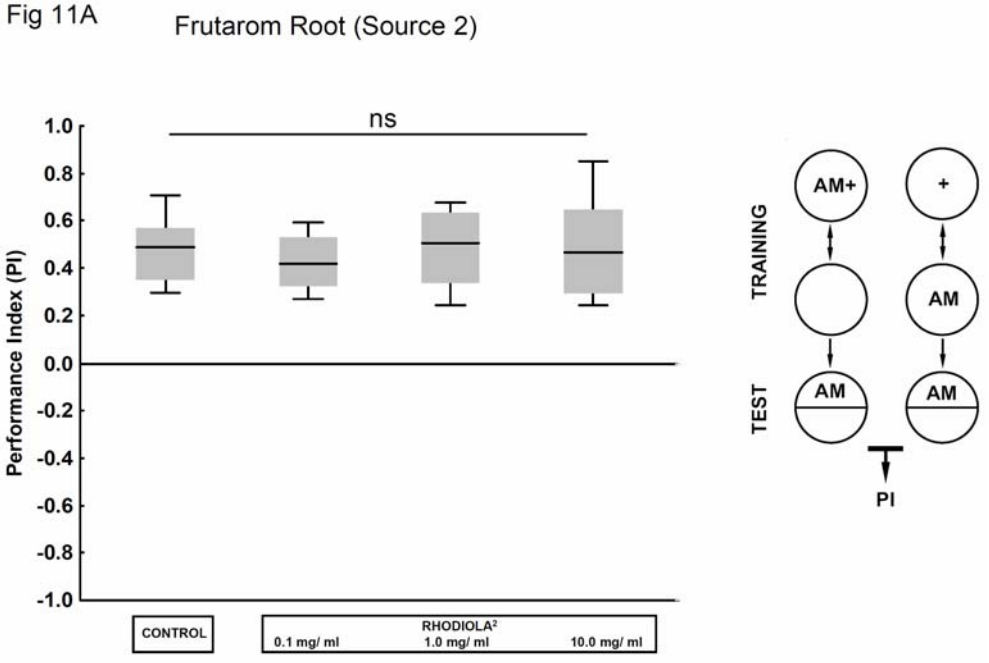

Fig 11B

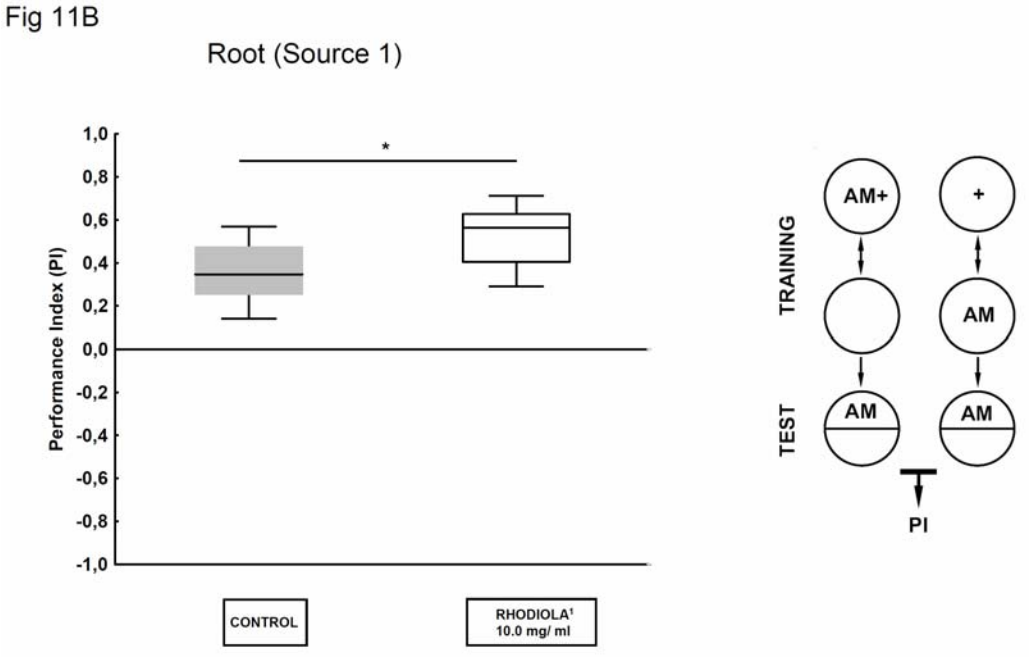

Fig 11C

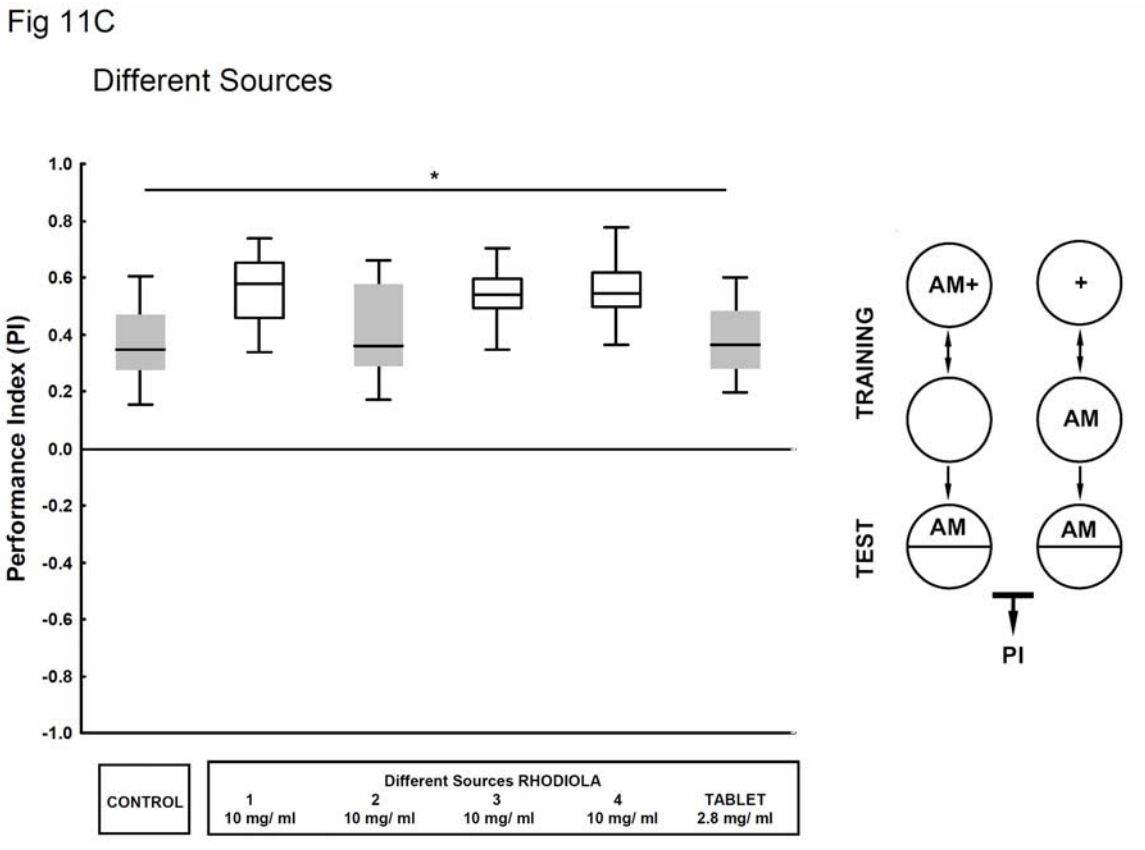

Figure 11 Comparing the 'cognitive enhancement' remedy of *Rhodiola* roots from different sources

(A) Shown are the Performance Indices (PI) of larvae reared on standard food (CONTROL) and of larvae reared with the indicated amounts of RHODIOLA[2] root.

There is no difference in associative function between the treatment groups (KW-test at $P > 0.05$).

(B) Using RHODIOLA[1], the previously observed cognitive enhancing effect replicates: Performance Indices (PI) of larvae reared on standard food (CONTROL) are lower than of larvae reared with 10 mg/ ml of RHODIOLA[1] root (MW-test at $P < 0.05$).

(C) Larvae reared on standard food (CONTROL) show lower PIs than larvae reared on 10 mg/ ml RHODIOLA[1] , RHODIOLA[3] , or RHODIOLA[4] root (MW-test: $P < 0.05/5$). There is no significant difference in PI values between CONTROL larvae and larvae reared on food containing either RHODIOLA[2] (MW-test: $P = 0.31$), or TABLET (MW-test: $P = 0.81$) (the KW- test across all groups yields $P < 0.05$).

Further details are as in legend of Fig. 6.

So what?

We have shown that RHODIOLA[1] treatment dose-dependently improves associative function; this improvement is neither due to alterations in those sensory and motor functions that are relevant for the employed odour-reward learning task, nor to alterations

in general locomotor parameters, nor to alterations induced by reward- or odour-exposure *per se*.

Given that the ability of animals to learn and remember is helpful for survival because it allows preparatory behaviour, for example during the search for food and mates, or in dangerous situations, it may be surprising that this ability does not apparently operate at its maximum. How come there is so much to gain in associative function? Interestingly, a similar question arises in evolutionary terms: There is apparently enough genetic variability to allow the experimental evolution of associative function (Mery and Kawecki, 2002). In both cases, one may ague that mnemonic function is costly in terms of establishing and maintaining the molecules and cellular structures supporting it (Mery and Kawecki, 2003; Mery and Kawecki, 2005). Thus, learning-and-memory systems operate at a level that reflects a multi-dimensional compromise between benefit and costs. Under such conditions, experimental interventions, such as RHODIOLA[1] treatment, can increase mnemonic ability- either directly by conferring a benefit, or indirectly, by curbing the costs. Interestingly, classical genetic approaches do not typically (Drier et al. 2002), if at all (Perazzona et al. 2004), uncover gain-of-function alleles for associative processing- either because such increases require the concerted alteration of two or more mutations, or because the respective behavioural paradigms were optimized for high-performance in the wild-type, precluding the detection of further increases in associative processing. In contrast, experience-based approaches have in some cases yielded increases in associative function (social interaction: Chabaud et al. 2009; reinforcer pre-exposure: Sitaraman et al. 2007).

In any event, our study may have two significant implications for further research: first, it may facilitate the identification of the effective 'cognitive enhancing' chemical compound(s) of *Rhodiola*: *Drosophila* as a genetically tractable study case should allow accelerated analyses of the molecular mechanism(s) that underlie the 'cognitive enhancement' conveyed by RHODIOLA[1], but not any of the closely related other agents used in this study. Given the homology of quite some of the hitherto identified molecular determinants of 'cognition' between animals and man (e.g. Pittenger and Kandel, 2003), such research may have bearings for humans as well. In this context, one should consider that under healthy conditions any increase in associative function (i) likely has hidden physiological 'costs', (ii) may induce too rapid learning and hence superstitious behaviour, i.e. behaviour not sufficiently grounded in experience, and (iii) may distort the balance

between learning and forgetting/extinction. Thus, one-dimensional 'optimization' strategies for cognitive function appear futile; also, in humans any such 'optimization' attempt is arguably unethical in general because it implies insufficient respect for between-individual differences. Still, with these caveats in mind such treatment may nevertheless be desirable in some cases, for example when under poor environmental conditions or under acute, heavy physiological demand mnemonic function needs to be maintained. Last but not least, it should be interesting to see whether RHODIOLA[1] is able to compensate ageing-related or pathological weaknesses of mnemonic systems in flies and/ or in man.

EXPERIMENTAL PROCEDURES

Food medium and fly keeping

For preparing standard food medium, 34 l of water was mixed to 5.9 kg cornmeal (Mühle Hofmann, Röthlein, Germany), boiled for 5 minutes and automatically stirred gently for 4 h; then, the mixture was left overnight. The next day, 400 g soya flour (Mühle Hofmann, Röthlein, Germany), 750 g dried yeast powder (Heirler Cenovis, Radolfzell, Germany) and 250 g agar-agar (Roth, Karlsruhe, Germany) was added to 6 l of water; after stirring, 1.8 l malt (Ulmer Spatz, Bingen am Rhein, Germany) and 1.8 l sugar beet molasses (Grafschafter Krautfabrik, Meckenheim, Germany) was added and together with the cornmeal mixture boiled for 5 min while gently stirred. Upon cooling down to 70- 80 °C, 100 g antifungal agent (methyl-4-hydroxyl benzoate; Merck, Darmstadt, Germany) was added.

To prepare fly-culture vials for our experiments, this food medium then was boiled in a microwave oven and, for CONTROL vials, aliquots of 20 ml were poured in plastic vials and kept at 4 °C for later use. For the experimental groups, either of the following substances were added 5 min after boiling to reach the specified concentrations; then, also these vials were stored for later use at 4 °C:

- RHODIOLA[1]: Dried *Rhodiola rosea* roots (collected in the Carpathian Mountains near lake Lazeshchyna) were grinded for appr. 60 s with a commercial coffee mill,

and the powder was added to the vials 5 min after boiling to reach the specified concentrations; then, vials were stored for later use at 4 °C.

- TABLET: We added grinded 'Arctic root' tablets (Swedish Herbal Institute, Gothenburg, Sweden; purchased via s.a.m. pharma, Viena, Austria) to the food to reach the indicated concentrations. According to the manufacturers specifications, 28 % of the tablets´ weight is of the patented SHR-5 extract of *Rhodiola rosea*. Assuming that this extract is enriched for dried-root ingredients by a factor of ten, a concentration of 2.8 mg/ml of TABLET should thus correspond to 10 mg/ml of RHODIOLA[1]; higher concentrations of TABLET compromise viability (not shown).
- EXTRACT: For these vials, a *Rhodiola rosea* extract was used, kindly provided by Frutarom (Londerzeel, Belgium); by the manufacturers specifications, this extract is enriched 7-fold, such that the chosen concentration range should cover effective RHODIOLA[1] concentrations.
- RHODIOLA[2] roots were also kindly supplied by Frutarom (Londerzeel, Belgium).
- RHODIOLA[3] refers to a second crop of ried *Rhodiola rosea* roots, collected in the Carpathian Mountains near lake Lazeshchyna.
- RHODIOLA[4] refers to dried *Rhodiola rosea* roots of Russian origin.

In all cases, vials were retrieved from the 4 °C store at around noon and two hours afterwards appr. 100 Canton-S wild-type flies were added into the vial which was then maintained at 25 °C, 60- 70 % relative humidity and a 14/ 10 hour light/ dark cycle. On the next day, these flies were removed; after additional four days, larvae were harvested from the food slurry for experiments.

Learning experiments: Two-odour paradigm

Learning experiments follow the procedures introduced by Scherer et al. (2003) and modified as an *en mass* assay by Neuser et al. (2005). In brief, Petri dishes (Sarstedt, Nümbrecht, Germany) with 85 mm inner diameter were filled with 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany), allowed to solidify, covered with their lids, and then left untreated at room temperature until the following day. As positive

reinforcer we used 2 mol fructose (FRU; purity: 99 %, Roth, Karlsruhe, Germany) added to 1 l of agarose 10 min after boiling.

Experiments were performed under natural light at 21- 24 °C. Before experiments, we replaced the regular lids of the Petri dishes with lids perforated in the centre by 15 one-mm holes to improve aeration.

Odour was applied by adding 10 µl of odour substance into custom-made teflon containers (inner diameter 5 mm; these could be closed by a perforated lid with seven holes, 0.5 mm in diameter each). As odours, we used *n*-amylacetate (AM; CAS: 628-63-7; purity: 98.5 %, diluted 1:50 in paraffin oil [Merck, Darmstadt, Germany]), and 1-octanol (OCT; CAS: 111-87-5; purity: 99 %, undiluted) all from Merck, Darmstadt, Germany, unless stated otherwise.

A spoonful of food medium containing larvae was taken from the food vial and transferred to a tap-water droplet on a Petri dish. Thirty animals were collected, briefly washed in tap water and as a group transferred to the assay plates for the start of training; in half of the cases we started with a FRU-containing Petri dish, and in the other half of the cases with a PURE, agarose-only containing Petri dish.

Immediately before a trial, two containers both loaded with the same odour were placed onto the assay plate on opposite sides of the plate, 7 mm from the edges. Within each reciprocal training condition, for half of the cases we started with AM, for the other with OCT, unless stated otherwise. Then, the Petri dish was closed and the larvae were allowed to freely move for 5 min. The larvae then were transferred to a Petri dish with the alternative odour and the respective other substrate for 5 min (for example, AM may be presented on a FRU-containing plate and OCT on an agarose-only Petri dish: AM+/ OCT training). This cycle was repeated three times. Fresh Petri dishes were used for each trial.

After such training, animals were tested for their choice between the odours. The larvae were placed in the middle of PURE Petri dish; unless mentioned otherwise, a container of AM was placed to one side and a container with OCT on other side to create a choice situation. After 3 min, the number of animals on the ‘AM’ or ‘OCT’ side was counted. After this test was completed, the next group animals was run and trained reciprocally (e.g. AM/ OCT+).

For both groups, we calculated the odour preference ranging from –1 to 1. To this end, we determined the number of animals observed on the AM side ($\#_{AM}$) minus the number of animals observed on the OCT side ($\#_{OCT}$), divided by the total number ($\#_{TOTAL}$):

$$(1) \qquad PREF = (\#_{AM} - \#_{OCT})/ \#_{TOTAL}$$

To determine whether these preferences are different depending on training regimen, we took the data from alternately run, reciprocally trained groups and calculated the performance index ranging from –1 to 1 as:

$$(2) \qquad PI = (PREF_{AM+/\ OCT} - PREF_{AM/\ OCT+})/ 2$$

Data for CONTROL and experimental groups were gathered alternately.

Learning experiments: One-odour paradigm

In two experimental series, we used a single-odour training regimen (Saumweber et al., 2011) where the animals in one group received presentations of either AM with the reward and alternately presentations of an empty odour container (EM) on an agarose-only Petri dish (AM+/ EM); the animals trained reciprocally received unpaired presentations of odour and reward (AM/ EM+). During the test, the animals were allowed to choose between AM *versus* EM; data then were treated analogously to what was described above.

Controls for specificity: Behaviour towards odours and sugar in experimentally naïve larvae

To test for the behavioural specificity of RHODIOLA[1] treatment, we determined the behaviour of the respectively reared larvae towards the to-be-associated stimuli:
To test behaviour towards FRU, we prepared split Petri dishes: One side contained agarose-only (PURE), while in the other FRU was present in addition (see sketch Fig. 2A).

Regarding the odours, we gave the larvae the choice between either AM *versus* an empty container, or between OCT versus an empty container (see sketch Fig. 2B, C).

In both cases, experimentally naïve larvae were placed to the middle and after 3 min the number of larvae on either side was counted; then PREF values were calculated as detailed above.

Controls for specificity: Olfactory behaviour after training-like stimulus exposure

As we have argued before (Gerber and Stocker, 2007; Michels et al., 2005), the mere exposure to the training stimuli, i.e. odour-exposure *per se* and reward-exposure *per se*, can have effects on test behaviour. We therefore assayed the behaviour of animals from the CONTROL and RHODIOLA[1] groups towards AM (diluted 1:50 in paraffin oil) and OCT, respectively, after either of two exposure treatments. Either the larvae were exposed to the reward but not the odours in an otherwise training-like way (see sketches in Fig. 3A, A'), or they were exposed to the odours but not the reward (Fig. 3B, B'). Then, PREF scores for the odours were determined as specified above.

Controls for specificity: Larval locomotion

The day before experiments, 145 mm-diameter Petri dishes (Sarstedt, Nümbrecht, Germany) were filled with agarose and stored at room temperature. A single larva was placed in the middle of the Petri dish, the lid was closed and the Petri dish was placed in a 70 cm x 40 cm x 40 cm dark box. For video recording (Fig 4A), the Petri dish was illuminated by a ring of 30 red LEDs (Flexible LED Leiste rot 30 x SMD-LED, 50 cm, 12 V, Lumitronix GmbH, Hechingen, Germany) separated from the Petri dish by a 5 mm thick light-dispensing opaque plastic. A PC-connected webcam was used to monitor larval behaviour at 5 Hz (software courtesy Andreas Eckart, Universität Würzburg, Germany, based on LABVIEW). Tracking was performed for 1 min; if the larva stayed at the edges of the Petri dish for more than 10 % of the passed runtime, tracking was automatically stopped. For each frame, the following values were determined:

- the position of the larva's centre of gravity; this served to estimate the SPEED of the larva across 5 frames (i.e. 1 sec; given in pixel x s^{-1}); for each larva, the median of all SPEED values across the 1-min recording period (60 values) is used for subsequent analyses;
- the area covered by the larva;

- the area and aspect ratio (height/ width) of a bounding box around the larva;
- the orientation of the axis through the larva in an arbitrarily defined 360° (increasing clockwise). From this axis value, we derived two further measures: (i) we determined "d" as the frame-to-frame angle between the orientation of the actual axis and the axis of the previous frame (in °); (ii) we determined "D" (in °) as summed d-values of the ten previous frames; thus D corresponds to the overall change in the larva's angle during the previous two seconds.

TURN: The criterion for a TURN was that the absolute value of D exceeds a threshold of 20° (|D| > 20°). As these TURNs typically last for less than 2 s, this criterion was disabled for the 2 s following a given TURN, making sure that one actual turning event was not considered twice (to distinguish TURNs from occasional turnings on the spot, no TURN was scored if the program detected both an aspect ratio larger than 0.8 [indicating a square-like box around the larva] and when the ratio of the area covered by the object divided by the area of the bounding box was larger than 0.6 [indicating the square-like box was largely covered by the larval body]). From this analysis, we derived for each individual larva the frequency of TURNs during the 1-min observation period.

Statistical analyses

All statistical analyses were performed with Statistica (version 8.0, StatSoft, Inc., Tulsa, OK, USA) on a PC; for the analyses of larval locomotion, some calculations were performed using Excel (version 2003, Microsoft Corporation, Washington, USA). In a conservative approach, non-parametric tests were used throughout: For multiple-group comparisons, Kruskal-Wallis (KW) tests were used, followed in case of significance by pair-wise comparisons with Mann-Whitney (MW) U-tests. The significance level used was 5 %, and was maintained at that level for follow-up pair-wise tests by a Bonferroni correction ($P< 0.05$ divided by the respective number of pair-wise tests). Data are displayed as box plots representing the median as the middle line, 25 and 75 % quantiles as box boundaries, as well as 10 and 90 % quantiles as whiskers, respectively.

Experimenters were blind with respect to treatment condition (food supplementation and content of the training-Petri dishes, respectively); these were decoded only after the experiments.

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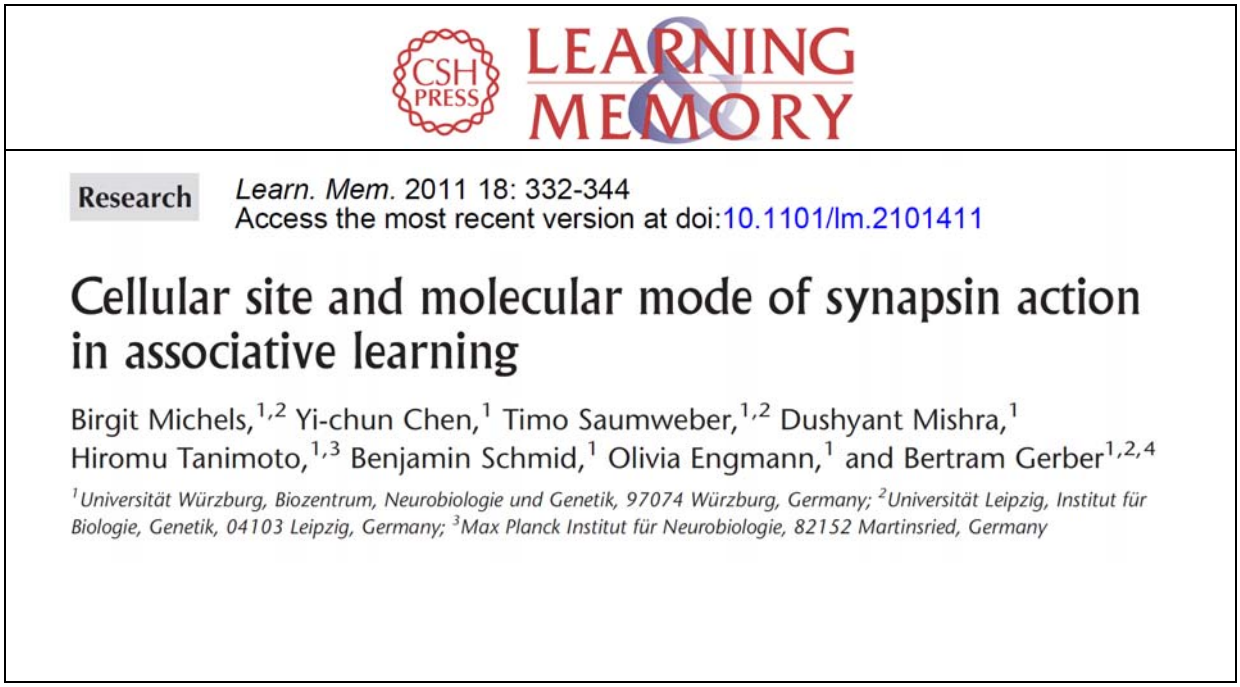
Chapter II.2

Local loss of function of Synapsin

Birgit Michels, Yi-chun Chen, Timo Saumweber, Dushyant Mishra, Hiromu Tanimoto, Benjamin Schmid, Olivia Engmann, Bertram Gerber



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Cellular site and molecular mode of synapsin action in associative learning

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Cellular Site and Molecular Mode of Synapsin Action in Associative Learning

Running title: Synapsin in associative learning

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ABSTRACT

Synapsin is an evolutionarily conserved, presynaptic vesicular phosphoprotein. Here, we ask where and how Synapsin functions in associative behavioural plasticity. Upon loss or reduction of Synapsin in a deletion mutant or via RNAi, respectively, *Drosophila* larvae are impaired in odour-sugar associative learning. Acute global expression of Synapsin and local expression in only the mushroom body, a third-order 'cortical' brain region, fully restores associative ability in the mutant. No rescue is found by Synapsin expression in mushroom body input neurons or by expression excluding the mushroom bodies. On the molecular level, we find that a transgenically expressed Synapsin with dysfunctional PKA-consensus sites cannot rescue the defect of the mutant in associative function, thus assigning Synapsin as a behaviourally-relevant effector of the AC-cAMP-PKA cascade. We therefore suggest that Synapsin acts in associative memory trace formation in the mushroom bodies, as a downstream element of AC-cAMP-PKA signaling. These analyses provide a comprehensive chain of explanation from the molecular level to an associative behavioural change.

INTRODUCTION

Associative, predictive learning is an essential and evolutionarily conserved function of the brain, enabling animals to prepare for defense against or timely escape from predators, and to search for food or other desiderata in an 'educated' way. Using larval *Drosophila*, we ask in which cells of the brain short-term odour-food associative memory traces are established, and what their molecular nature is.

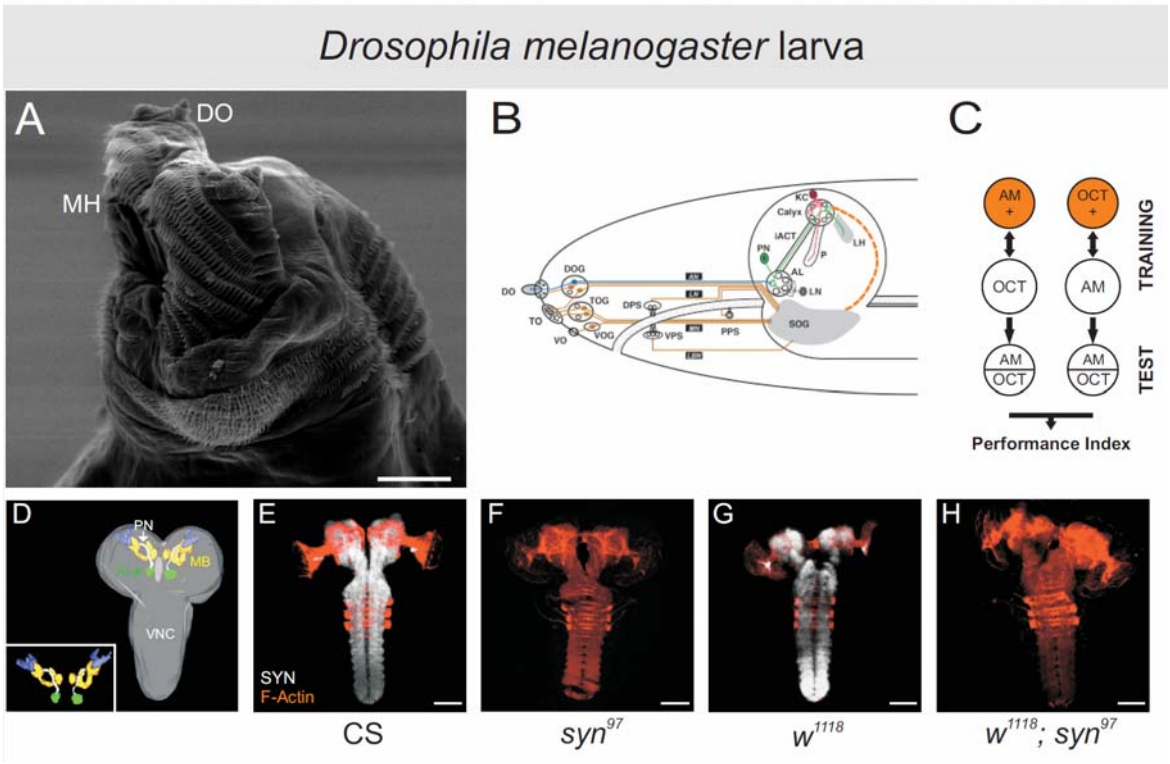
The basic architecture of the larval olfactory pathway is simple (Fig. 1; Movie S1) (Hallem and Carlson 2006; Gerber and Stocker 2007; Vosshall and Stocker 2007; Gerber et al. 2009; Masse et al. 2009): 21 olfactory receptor genes of the *Or* family are expressed, one in each of the 21 olfactory sensory neurons, each innervating one of 21 anatomically identifiable antennal lobe glomeruli. Within the antennal lobe, lateral connections shape information flow to ~ 21 uniglomerular projection neurons, which convey signals to two target areas, the calyx of the mushroom body and the lateral horn, each entertaining connectivity to premotor centers. In the calyx, which consists of ~ 600 mature Kenyon cells, projection neurons typically innervate but one anatomically-identifiable calycal glomerulus. In turn, Kenyon cells receive input from 1- 6 randomly chosen glomeruli, establishing a divergence-convergence architecture suitable for combinatorial coding. Output from the mushroom body then is carried to premotor centers via few mushroom body output neurons. As for the second target area of the uniglomerular projection neurons, they innervate the lateral horn, which relays to premotor centers, too. Thus, dependent on the ligand profiles of the olfactory receptors and the connectivity within this system, odours activate specific combinations of neurons along the olfactory pathways. Regarding taste, ~ 90 gustatory sensory neurons are distributed across three external and three internal sense organs, projecting to distinct areas in the suboesophageal ganglion, according to the receptor gene they express and their sense-organ of origin. From the suboesophageal ganglion, reflexive gustatory behaviours can be driven via the ventral nerve cord, and modulatory neurons (e.g. octopaminergic and dopaminergic neurons) are sent off to the brain, including the mushroom bodies, to signal reinforcement (Schroll et al. 2006; Selcho et al. 2009).

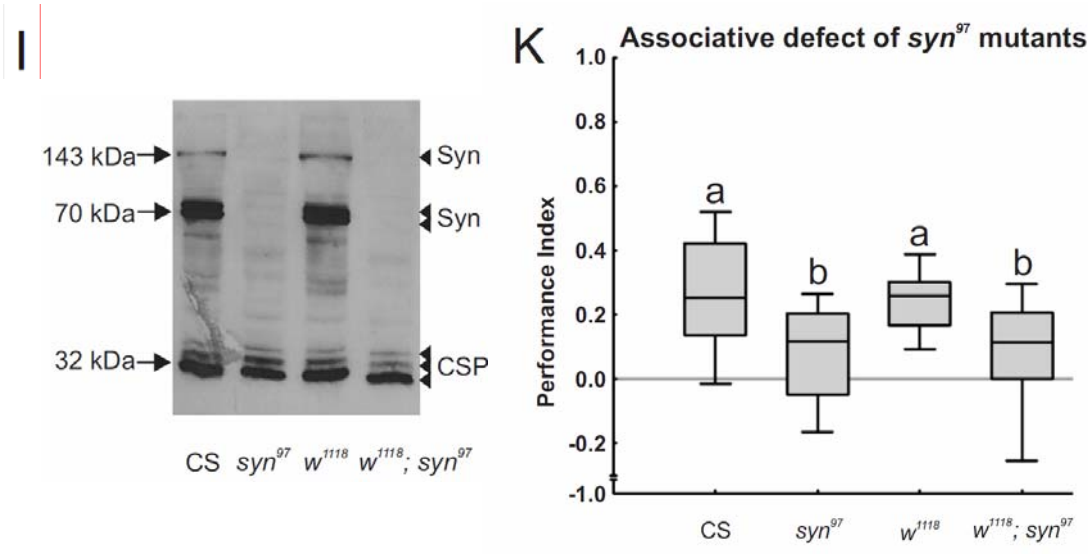
On the molecular level, mutant screens for associative ability in *Drosophila* (Dudai et al. 1976; Aceves-Pina and Quinn 1979) (regarding *Aplysia* see Brunelli et al. 1976) identified the adenylyl cyclase-cAMP-PKA pathway as what turned out to be an evolutionarily

conserved determinant for synaptic and behavioural plasticity (Pittenger and Kandel 2003; Davis 2005; for larval *Drosophila*: Aceves-Pina and Quinn 1979; Zhong and Wu 1991; Khurana et al. 2009). However, the actual effector proteins that are phosphorylated by PKA to support fly short-term memory remained clouded (for *Aplysia* see Hawkins 1984). Here, we test whether the Synapsin protein may be one such PKA target.

Synapsin is an evolutionarily conserved phosphoprotein associated with synaptic vesicles (Hilfiker et al. 1999; Sudhof 2004), which in flies is dispensable for basic synaptic transmission (Godenschwege et al. 2004). In *Drosophila*, Synapsin is encoded by a single gene (Klagges et al. 1996). It can bind to both synaptic vesicles and cytoskeletal actin (Greengard et al. 1993; Hilfiker et al. 1999; Hosaka et al. 1999), forming a so-called reserve pool of vesicles. Importantly, phosphorylation of Synapsin allows synaptic vesicles to dissociate from this reserve pool and to translocate towards the active zone, making them eligible for release upon a future action potential (Li et al. 1995; Hilfiker et al. 1999; Akbergenova and Bykhovskaia 2007; Gitler et al. 2008; Akbergenova and Bykhovskaia 2010). Candidate phosphorylation sites to mediate such plasticity in *Drosophila* include the evolutionarily conserved PKA/CaM kinase I/IV consensus site in domain A, and an evolutionarily not conserved PKA-consensus site near domain E (Kao et al., 1999; Klagges et al., 1996; Hilfiker et al., 1999), as well as seven recently identified phosphorylation sites of *Drosophila* Synapsin (Nuwal et al. 2010) (regarding *Helix*, see also Giachello et al. 2010). On the behavioral level, the protein-null deletion mutant syn^{97} suffers from a 50 % reduction in odour-sugar reward memory (Michels et al. 2005) (adult odour-shock learning: Godenschwege et al. 2004; Knapek et al. 2010), whereas the ability to recognize gustatory and olfactory stimuli, motor performance, sensitivity to experimental stress, sensory adaptation, habituation, and satiation all remain intact in these mutants (Michels et al. 2005). However, attributing the defect in associative function in the deletion mutant to the lack of the Synapsin protein requires a rescue, which had not been attempted to date, neither in adults, nor in larvae. Using a series of such rescue as well as RNAi experiments, we analyze on the cellular level where in the larval brain a Synapsin-dependent memory trace is localized. On the molecular level, we test whether mutated forms of the Synapsin protein, which lack functional PKA-consensus motifs, are able to support associative function.

Fig.1


Drosophila melanogaster larva
A
DO
MH
B
C
AM
OCT
TRAINING
TEST
Performance Index
D
PN
MB
VNC
E
SYN
F-Actin
F
G
H
CS
syn97
w1118
w1118; syn97


I
143 kDa
70 kDa
32 kDa
Syn
Syn
CSP
CS syn97 w1118 w1118; syn97
K
Associative defect of syn97 mutants
Performance Index
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0.8
0.6
0.4
0.2
0.0
-0.2
-1.0
a
b
a
b
CS
syn97
w1118
w1118; syn97

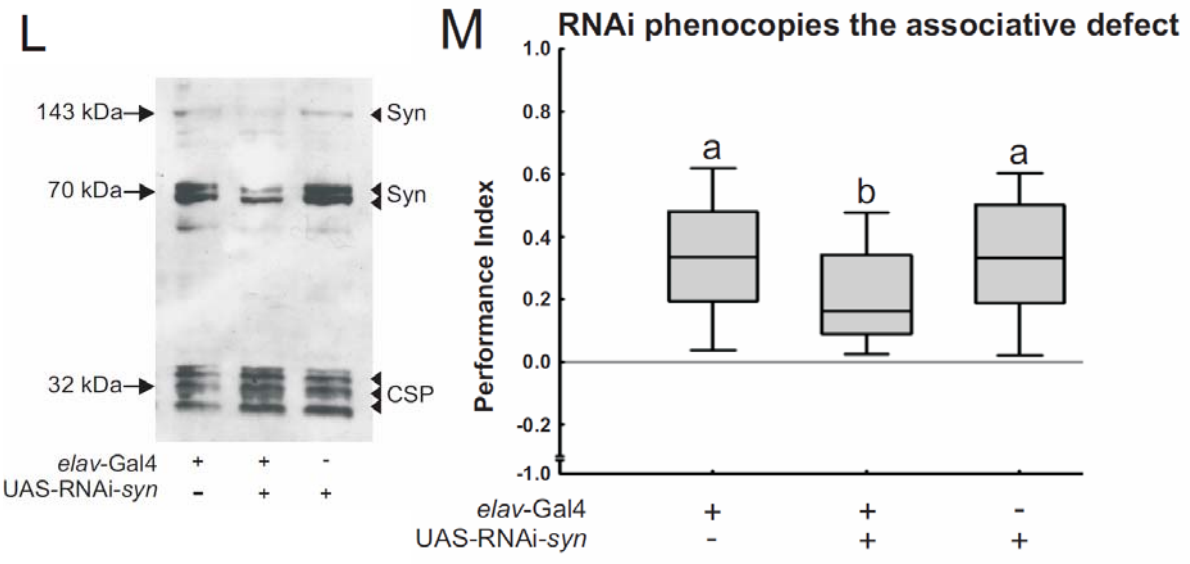


Figure 1: The chemosensory pathways of *Drosophila* larva and the requirement of Synapsin for associative function. (A) SEM image of the larval head; courtesy of M. Koblofsky. (B) Cephalic chemosensory pathways in the larva (modified from Gerber and Stocker, 2007). (C) The odour-sugar associative learning paradigm. Circles represent petridishes containing a sugar reward (orange, +) or only pure agarose (white). Animals are trained either AM+/OCT or OCT+/AM, and are then tested for choice between AM *versus* OCT (for half of the cases, the sequence of training trials is reversed: OCT/AM+ and AM/OCT+). (D) Dorsal view of a *Drosophila* larval brain with the major brain regions reconstructed. The inset shows a magnified view of the MB (see also Movie S1). (E-K) Associative impairment of syn^{97} mutants is interpretable without reference to *white* function. (E-I) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes; the western blot shows the expected bands at 74 and 143 kDa. (K) In syn^{97} and w^{1118}; syn^{97} mutants, associative function is reduced by half; the w^{1118} mutation has no effect. Box plots marked with different letters indicate significant differences in associative ability ($P< 0.05/ 4$). (L, M) Associative function is impaired upon knock-down of Synapsin by RNAi**.** (L) Western blot from brains of larval *Drosophila* of the indicated genotypes. Synapsin expression is reduced in the brain-wide KNOCK-DOWN larvae. (M) Associative function is impaired in the brain-wide KNOCK-DOWN strain. Box plots marked with different letters indicate significance ($P< 0.05/ 2$).

MH Mouth hook; dorsal, terminal, ventral organ (DO, TO, VO) and their ganglia (DOG, TOG, VOG); AL antennal lobe, PN projection neurons, MB mushroom body, P peduncle of the MB, KC Kenyon cells comprising the MB, LH lateral horn; antennal, labral, maxillary, labial nerve (AN, *LN*, MN, LBN); dorsal, ventral, posterior pharyngeal sense organ (DPS, VPS, PPS); LN local interneurons, PN projection neurons, iACT inner antennocerebral tract, SOG subesophageal ganglion; the orange arrowheads indicate aminergic reinforcement neurons towards the mushroom bodies; the pharynx is shown stippled; VNC ventral nerve cord. Scale bars: 50µm.

RESULTS

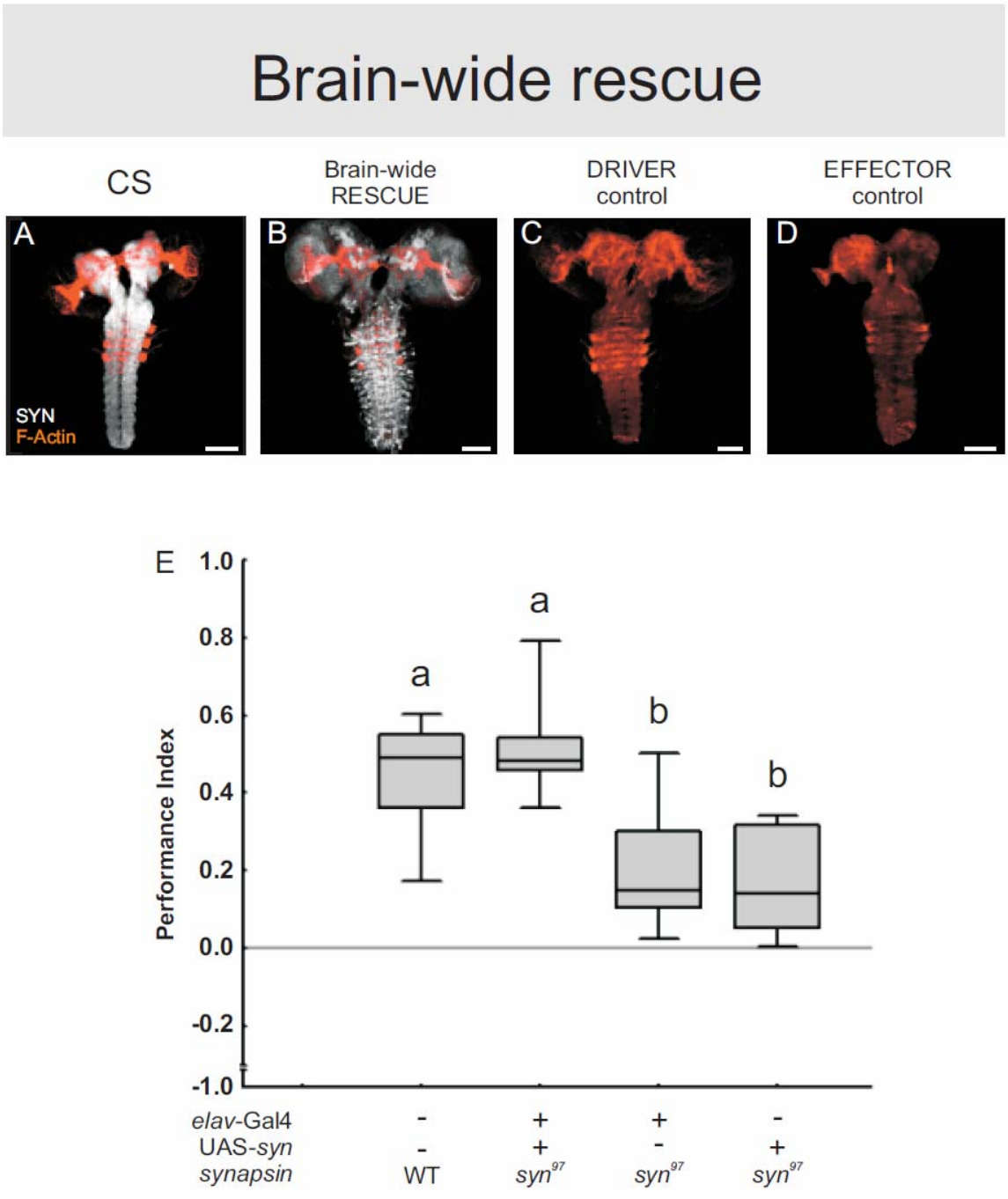
Associative defect of syn^{97} mutants phenocopied by RNAi

We have shown (Michels et al. 2005) that larvae lacking Synapsin (syn^{97}) show a 50 % reduction in an odour-sugar associative learning paradigm but show intact ability to (i) taste, (ii) smell, and (iii) to move about the test arena; also, susceptibility to (iv) the stress of handling, (v) olfactory adaptation, and (vi) changes of motivation as caused by the

experimental regimen are unaltered. Here, we first confirm the lack of Synapsin (Fig. 1F, H, I) and the associative defect of syn^{97} larvae: Wild-type CS show about twice as high associative performance indices as compared to syn^{97} mutants (Fig. 1K; MW: P< 0.05/ 4; U= 106; N= 28, 16). The same defect is uncovered comparing between w^{1118} and w^{1118}; syn^{97} larvae (Fig. 1K; MW: P< 0.05/ 4; U= 44; N= 16, 13). This shows that the defect of syn^{97} larvae in odour-sugar associative learning – and thus performance of transgenic larvae carrying w^{1118} as marker - can be interpreted without reference to *white* function.

Next, using RNAi, we find that Synapsin levels are indeed reduced (Fig. 1L), and concomitantly associative performance scores in the KNOCK-DOWN larvae are about 50 % lower than in EFFECTOR control (Fig. 1M; MW: P< 0.05/ 2, U= 408), and in DRIVER control larvae (Fig. 1M; MW: P< 0.05/ 2, U= 441) (KW: P< 0.05; H= 8.00; df= 2; N= 36, 37, 34). Thus, a reduction of Synapsin by means of RNAi causes an associative impairment which phenocopies the defect in the syn^{97} null mutant.

Fig. 2


Brain-wide rescue
CS
Brain-wide RESCUE
DRIVER control
EFFECTOR control
A
B
C
D
SYN
F-Actin
E
Performance Index
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0.8
0.6
0.4
0.2
0.0
-0.2
-1.0
a
a
b
b
elav-Gal4
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synapsin
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+
syn97
+
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+
syn97

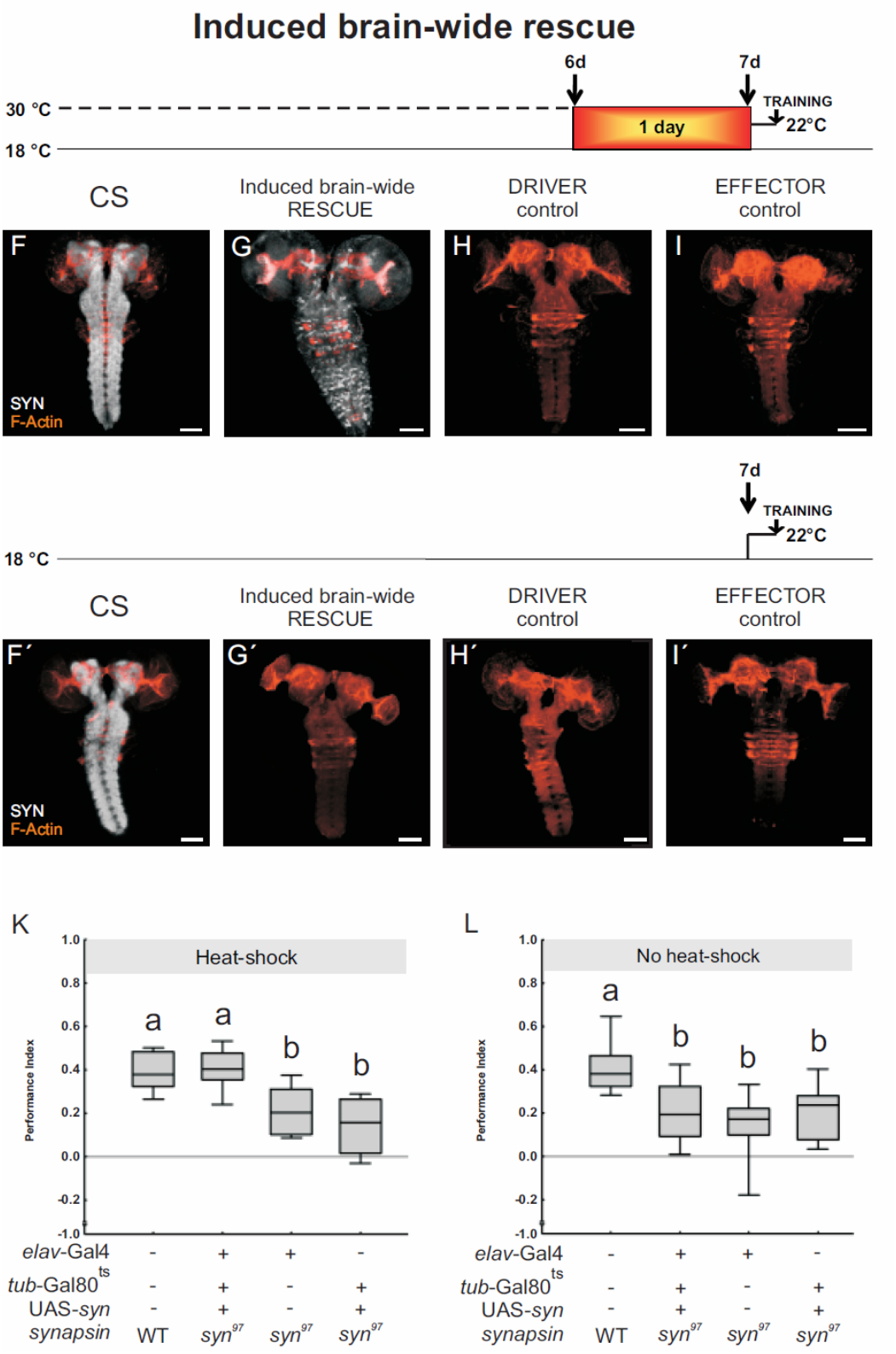


Figure 2: Brain-wide and induced rescue. (A-E) Constitutive and (F-L) induced expression of Synapsin. (A-D, F-I´) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. (A-D) Synapsin expression is detected in wild-type CS and in the brain-wide RESCUE strain. (E) Associative function is fully rescued in the brain-wide RESCUE strain. (F-I) With

heat-shock, Synapsin expression is seen in wild-type CS and induced brain-wide RESCUE larvae; (F´-I´) without heat-shock, Synapsin staining is detected only in the wild-type CS strain. (K) Associative function is fully rescued by induced Synapsin expression; without heat-shock (L), no rescue is observed. Scale bars: 50µm.

All other details as in the legend of Fig. 1.

Brain-wide rescue

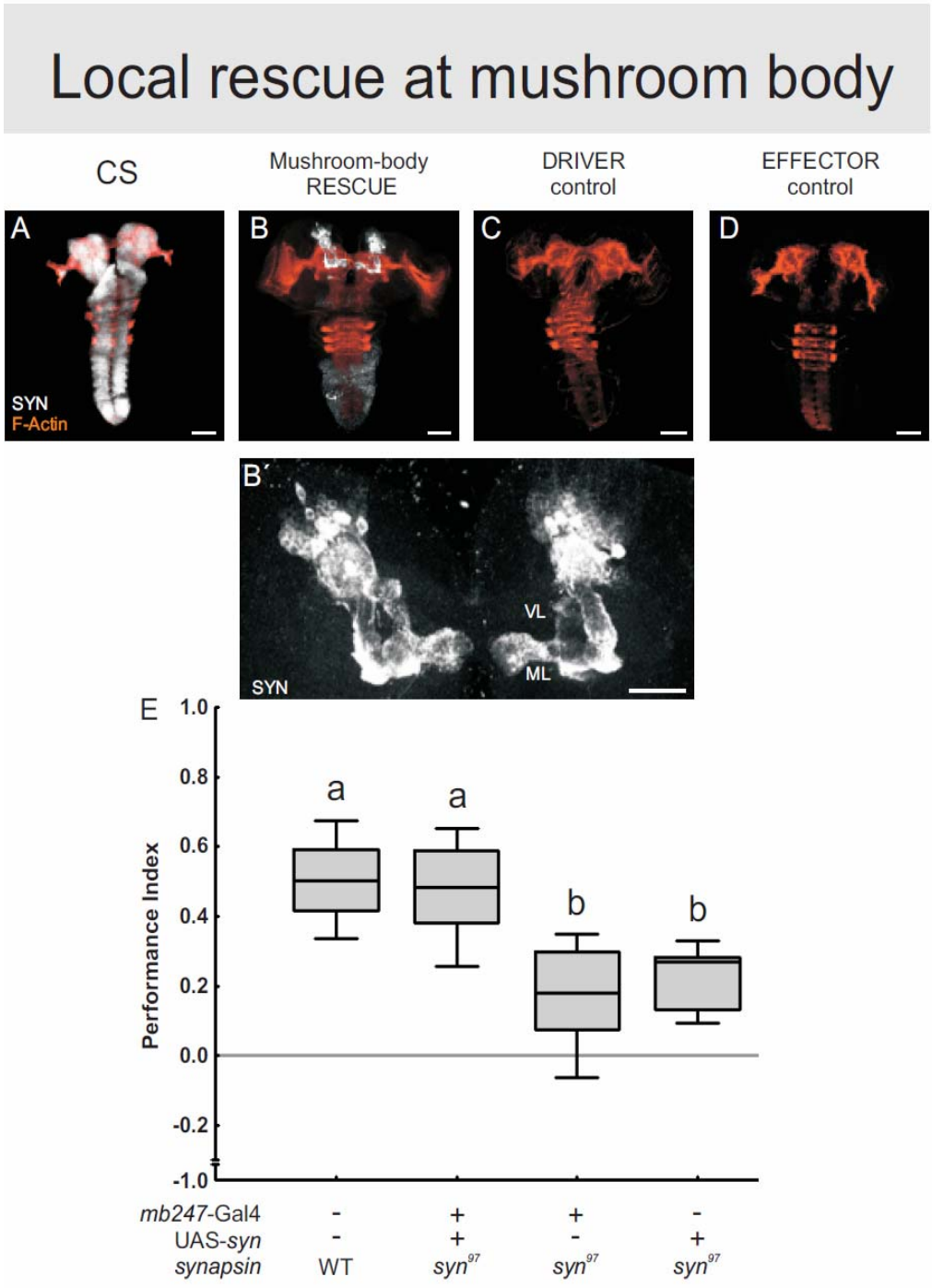
In brain-wide RESCUE larvae, Synapsin expression is restored throughout the brain (Fig. 2B; S1B-D; Movie S2). Comparing performance scores between genotypes shows a difference in associative ability (Fig. 2E; KW: P< 0.05; H= 19.03; df= 3; N= 9, 7, 7, 10). Specifically, the brain-wide RESCUE larvae perform better than EFFECTOR control larvae (Fig. 2E; MW: P< 0.05/ 3, U= 0) and DRIVER control larvae (Fig. 2E; MW: P< 0.05/ 3, U= 4.5). Importantly, associative ability is restored fully in the brain-wide RESCUE larvae, i.e. they do as well as wild-type CS larvae (Fig. 2E; MW: P> 0.05/ 3; U= 28). Thus, a brain-wide rescue of Synapsin is sufficient to fully restore the *syn*^{97} mutant associative defect.

Induced rescue

To see whether the defect in associative function upon lack of Synapsin is indeed due to an acute requirement of Synapsin, we induce expression acutely before the behavioural experiment. Upon heat shock (HS) to induce Synapsin expression, both wild-type CS and induced brain-wide RESCUE larvae show Synapsin expression throughout the brain (Fig. 2F, G). However, the genetic controls do not show Synapsin expression (Fig. 2H, I). When no heat shock is applied, Synapsin is found only in the wild-type CS, but in neither of the other genotypes (Fig. 2F´-I´). With regard to associative ability, the four genotypes differ after heat shock (Fig. 2K; KW: P< 0.05; H= 18.37; df= 3; N= 8, 10, 8, 12). Importantly, induced brain-wide RESCUE larvae show the same associative performance indices as wild-type CS larvae (Fig. 2K; MW: P= 0.79; U= 37). Also, upon heat shock the induced brain-wide RESCUE larvae perform significantly better than EFFECTOR control (Fig. 2K; MW: P< 0.05/ 3, U= 11) and than brain-wide DRIVER control larvae (Fig. 2K; MW: P< 0.05/ 3, U= 11). When no heat shock is given, associative performance scores expectedly also show a significant difference between the four genotypes (Fig. 2L; KW: P< 0.05; H= 12.95; df= 3; N= 9, 12, 9, 8); however, without heat shock the induced brain-

wide RESCUE larvae show significantly lower scores than wild-type CS (Fig. 2L; MW: P< 0.05/ 3; U= 16) and do not differ from EFFECTOR control (Fig. 2L; MW: P> 0.05/ 3, U= 47) and brain-wide DRIVER control larvae (Fig. 2L; MW: P> 0.05/ 3, U= 44). Therefore, associative function is restored fully when Synapsin expression is acutely induced, suggesting an acute function of Synapsin in associative processing.

Fig.3



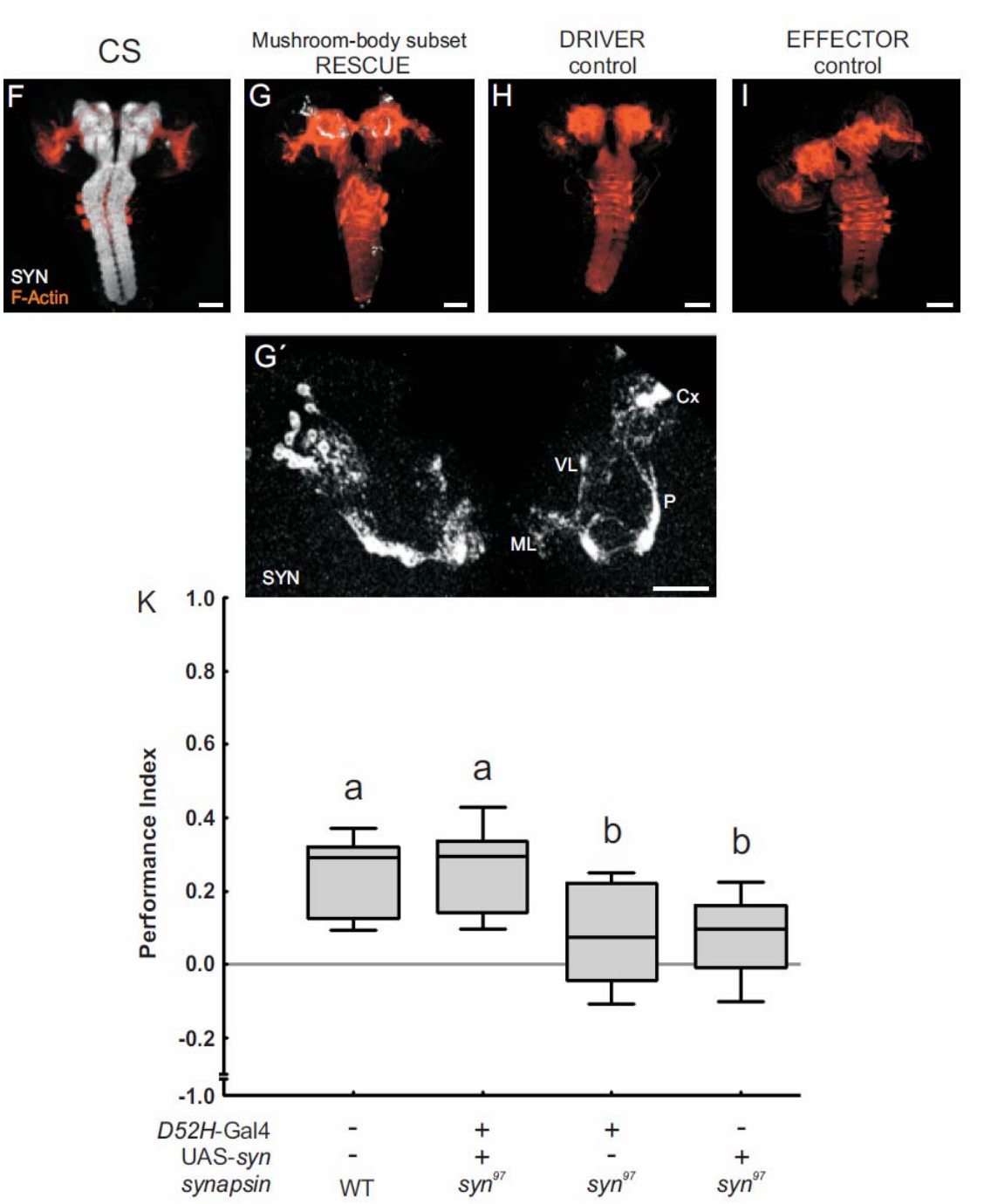


Figure 3: Local rescue at the mushroom bodies. (A-D, F-I) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes; in (B´) and (G´), a magnified view of the mushroom bodies from the RESCUE strain is presented. (E) Associative function is fully rescued in the mushroom-body RESCUE strain. (F-K) Local rescue in a small subset of mushroom body neurons by using a mushroom-body subset driver (*D52H*-Gal4). Associative function is fully rescued in the mushroom-body subset RESCUE strain. Calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML). Scale bars: 50µm in A-D and F-I, 25µm in B' and G'.

All other details as in the legend of Fig. 1.

Local rescue at mushroom body

We next ask whether Synapsin expression in only the mushroom bodies will restore the defect of the *syn*^{97} mutants in associative function. Associative performance scores differ between wild-type CS, mushroom-body RESCUE strain, DRIVER control, and EFFECTOR control (Fig. 3E; KW: P< 0.05; H= 21.39; df= 3; N= 10, 11, 10, 11). Mushroom-body RESCUE larvae show associative scores indistinguishable from wild-type CS (Fig. 3E; MW: P= 0.62; U= 48), but better than mushroom-body DRIVER control (Fig. 3E; MW: P< 0.05/ 3; U= 11) and EFFECTOR control larvae (Fig. 3E; MW: P< 0.05/ 3; U= 18). We therefore conclude that Synapsin expression in the mushroom body, as covered by the *mb247*-Gal4 driver (Fig. 3B, B´), is sufficient to fully rescue the *syn*^{97}- mutant defect in an odour-sugar associative learning paradigm.

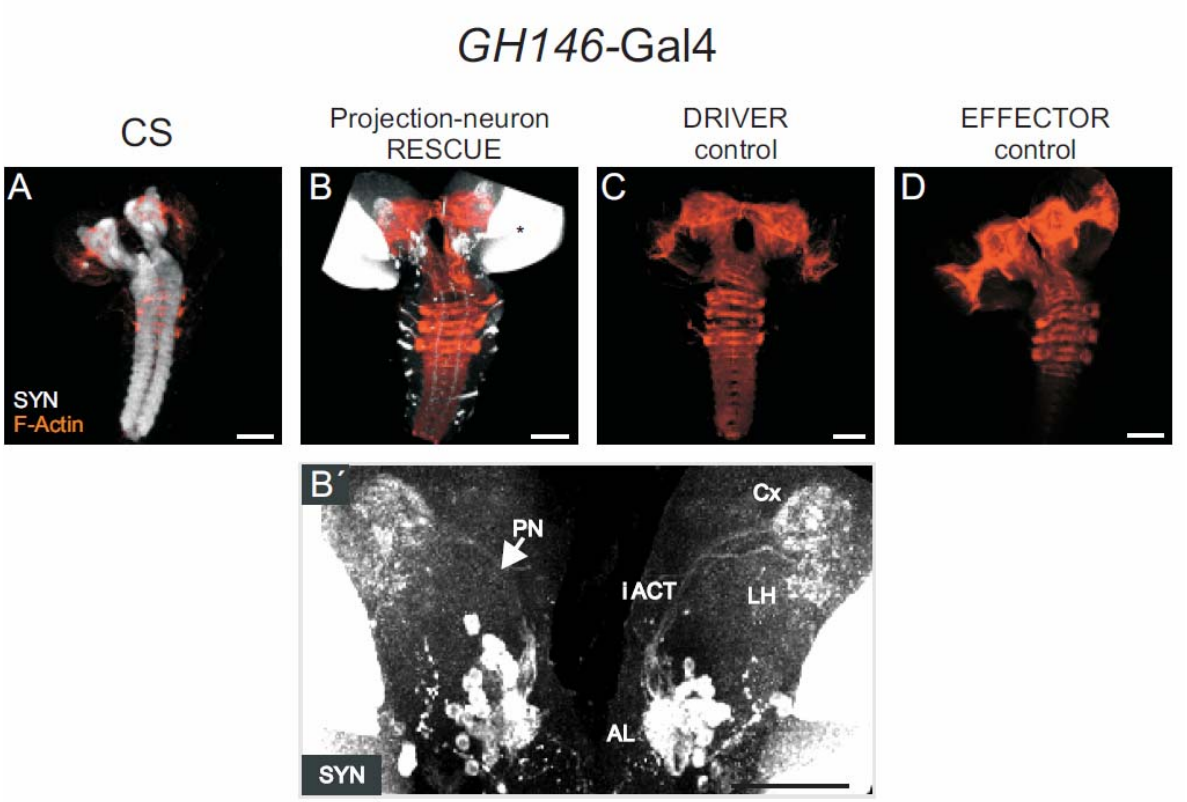
In terms of expression pattern, *mb247*-Gal4 leads to Synapsin expression in all basic compartments of the larval mushroom body, i.e. calyx, peduncle and lobes (Fig. 3B, B´; S1E, F; Movie S3), covering ~ 300 larval mushroom body neurons.

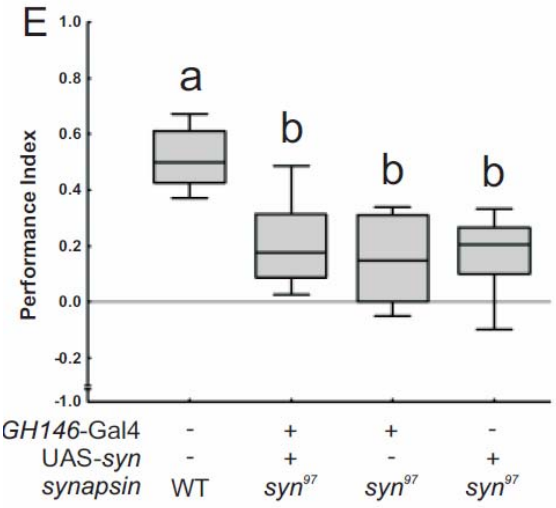
We next ask whether a rescue of associative function can also be found if drivers are used that cover fewer mushroom body neurons. Crossing the *D52H*-Gal4 driver to a UAS-*GFP* effector strain, we observe that expression is found in indeed few mushroom body neurons (7 mushroom body neurons per hemisphere: Fig. S1G, H). Notably, although only so few mushroom body neurons are covered, GFP expression reveals the basic compartments of the larval mushroom bodies; in particular the mushroom body input regions (the calyx) seem to be covered fairly well (Fig. S1G, H; Movie S4). The same holds true for Synapsin expression if the *D52H*-Gal4 driver strain is recombined into the *syn*^{97}- mutant background and crossed to our rescue effector strain (Fig. 3G, G´).

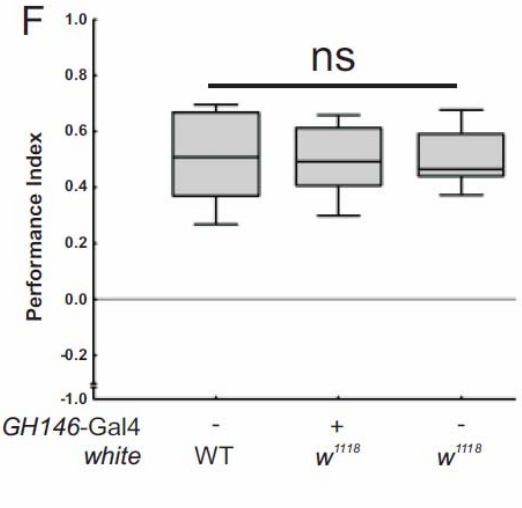
Using the *D52H*-Gal4 driver, we find that wildtype CS, the mushroom-body-subset RESCUE strain and its genetic controls differ in associative performance indices (Fig. 3K; KW: P< 0.05; H= 13.85; df= 3; N= 12, 10, 12, 12). Mushroom-body–subset RESCUE larvae do just as well as wild-type CS (Fig. 3K; MW: P= 0.55; U= 51), whereas they perform better than either mushroom-body-subset DRIVER control (Fig. 3K; MW: P< 0.05/ 3; U= 18) or EFFECTOR control larvae (Fig. 3K; MW: P< 0.05/ 3; U= 21.0). This suggests that Synapsin expression in only a handful of mushroom body neurons, defined by expression from the *D52H*-Gal4 driver, can be sufficient to rescue the *syn*^{97}- mutant defect in associative function.

Fig.4

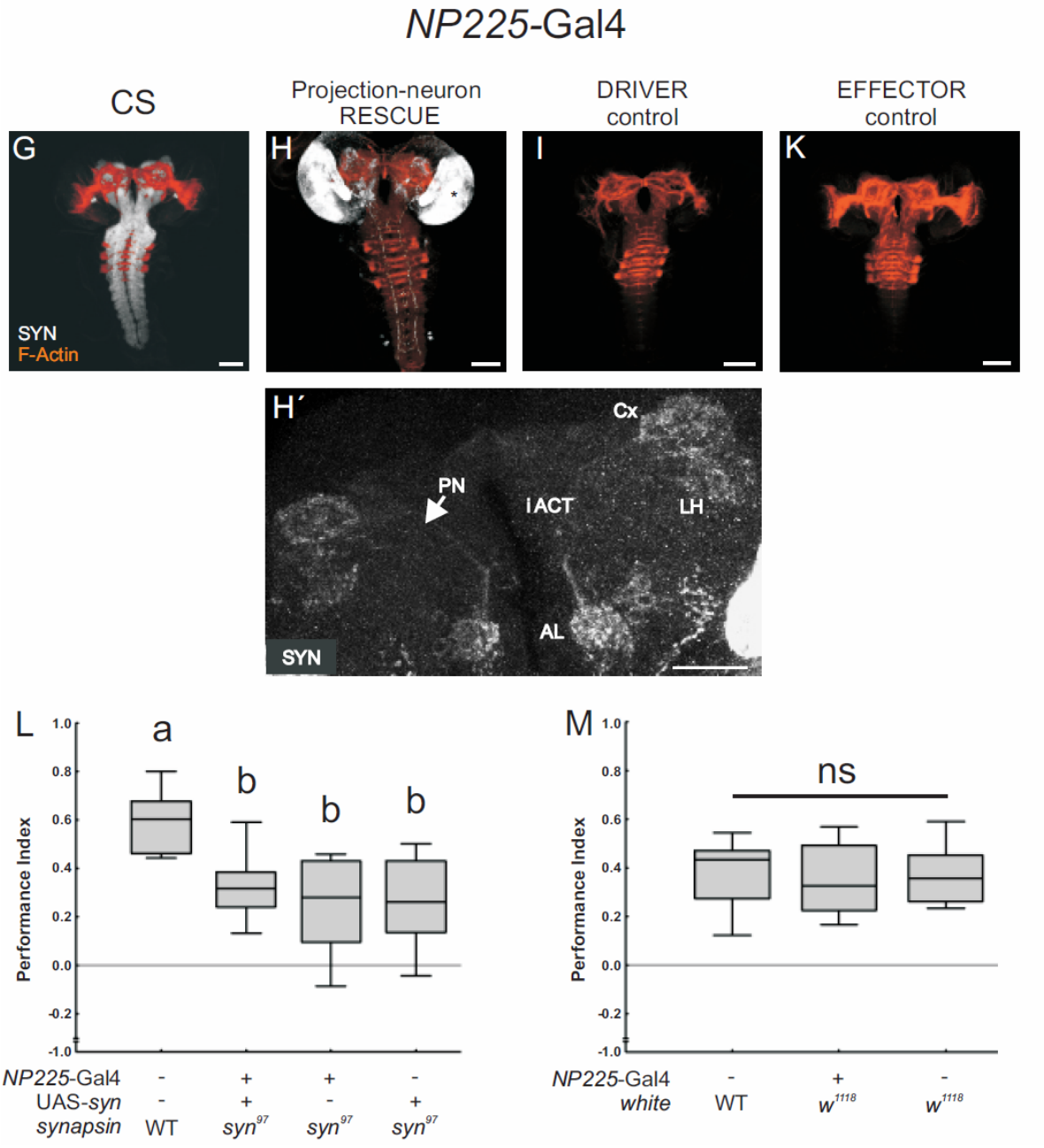
No projection neuron rescue







No projection neuron rescue



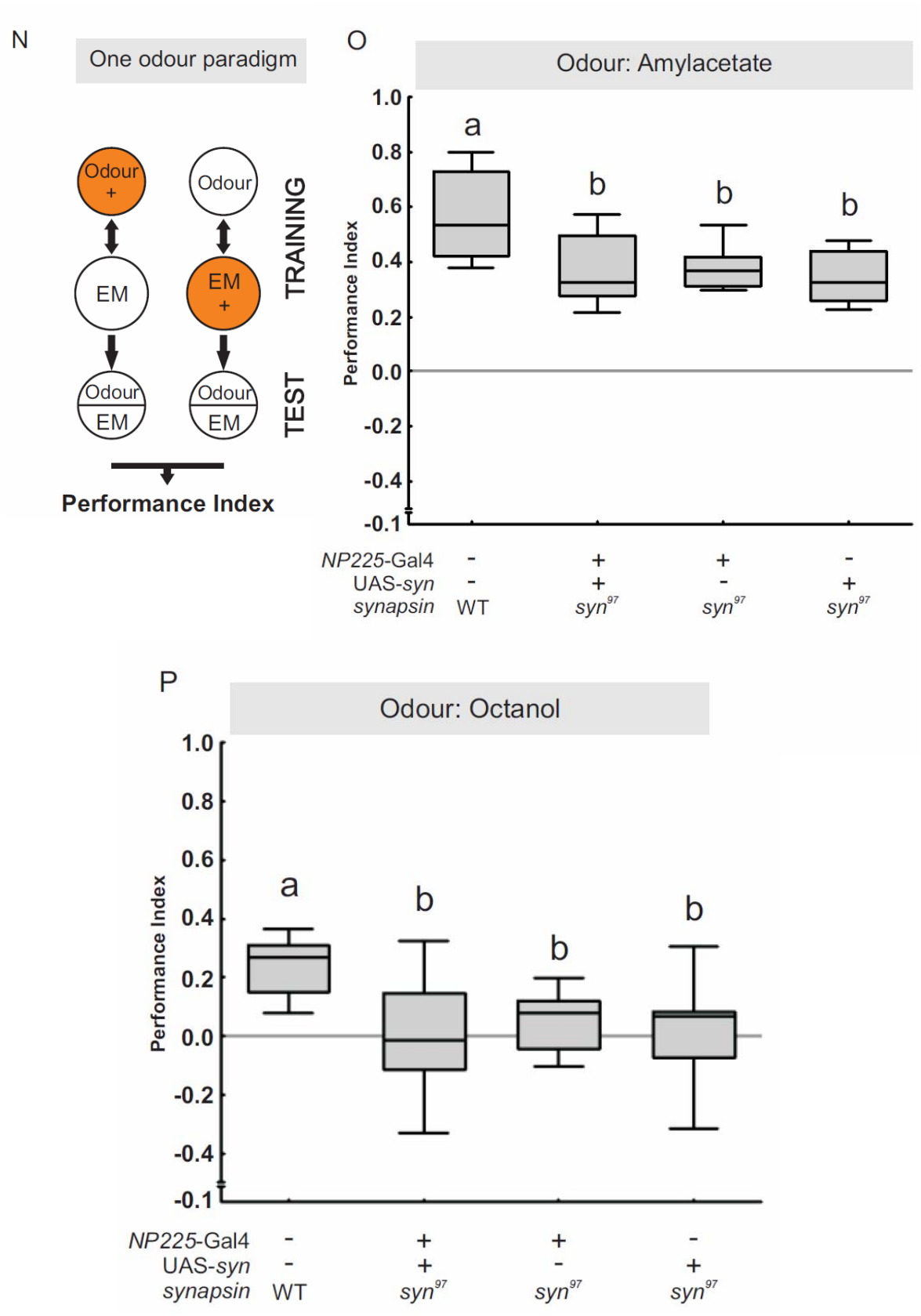


Figure 4: No rescue in the projection neurons. (A-D, G-K) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. In (B´, H´), magnified views of the projection neurons from the RESCUE strains are presented. (E)

Synapsin expression in projection neurons (driver *GH146*-Gal4) is not sufficient to restore associative function. (F) No haploinsufficiency caused by the insertion of the *GH146*-Gal4 construct. (G-M) Also another projection neuron driver (*NP225*-Gal4) is not sufficient to restore associative ability (L), and also does not entail haploinsufficiency (M). (N) Schematic of the one-odour learning paradigm. Larvae receive either paired or unpaired presentations of odour and reward (orange label, +), and then are assayed for their preference for the trained odour. (O, P) No rescue of associative function by Synapsin expression (driver *NP225*-Gal4) in projection neurons in the one-odour paradigm using either AM (O) or OCT (P). Optic lobe Anlagen (*), projection neuron (PN), antennal lobe (AL), inner antennocerebral tract (iACT), calyx (Cx), lateral horn (LH). Scale bars: 50µm in A-D and G-K, 25µm in B' and H'.

All other details as in the legend of Fig. 1.

No rescue at projection neurons

Given that in bees (reviewed in Menzel 2001) and adult flies (Thum et al. 2007) the projection neurons have been suggested as an additional site of an odour-sugar memory trace, we next test whether associative function is restored in projection-neuron RESCUE larvae as compared to their genetic controls and wild-type CS. Associative performance indices between these genotypes are different (Fig. 4E; KW: $P< 0.05$; $H= 19.15$; $df= 3$; $N= 10, 10, 10, 10$). Importantly, however, projection-neuron RESCUE larvae show scores significantly smaller than wild-type CS (Fig. 4E; MW: $P< 0.05/ 3$; $U= 9$) and indistinguishable from either genetic control (Fig. 4E; projection-neuron RESCUE versus projection-neuron DRIVER control: MW: $P> 0.05/ 3$; $U= 43.5$; projection-neuron RESCUE versus EFFECTOR control: MW: $P> 0.05/ 3$; $U= 46$).

However, as is the case for any lack-of-rescue, the insertion of the driver construct may produce haploinsufficiency in the gene(s) neighbouring it, and this haploinsufficiency may lead to a learning defect masking an actually successful rescue. Therefore, we compare larvae heterozygous for the used projection-neuron driver construct (*GH146*-Gal4) to wild-type CS and w^{1118} mutant larvae. Associative performance indices of these three genotypes are indistinguishable (Fig. 4F; KW: $P> 0.05$; $H= 0.04$; $df= 2$; CS: $N= 10, 10, 10$). Thus, expression of Synapsin in projection neurons, as covered by *GH146*-Gal4, is not sufficient for rescuing the syn^{97} mutant defect in a larval odour-sugar associative learning paradigm. This lack-of-rescue cannot be attributed to a haploinsufficiency caused by the insertion of the *GH146*-Gal4 construct.

Regarding the expression pattern of Synapsin supported by *GH146*-Gal4, we note that consistent with what has been reported previously (Marin et al. 2005; Masuda-Nakagawa et al. 2005; Ramaekers et al. 2005), a substantial fraction of the projection neurons (at

least 13-16 of the total of about 21) are expressing Synapsin. Correspondingly, we observe expression throughout the input and output regions of the projection neurons (antennal lobe, mushroom body calyx, lateral horn: Fig. 4B, B´). Obviously, however, expression is not restricted to the projection neurons (see also Heimbeck et al. 2001; Thum et al. 2007): Strong expression is seen in the optic lobe Anlagen, a site where in the wild-type CS strain no Synapsin is expressed (* in Fig. 4B). As synapse formation in the lamina emerges at the earliest in the midpupal period, this expression likely is without consequence in our paradigm. Finally, when assayed via GFP-expression, we uncover expression in a mushroom body-extrinsic neuron (Fig. S1I-L; Movie S5; see also Heimbeck et al. 2001). Possibly, such expression remains unrecognized in terms of Synapsin immunoreactivity. Given that all these behavioural and histological conclusions are confirmed using *NP225*-Gal4 as another projection-neuron RESCUE strain (Fig. 4G-M; S1M-O; Movie S6), a rescue of the associative defect in the syn^{97}-mutant does not appear to be possible in the projection neurons.

Scrutinizing the lack-of-rescue at projection neurons

Of all available fly strains, *GH146*-Gal4 and *NP225*-Gal4 express broadest and strongest in the projection neurons. Still, about one third of the projection neurons of the larva are not covered. Therefore, it is possible that within the Gal4-expression pattern, activity evoked by both odours is the same, whereas those projection neurons that allow making a difference between both odours could be spared from Gal4 expression. We therefore tested the projection neuron rescue larvae in a one-odour paradigm (Saumweber et al. 2010), such that one of the two odours is omitted. That is, larvae receive either paired or unpaired presentations of odour and reward, and then are assayed for their preference for the trained odour (Fig. 4N). In such an experiment, projection-neuron RESCUE larvae show associative performance indices significantly smaller than wild-type CS (for AM: Fig. 4O; MW: $P< 0.05/ 3$; $U= 23$; $N= 12, 12$; for OCT: Fig. 4P; MW: $P< 0.05/ 3$; $U= 32$; $N= 13, 13$) and indistinguishable from either genetic control (for AM: Fig. 4O; projection-neuron RESCUE versus projection-neuron DRIVER control: MW: $P> 0.05/ 3$; $U= 63$; projection-neuron RESCUE versus EFFECTOR control: MW: $P> 0.05/ 3$; $U= 66.5$; $N= 12, 12, 12$; for OCT: Fig. 4P; projection-neuron RESCUE versus projection-neuron DRIVER control: MW: $P> 0.05/ 3$; $U= 69$; projection-neuron RESCUE versus

EFFECTOR control: MW: P> 0.05/ 3; U= 80; N= 13, 13, 13) (KW: for AM, Fig. 4O: P< 0.05; H= 13.35; df= 3; N= 12 for all groups; for OCT, Fig. 4P: P< 0.05; H= 12.00; df= 3; N= 13 for all groups). Thus, despite sincere efforts, there is no evidence that Synapsin expression in the projection neurons, as covered by the broadest- and strongest-expressing driver strains available, were sufficient to restore associative function in *syn*^{97}-mutants.

Fig.5

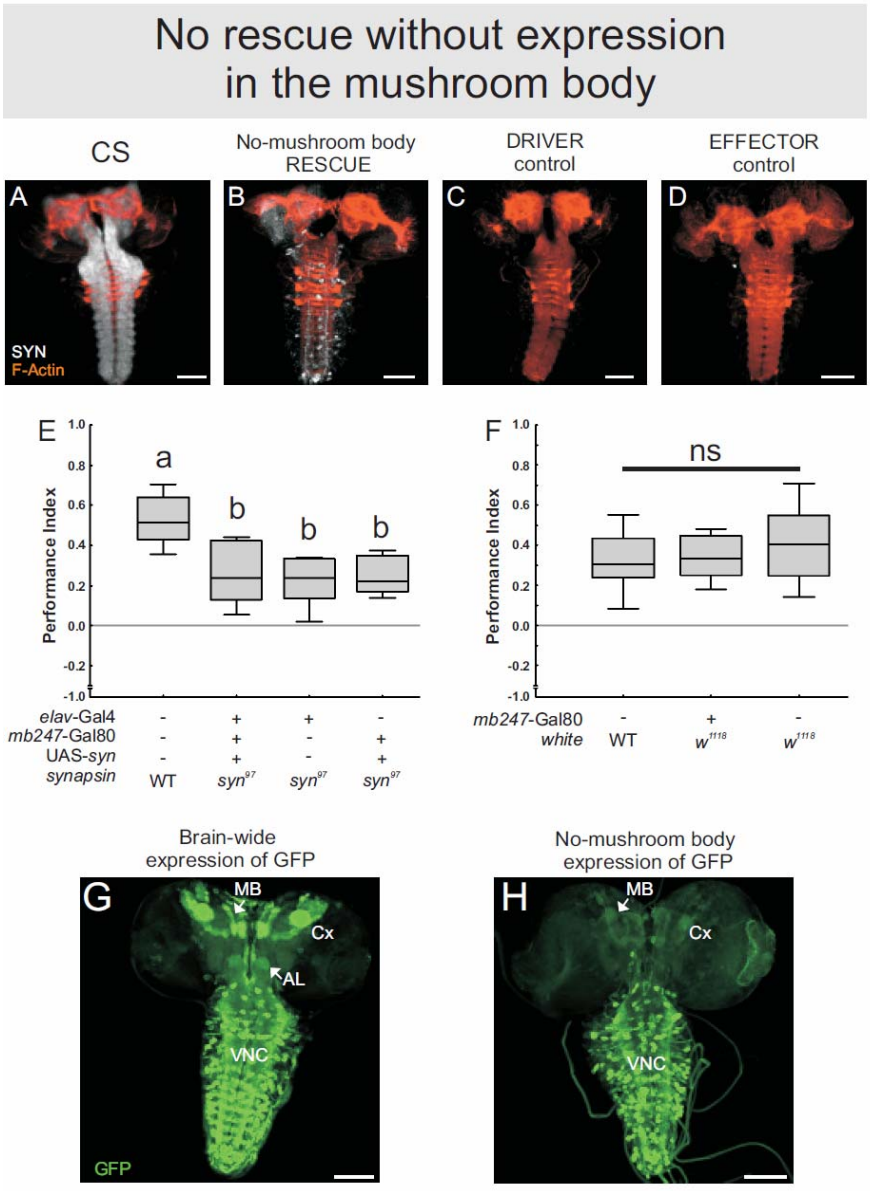


Figure 5: No rescue by Synapsin expression outside of the mushroom bodies. (A-D) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. (G, H) Expression of GFP in *elav*-Gal4 flies (G) and e*lav*-Gal4, *mb247*-Gal80 flies (H), each crossed to UAS-*GFP* flies. Antennal lobe (AL), mushroom body (MB), calyx (Cx) ventral nerve cord (VNC). (E) Synapsin expression outside the mushroom bodies is not sufficient for restoring associative ability. (F) No haploinsufficiency caused by the insertion of the *mb247*-Gal80 construct. Scale bars: 50µm.

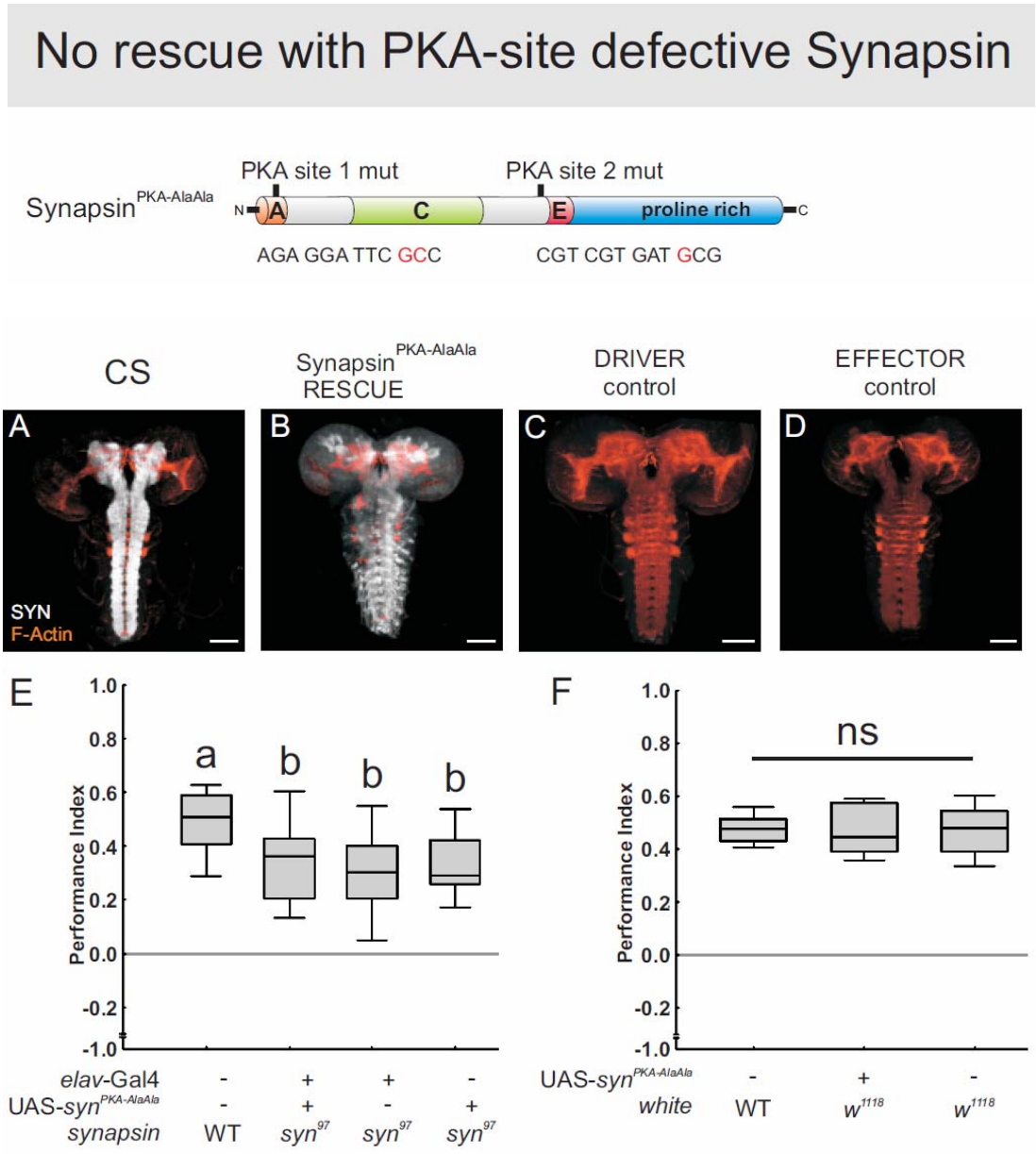
All other details as in the legend of Fig. 1.

No rescue without mushroom body expression

Given that Synapsin expression in the mushroom body, but not in projection neurons, is sufficient to restore the defect of the *syn*^{97}-mutant in associative function, we asked whether mushroom body expression of Synapsin in turn would be required. Comparing associative ability in no-mushroom body RESCUE larvae to wild-type CS and to their genetic controls (no-mushroom body DRIVER control and EFFECTOR control) reveals a significant difference (Fig. 5E; KW: P< 0.05; H= 14.40; df= 3; N= 12, 12, 12, 12). Importantly, the no-mushroom body RESCUE larvae do not show associative performance scores as high as wild-type CS (Fig. 5E; MW: P< 0.05/ 3; U= 24); rather, associative ability is as poor as in the genetic controls (Fig. 5E; no-mushroom body RESCUE versus EFFECTOR control: MW: P> 0.05/ 3; U= 68; no-mushroom body RESCUE versus DRIVER control: MW: P> 0.05/ 3; U= 69.5). Such lack-of-rescue cannot be attributed to a haploinsufficiency caused by the insertion of the *mb247*-Gal80 construct (Fig. 5F; KW: P> 0.05; H= 1.15; df= 2; N= 13, 11, 12).

A comparison of Synapsin expression with repression in the mushroom bodies (by virtue of *mb247*-Gal80) (Fig. 5B) to Synapsin expression without such repression (i.e. without *mb247*-Gal80) (Fig. 2B) reveals a full abolishment of expression in the mushroom bodies. Considering expression of a GFP reporter (Fig. 5G, H), however, suggests that *mb247*-Gal80 (i) may spare some mushroom body expression and (ii) leads to a reduction of expression also outside the mushroom body (as previously noted by Ito et al. 2003). Such possible discrepancies must remain unrecognized if the expression of the actual effector is not documented. In our case, it is possible that (i) detection of GFP is more sensitive than detection of Synapsin; (ii) the *mb247*-element supports different expression patterns in the *mb247*-Gal4 strain as compared to the *mb247*-Gal80 strain; or that (iii) Gal80 has non-cell autonomous effects. We conclude that Synapsin expression outside of the coverage of *mb247*-Gal80 is not sufficient to rescue the associative defect in the *syn*^{97}-mutant. In turn, those neurons which are covered by *mb247*-Gal80 do need to express Synapsin to support associative function.

Fig.6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E
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CS
Synapsin PKA-AlaAla RESCUE
DRIVER control
EFFECTOR control
A
B
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SYN
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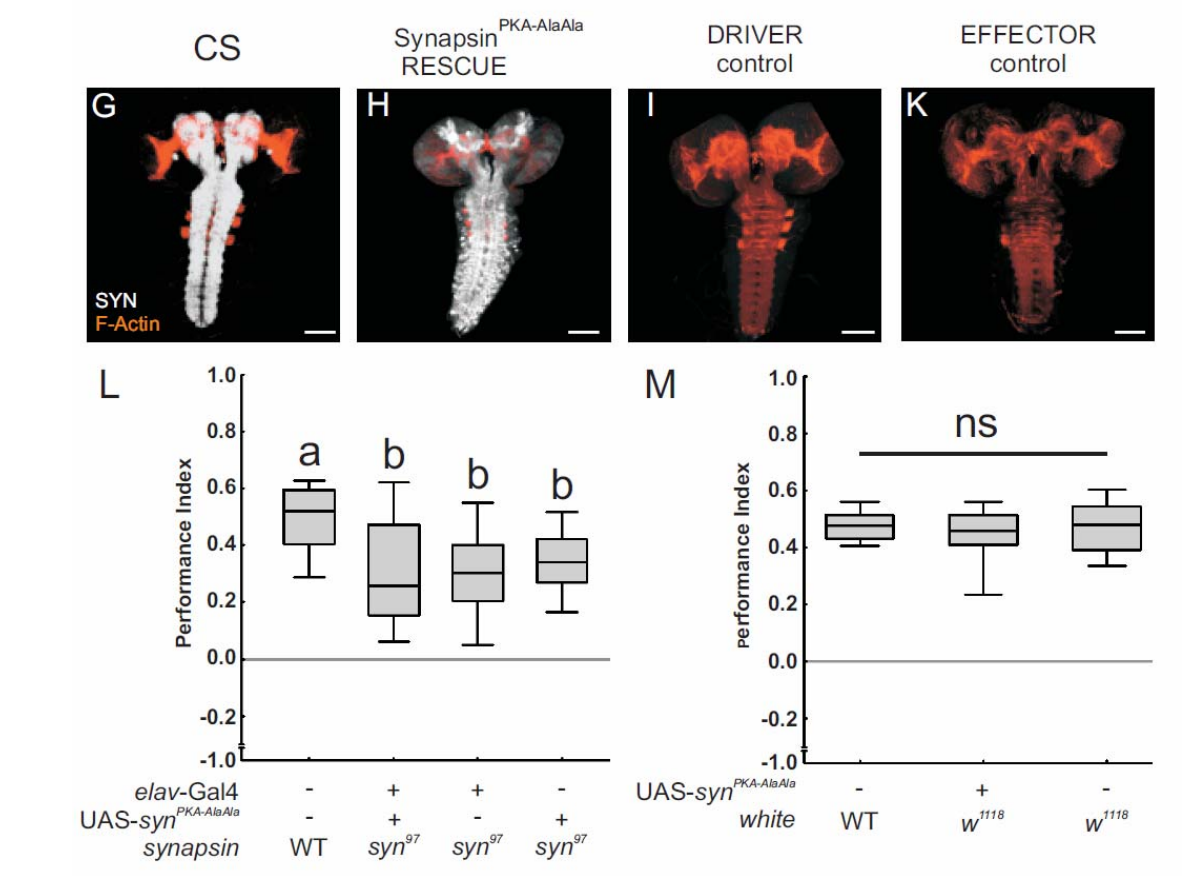
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a
b
b
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synapsin WT syn97 syn97 syn97
UAS-syn PKA-AlaAla - + -
white WT w1118 w1118

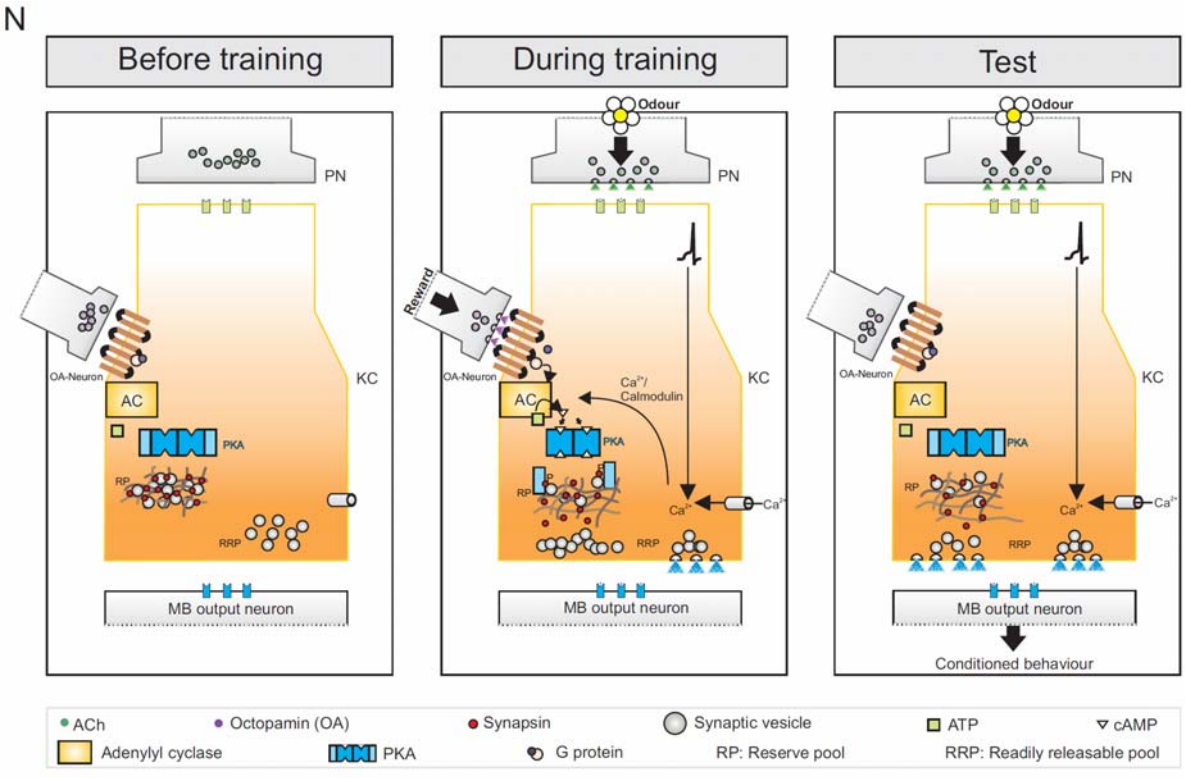
N
Before training
During training
Test
Odour
PN
KC
OA-Neuron
Reward
AC
PKA
RP
RRP
Ca2+/ Calmodulin
Ca2+
MB output neuron
Conditioned behaviour
ACh
Octopamin (OA)
Synapsin
Synaptic vesicle
ATP
cAMP
Adenylyl cyclase
PKA
G protein
RP: Reserve pool
RRP: Readily releasable pool

Figure 6: No rescue by a Synapsin protein with mutated PKA-sites. The upper panel shows the organization of transgenically expressed Synapsin$^{PKA\text{-}AlaAla}$ with both PKA-sites mutated. (A-D, G-K) Anti-Synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. (E) Expression of Synapsin with mutated PKA-sites does not rescue associative function in *syn*^{97}-mutant larvae. (F) No haploinsufficiency caused by the the *UAS-syn*$^{PKA\text{-}AlaAla}$ insertion. (G-M) Using an independent EFFECTOR fly strain, with the *UAS-syn*$^{PKA\text{-}AlaAla}$ construct inserted at a different site, yields the same results. Scale bars: 50µm.

All other details as in the legend of Fig. 1.

(N) Working hypothesis of the molecular mode of Synapsin action in associative learning. Our results suggest a memory trace for the association between odor and reward to be localized within the Kenyon cells (KC). The type I adenylyl cyclase (AC) acts as a molecular coincidence detector: The odour leads to presynaptic calcium influx, and hence to an activation of calmodulin, whereas the reward leads to an activation of likely octopaminergic neurons and the corresponding G-protein coupled receptors (Hauser et al. 2006). Only if both these signals are present, the AC-cAMP-PKA cascade is triggered, and the respective effector proteins, including Synapsin, are phosphorylated. This allows a recruitment of synaptic vesicles from the reserve pool to the readily releasable pool. Upon a subsequent presentation of the learnt odour, more transmitter can be released (Hilfiker et al. 1999). This strengthened output is proposed to mediate conditioned behavior towards the odor at test.

No rescue with PKA-site defective Synapsin

Since properly regulated AC-cAMP-PKA signalling has been shown to be necessary for olfactory short term memory in *Drosophila* (see Discussion), we decided to test whether the two predicted PKA-sites of the Synapsin protein are required for normal learning. Therefore, we expressed a mutated Synapsin protein that cannot be phosphorylated at these two predicted PKA-sites because the serines of these PKA-consensus sites (S-6 and S-533) were replaced by alanine (PKA-AlaAla; for details see sketch in Fig. 6). Comparing associative ability in such Synapsin$^{PKA\text{-}AlaAla}$-RESCUE larvae to wild-type CS and to their genetic controls reveals a significant difference (Fig. 6E; KW: $P< 0.05$; $H= 12.24$; $df= 3$; $N= 17$ of all groups). Importantly, the Synapsin$^{PKA\text{-}AlaAla}$-RESCUE larvae do not perform as well as wild-type CS (Fig. 6E; MW: $P< 0.05/ 3$; $U=70$); rather, associative ability is as poor as in the genetic controls (Fig. 6E; Synapsin$^{PKA\text{-}AlaAla}$-RESCUE versus EFFECTOR control: MW: $P> 0.05/ 3$; $U= 130.5$; Synapsin$^{PKA\text{-}AlaAla}$-RESCUE versus DRIVER control: MW: $P> 0.05/ 3$; $U= 121$). Such lack-of-rescue cannot be attributed to a haploinsufficiency caused by the insertion of the UAS-*syn*$^{PKA\text{-}AlaAla}$ construct (Fig. 6F; KW: $P> 0.05$; $H= 0.04$; $df= 2$; $N= 12$ for all groups) (for a repetition of these experiments with an independent insertion of the same effector construct see Fig. 6G-M). Thus, intact PKA-sites of Synapsin are required to restore associative ability in the *syn*^{97}-mutant.

DISCUSSION

The associative defect in the *syn*^{97}-mutant (Fig. 1K; Michels et al. 2005) can be phenocopied by an RNAi-mediated knock-down of Synapsin (Fig. 1M), and can be rescued by acutely restoring Synapsin (Fig. 2K, L). In terms of site of action, locally restoring Synapsin in the mushroom bodies fully restores associative ability (Fig. 3E, K), whereas restoring Synapsin in the projection neurons does not (Fig. 4E, L). If Synapsin is restored in wide areas of the brain excluding the mushroom bodies, learning ability is not restored, either (Fig. 5E). We therefore conclude that a Synapsin-dependent memory trace is located in the mushroom bodies, and suggest that this likely is the only site where such a trace is established regarding odour-sugar short-term memory in larval *Drosophila*. In terms of mode of action, we find that a Synapsin protein that carries dysfunctional PKA-sites (Fig. 6E, L) cannot rescue the *syn*^{97}-mutant learning defect. We therefore suggest that Synapsin functions as a downstream element of AC-cAMP-PKA signaling in associative function.

Mode of action: Synapsin as target of the AC-cAMP-PKA cascade

Arguably, the Rutabaga type I adenylyl cyclase acts as a detector of the coincidence between an aminergic reinforcement signal (appetitive learning: octopamine; aversive learning: dopamine; Schwaerzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006) and the odour-specific activation of the mushroom body neurons (Fig. 6N). Initially, this notion had been based on mutant and biochemical analyses in *Drosophila* (Livingstone et al. 1984; Dudai 1985; Heisenberg et al. 1985) and physiology in *Aplysia* (Brunelli et al. 1976; Hawkins 1984; Yovell et al. 1992; Byrne and Kandel 1996; Abrams et al. 1998). Indeed, activation of mushroom body neurons in temporal coincidence with dopamine application increases cAMP levels in wild-type, but not AC-deficient flies (*rut*^{2080}) (Tomchik and Davis 2009), and Gervasi et al. (2010) show a corresponding AC-dependence of PKA activation by mushroom body co-stimulation with octopamine. However, the downstream effects of the AC-cAMP-PKA cascade remained clouded. We here suggest that, similar to the situation in snails (Fiumara et al. 2004), one of these PKA-effectors is Synapsin, such that Synapsin phosphorylation allows a transient recruitment of synaptic vesicles from the reserve pool to the readily releasable pool. A subsequent presentation of the learnt odour could then draw upon these newly-recruited

vesicles. This scenario also captures the lack of additivity of the *syn*^{97} and *rut*^{2080} mutations in adult odour-shock associative function, and the selective defect of the *syn*^{97}-mutation in short- rather than longer-term memory (Knapek et al. 2010).

Given that the memory trace established in our paradigm likely is localized to few cells relative to the brain as a whole (see following section), given that these are transient, short-term memory traces (Neuser et al. 2005), and given the possibility of de-phosphorylation, it is not unexpected that Nuwal et al. (2010) have not uncovered either predicted PKA-site of Synapsin as being phosphorylated in a biochemical approach, using whole brain homogenates from untrained animals. Given the likely spatial and temporal restriction of these events *in vivo*, immunohistological approaches are warranted to see whether, where, and under which experimental conditions Synapsin phosphorylated at either of its PKA-sites indeed can be detected.

Interestingly, the evolutionarily conserved N-terminal PKA-1 site undergoes ADAR-dependent mRNA editing (Diegelmann et al. 2006) which despite the genomically coded RRFS motif yields a protein carrying RGFS. This editing event, as judged from whole-brain homogenates, occurs for most but not all Synapsin and, as suggested by *in vitro* assays of an undecapeptide with bovine PKA, may reduce phosphorylation rates by PKA. Given that the successfully rescuing UAS-*syn* construct (Fig.s 2, 3) codes for the edited RGFS sequence, it should be interesting to see whether this rescue is conferred by residual phosphorylation at PKA-1, and/or by phosphorylation of the evolutionaryily non-conserved PKA-2 site. Last, but not least, one may ask whether an otherwise wildtype Synapsin protein featuring a non-edited RRFS motif is rescuing associative function, too.

In any event, our finding that the PKA-consensus sites of Synapsin are required to restore learning in the *syn*^{97}-mutant (Fig. 2E versus Fig. 6E, L) is the first functional argument to date, in any experimental system, to suggest Synapsin as an effector of the AC-cAMP-PKA cascade in associative function.

Cellular site: A memory trace in the projection neurons?

In contrast to our current results in larvae, Thum et al. (2007) argue that not only the mushroom bodies but also projection neurons accommodate appetitive short-term

memory traces in adult *Drosophila* (see also Menzel [2001] for the situation in bees). How can this be reconciled?

- *Projection neurons may house such a memory trace in adults, but not in larvae.* However, despite the reduced cell number in larvae, the general layout of the olfactory system appears strikingly similar to adults (Gerber et al. 2009).
- *A projection neuron memory trace may be Rutabaga-dependent, but Synapsin-independent.* As Rutabaga and Synapsin are present within most if not all neurons, with Rutabaga arguably acting upstream of Synapsin (Fig. 6N), this would need to assume that the AC-cAMP-PKA cascade is specifically disconnected from Synapsin in the projection neurons.
- *The Rutabaga rescue in projection neurons may be non-associative.* Appetitive training may non-associatively increase the gain of all projection neuron-to-mushroom body synapses, and this may be Rutabaga-dependent. As Rutabaga expression in the projection neurons rescues associative performance, however, one would need to additionally assume that residual Rutabaga function in the mushroom bodies of the rut^{2080}-mutants (the rut^{2080} allele is not a null-allele: Pan et al. [2009]) is only able to support an associative memory trace in the mushroom bodies if the mushroom bodies are driven sufficiently strong, by virtue of the non-associative facilitation of their input. This would integrate two further observations that argue against a functionally independent, appetitive associative short-term memory trace in the projection neurons: (i) Expression of a constitutively active $G\alpha_s$ in only the mushroom body impairs adult odour-sugar learning (Thum 2006; loc. cit. Fig. 13). (ii) Blocking projection neuron output during training prevents appetitive associative memory formation (HT, unpubl.).
- *We may have overlooked a projection neuron rescue.* (i) As argued above (Fig. 4F, M), a haploinsufficiency caused by the *GH146*-Gal4 and *NP225*-Gal4 insertions can be ruled out as reason for such inadvertence. (ii) Both employed odours may be processed only outside the covered projection neurons. Thus, blocking synaptic output from these neurons should leave olfactory behaviour unaffected- we find, however, that odour preferences in such an experiment are massively reduced (for *NP225*-Gal4: Fig. S2). (iii) Within the subset of covered projection neurons, the activity patterns evoked by both odours may actually be the same. Discrimination between them may rely on between-odour differences outside of covered

projection neuron subset. However, even in a one-odour paradigm, which does not require discrimination, we find no projection neuron rescue, either (Fig. 4N-P).

- *Adult Rutabaga expression by GH146-Gal4 and NP225-Gal4 may include neurons that are not covered in the larva.* A careful assessment of anti-Rutabaga immunohistochemistry is a prerequisite to see whether this is true.
- Adults, but not larvae, need to be starved before appetitive learning, such that a discrepancy between larvae and adults may be affected by motivational differences.

To us, none of these scenarios seems fully compelling; it therefore appears that for the time being it must remain unresolved whether indeed there is a discrepancy between larvae and adults regarding a projection neuron memory trace, and if so, why this would be the case. In any event, from the present data on the larva, a Synapsin-dependent memory trace in the projection neurons does not need to be reckoned with.

Cellular site: A role for mushroom body subsystems?

Are the mushroom bodies necessary for olfactory associative function in larvae, as is arguably the case in adults (reviewed in Gerber et al. 2009)? Heisenberg et al. (1985) found that the *mbm*^{1} mutation, which causes miniaturized mushroom bodies, is strongly impaired in an odour-electric shock associative paradigm. Twenty-five years later, Pauls et al. (2010) reported that blocking synaptic output of mushroom body neurons by means of *shibire*^{ts} throughout training and testing reduces odour-sugar associative function. Interestingly, this effect differed between driver strains used. Using GFP expression as a stand-in for *shibire*^{ts} expression and assuming that all mushroom body neurons are sensitive to the effects of *shibire*^{ts}, Pauls et al. (2010) argued that intact output from specifically embryonic-born mushroom body neurons is necessary for associative function. In turn, embryonic-born mushroom body neurons are apparently sufficient for associative function, as already stage one larvae, not yet equipped with larval-born mushroom body neurons, can perform in the task, and because ablating larval-born mushroom body neurons by means of hydroxy urea treatment was without effect. Thus, embryonic-born mushroom body neurons appear sufficient, and intact synaptic out from them required, for proper odour-reward associative function in the larva.

Our present analysis shows that restoring Synapsin in the mushroom bodies is sufficient to fully restore associative function. Strikingly, expression of Synapsin in only a handful of mushroom body neurons is sufficient in this regard (Fig. 3K; using *D52H*-Gal4). Despite the low number of covered cells, the majority of the 36 mushroom body-glomeruli appear innervated (Masuda-Nakagawa et al. 2005; Masuda-Nakagawa et al. 2009). Indeed, Masuda-Nakagawa et al. (2005) showed that each mushroom body neuron on average receives input in a random subset of six from the total ~ 36 glomeruli. Thus, if more than six randomly chosen mushroom body neurons are included by a Gal4 strain, fairly broad aspects of the olfactory input space should be covered (see also Murthy et al. 2008). We note, however, that the *D52H*-Gal4 element includes a *dunce* enhancer sequence (Qiu and Davis 1993). The *dunce* gene codes for a cAMP-specific phosphodiesterase required for associative function in adult and larval *Drosophila* (Aceves-Pina and Quinn 1979; Tully and Quinn 1985) and is expressed in the mushroom bodies of both stages (Nighorn et al. 1991). Thus, it may be that these neurons are of peculiar role for establishing a memory trace.

Our present analysis, with an important caveat, also suggests a requirement of the mushroom bodies. Restoring Synapsin throughout the brain, but excluding the mushroom bodies, does not restore associative function (Fig. 5). The caveat, however, is that global Synapsin expression (by *elav*-Gal4) with an intended local repression in the mushroom bodies (by *mb247*-Gal80) apparently reduces Synapsin expression also outside the expression pattern expected from the mb247-element (an effect that can unwittingly be overlooked if using GFP expression as stand-in for the experimental agent; Fig. 5G, H). Unfortunately, an independent assault towards necessity, namely to locally reduce Synapsin expression by RNAi, does not appear feasible, as we could not document an actual local reduction of Synapsin expression in larval mushroom bodies in whole mount brains, likely because mushroom body neurons expressing the transgene are too closely intermingled with mushroom body neurons that do not (not shown).

Outlook

We have identified the mushroom bodies (Fig. 3), but not the projection neurons (Fig. 4), as a cellular site of action of Synapsin in odour-sugar associative function of larval *Drosophila*. We provide experimental evidence to suggest that the molecular mode of

action of Synapsin is as a substrate of the AC-cAMP-PKA pathway (Fig. 6). This analysis brings us closer towards an unbroken chain of explanation from the molecular to the cellular level and further to a learnt change in behaviour. Given the homology of many of the molecular determinants for synaptic and behavioural plasticity (Pittenger and Kandel 2003; Davis 2005) this may become relevant for biomedical research. Last but not least, on the cellular level, an understanding of which specific sites along a sensory-motor circuit are altered to accommodate behavioural changes may be inspiring for the design of 'intelligent' technical equipment.

MATERIALS AND METHODS

Third-instar feeding-stage larvae aged 5 days after egg laying were used throughout. Animals were kept in mass culture, maintained at 25 °C (unless mentioned otherwise), 60-70 % relative humidity and a 14/ 10 hour light/ dark cycle. Experimenters were blind with respect to genotype and treatment condition in all cases; these were decoded only after the experiments.

Fly strains

We used the wild-type CS strain (Michels et al. 2005) as reference throughout. The syn^{97CS} mutant strain, carrying a 1.4 kb deletion in the *synapsin* gene and lacking all Synapsin, had been outcrossed to wild-type CS for 13 generations (Godenschwege et al. 2004; Michels et al. 2005) and will be referred to as syn^{97} for simplicity.

In all cases when transgenic strains were involved, these strains all were in the w^{1118}-mutant background and carry a mini-*white* rescue construct on their respective transgene to keep track of those transgenes. The w^{1118} mutation is without effect in our associative learning paradigm (Fig.s 1K, 4F, M; see also Yarali et al. 2009).

Driver and effector strains

We recombined various transgenic Gal4 driver strains into the syn^{97}- mutant background by classical genetics (roman numerals refer to the chromosome carrying the construct):

- *elav*-Gal4; syn^{97} [X] (c155 in Lin and Goodman 1994) for brain-wide transgene expression;
- *mb247*-Gal4, syn^{97} [III] (Zars et al. 2000) for transgene expression in many mushroom body neurons;
- *D52H*-Gal4; syn^{97} [X] (Qiu and Davis 1993; Tettamanti et al. 1997) (kindly provided by R. Davis), for transgene expression in a small subset of mushroom body neurons;
- *GH146*-Gal4; syn^{97} [II] (Heimbeck et al. 2001) for transgene expression in projection neurons;
- *NP225*-Gal4; syn^{97} [II], (Tanaka et al. 2004) also for transgene expression in projection neurons.

As effector strains we used the transgenic UAS-*syn,* syn^{97} [III] strain (generated on the basis of Löhr et al. 2002), a UAS-RNAi-*syn* [III] strain (see below), or UAS-shi^{ts1} [III] to block neurotransmitter release (Kitamoto 2001).

Rescue

Three kinds of crosses were performed, of flies all in the w^{1118} mutant background:

- RESCUE: we crossed a homozygous driver strain, e.g. *elav*-Gal4; syn^{97} to a homzygous UAS-*syn*, syn^{97} effector strain, yielding double heterozygous larvae, in the *synapsin*-mutant background: *elav*-Gal4/ +; ; UAS-*syn,* syn^{97}/ syn^{97};
- DRIVER control: we correspondingly crossed e.g. *elav*-Gal4; syn^{97} to syn^{97} yielding single-heterozygous *elav*-Gal4/ +; ; syn^{97}/ syn^{97};
- EFFECTOR control: we crossed UAS-*syn*, syn^{97} to syn^{97} yielding single-heterozygous ; ; UAS-*syn,* syn^{97}/ syn^{97}.

When other expression patterns were desired, the respective other Gal4-strains were used.

Excluding the mushroom bodies from the rescue-expression pattern

To restore Synapsin expression throughout the brain, but not in the mushroom body, a *mb247*-Gal80; UAS-*syn*, syn^{97} effector strain was generated (generous gift from S. Knapek) by classical genetics from *mb247*-Gal80 [II] (Krashes et al. 2007) and UAS-*syn*,

syn^{97} (see above). Because Gal80 is an inhibitor of Gal4, Gal80 can suppress Gal4 in the mushroom body and thus prevent Synapsin expression in the mushroom bodies. The following crosses were performed, of flies all in the *w*^{1118} mutant background:

- no-mushroom body RESCUE: flies of the *mb247*-Gal80; UAS-*syn*, *syn*^{97} effector strain were crossed to *elav*-Gal4; *syn*^{97} as driver strain. This yielded triple-heterozygous *elav*-Gal4/ +; *mb247*-Gal80/ +; UAS-*syn, syn*^{97}/ *syn*^{97};
- DRIVER control: we crossed *elav*-Gal4; *syn*^{97} to *syn*^{97} yielding *elav*-Gal4/ *+; ; syn*^{97}/ *syn*^{97};
- EFFECTOR control: we crossed *mb247*-Gal80; UAS-*syn*, *syn*^{97} to *syn*^{97} yielding ; *mb247*-Gal80/ +; UAS-*syn, syn*^{97}/ *syn*^{97}.

Induced rescue

For induced expression of Synapsin, we generated a fly strain carrying *tub*-GAL80^{ts} [II] (McGuire et al. 2003) and UAS-*syn* in the *syn*^{97}- mutant background (*tub*-GAL80^{ts}; UAS-*syn*, *syn*^{97}). The following crosses were performed, of flies all in the *w*^{1118} mutant background:

- induced brain-wide RESCUE: *tub*-GAL80^{ts}; UAS-*syn*, *syn*^{97} flies were crossed to *elav*-Gal4; *syn*^{97} to yield *elav*-Gal4/ +; *tub*-Gal80^{ts}/ +; UAS-*syn*, *syn*^{97}/ *syn*^{97};
- DRIVER control: *elav*-Gal4; *syn*^{97} was crossed to *syn*^{97} yielding *elav*-Gal4/ +; ; *syn*^{97}/ *syn*^{97};
- EFFECTOR control: we crossed *tub*-Gal80^{ts}; UAS-*syn*, *syn*^{97} to *syn*^{97} yielding ; *tub*-Gal80^{ts}/ +; UAS-*syn, syn*^{97}/ *syn*^{97}.

These crosses were cultured at 18 °C. To induce Synapsin expression, a 30 °C heat-shock was applied for 24 hours on day 6 AEL. Then, vials were kept at room temperature for 2 hours before experiments were performed. Thus, Synapsin expression is expected only in the induced brain-wide RESCUE strain and only when a heat shock was applied. This is because Gal80^{ts} suppresses Gal4-mediated transgene expression at 18 °C but not at 30 °C.

RNAi

To yield an RNAi-mediated knock-down of Synapsin, a UAS-RNAi-*syn* [III] strain was generated. A 497 nt coding fragment of the *syn*-cDNA was amplified by PCR with primers containing unique restriction sites: the primer pair 5'-GAG CTC TAG AAC GGA TGC AGA ACG TCT G-3' and 5'-GAG CGA ATT CTG CCG CTG CTC GTC TC-3' was used for the sense cDNA fragment and 5'-GAG CGG TAC CAC GGA TGC AGA ACG TCT G-3' and 5'-GAG CGA ATT CGC CCG CTG CCG CTG CTC-3' were used for the anti-sense cDNA fragment, respectively. The PCR-amplified fragments were digested with *XbaI/EcoRI* and *EcoRI/KpnI* respectively and subcloned into *XbaI/KpnI* pBluescript KSII (Stratagene, La Jolla, USA). The resulting inverted repeat sequence was excised as a 1kb *NotI/KpnI* fragment, ligated into *NotI/KpnI*-cut pUAST (Brand and Perrimon 1993) and transformed into recombination-deficient SURE2 supercompetent cells (Stratagene, La Jolla, USA). Germ-line transformation was performed into a w^{1118} strain (Bestgene, Chino Hills, USA). For experiments, the following crosses, all in the w^{1118} mutant background, were performed:

- KNOCK-DOWN: UAS-RNAi-*syn* was crossed to UAS-*dcr-2*; *elav*-Gal4 (generated by classical genetics from the UAS-*dcr-2* [X] strain [Dietzl et al. 2007] and the *elav*-Gal4 [III] strain, both from Bloomington stock center); this yielded triple-heterozygous animals of the genotype UAS-*dcr-2*/ +; ; *elav*-Gal4/ UAS-RNAi-*syn.*
- DRIVER control: we crossed UAS-*dcr-2*; *elav*-Gal4 to no-transgene carrying flies yielding UAS-*dcr-2*/ +; ; *elav*-Gal4/ +;
- EFFECTOR control: we correspondingly generated ; ; UAS-RNAi-*syn*/ +.

Expression of mutated transgenes

In order to generate loss-of-function mutations in both putative PKA phosphorylation sites of Synapsin, site-directed mutagenesis was performed (see sketch in Fig. 6). The *syn*-cDNAs containing Ser^{PKA-1}→Ala and Ser^{PKA-2}→Ala were amplified by PCR using the following primers: For amplifying the non-phosphorylatable PKA-1, the primer pair Ser→Ala PKA 1 forward, 5′-GAG CTC CAC CGC GGT GGC GGC CGC TCT AGA ACT AGT-3′ and Ser→Ala PKA 1 reverse 5′-GGA TCG ACA TCG TCT ACC TCG

GAA GAC AAG TCT CCC GAG GCG AAT CCT CT-3 were used. For amplifying the non-phosphorylatable PKA-2, a PCR was carried out with the primer pair Ser→Ala PKA 2 forward, 5´-TCG TCG GGA CCC AGC ACA GTG GGT GGG GTG CGT CGT GAT GCG CAG A-3 and Ser→Ala PKA 2 reverse, 5´-GGA ACA AAA GCT GGG TAC CGG GCC CCC CCT CGA GGT CGA CGG TAT-3´. The PCR-amplified fragments were digested with *SpeI/PflFI* and *PpUMI/XhoI*, respectively, subcloned successively into *SpeI/PflFI* and *PpUMI/XhoI* digested pBluescript KSII vector (Stratagene, La Jolla, USA) containing the syn-cDNA over *EcoRI*, and sequenced. The resulting mutated *syn*-cDNA sequence was excised as a 3.4 kb *EcoRI* fragment, ligated into the *EcoRI*-cut pUAST vector (Brand and Perrimon 1993) and transformed into recombination-deficient TOP10 chemically competent *E. coli* cells (Invitrogen GmbH, Karlsruhe, Germany). Germ-line transformation then was performed into the w^{1118}*;* syn^{97} strain (Bestgene, Chino Hills, USA), yielding two effector strains, namely UAS-$syn^{PKA\text{-}AlaAla}$*,* syn^{97} (1) [III] and UAS-$syn^{PKA\text{-}AlaAla}$*,* syn^{97} (2) [III]. The latter strain is an independent insertion strain of the same UAS-$syn^{PKA\text{-}AlaAla}$ construct. The following genotypes could thus be generated:

- RESCUE$^{\text{PKA-AlaAla}}$: UAS-$syn^{PKA\text{-}AlaAla}$*,* syn^{97} flies were crossed to *elav*-Gal4; syn^{97}, resulting in double heterozygous *elav*-Gal4/+; ; UAS-$syn^{PKA\text{-}AlaAla}$, syn^{97}/ syn^{97} larvae;
- DRIVER control: we correspondingly crossed *elav*-Gal4; syn^{97} to syn^{97} yielding single-heterozygous *elav*-Gal4/ +; ; syn^{97}/ syn^{97};
- EFFECTOR control: we crossed UAS-$syn^{PKA\text{-}AlaAla}$, syn^{97} to syn^{97} yielding ; ; UAS-$syn^{PKA\text{-}AlaAla}$, syn^{97}/ syn^{97}.

Western blotting

For each lane in the Western blots, 10 larval brains were homogenized in 10 µl 2 x SDS gel loading buffer. The sample was heated to 70 °C for 5 min and centrifuged for 2 min before electrophoresis. Proteins were separated by 12.5% SDS-PAGE in a Multigel chamber (100 mA, 3 h; PEQLAB, Erlangen, Germany) and transferred to nitrocellulose membranes (Kyhse-Andersen 1984). Immunoreactions were successively performed with two monoclonal mouse antibodies: SYNORF1 for Synapsin detection (Klagges et al. 1996) (dilution 1:100), and ab49 (Zinsmaier et al. 1990; Zinsmaier et al. 1994) (dilution 1:400) for detection of the Cysteine String Protein (CSP; Arnold et al. 2004) as loading

control. Visualization was achieved with the ECL Western blot detection system (Amersham, GE Healthcare, Ismaning, Germany).

Immunohistochemistry

Larval brains were dissected in phosphate-buffered saline containing 0.3 % Triton X-100 (PBST) and fixed in 4 % paraformaldehyde dissolved in PBST for 1 h. After three washes (each 10 min) in PBST, the brains were treated in blocking solution containing 3 % normal goat serum (Dianova, Hamburg, Germany) in PBST for 1½ h. Tissue was then incubated overnight with the primary monoclonal anti-Synapsin mouse antibody (SYNORF1, diluted 1: 10 in blocking solution) (Klagges et al. 1996). Six washing steps in PBST (each 10 min) were followed by incubation with a secondary rabbit anti-mouse antibody conjugated with Alexa 488 (diluted 1:200) (Molecular Probes, Invitro Detection Technologies, Karlsruhe, Germany). For orientation in the preparation, in particular in cases when no Synapsin was expected to be present, we used overnight staining with Alexa Fluor 568 Phalloidin (diluted 1:200) (Molecular Probes; Lot 41A1-4; Eugene; Oregon; USA), which visualizes filamentous actin. After final washing steps with PBST, samples were mounted in Vectashield (Linaris, Wertheim, Germany).

In cases when we sought for an independent approximation of transgene expression supported by the various driver strains, we crossed the respective driver strains to UAS-mCD8::*GFP* flies (labelled as UAS-*GFP* for simplicity throughout) (Lee and Luo 1999) and probed for GFP expression. To this end, larval brains were incubated with a primary polyclonal rabbit anti-GFP serum (A6455, diluted 1:1000) (Invitrogen, Eugene, USA). After washing with PBST, samples were incubated with a secondary goat anti-rabbit serum (Alexa Fluor 488, anti-rabbit Ig, diluted 1:100) (MoBiTech, Göttingen, Germany).

Three-dimensional reconstructions of larval brain stainings were accomplished with the ImageJ 3D Viewer and Segmentation Editor (Schmid et al. 2010).

Scanning electron microscopy

For scanning electron microscopy (SEM), larvae were collected in water and cooled to immobility for 30 min. The last third of the animal was cut off and larvae were fixed

overnight in 6.25 % glutaraldehyde with 0.05 mol 1:1 Sörensen phosphate buffer (pH 7.4). Fixed specimens were washed five times in buffer for 5 min each and dehydrated through a graded series of acetone. After critical-point drying in CO_2 (BALTEC CPD 030; Schalksmühle, Germany), larvae were mounted on a table and sputtered with Au/Pd (BALTEC SCD 005; Schalksmühle, Germany). Specimens were viewed using a scanning electron microscope (Zeiss DSM 962, Oberkochen, Germany).

Associative learning experiments

Learning experiments follow standard methods (Scherer et al. 2003; Neuser et al. 2005; for a detailed protocol see Gerber et al. 2010) (sketch in Fig. 1C), employing a two-odour, reciprocal conditioning paradigm, unless mentioned otherwise. In brief, olfactory choice performance of larvae was compared after either of two reciprocal training regimen: During one of these regimen, larvae received *n*-amylacetate (CAS: 628-63-7; AM; Merck, Darmstadt, Germany) with a sugar reward (+) and 1-octanol (CAS: 111-87-5; OCT; Sigma-Aldrich, Seelze, Germany) without reward (AM+/ OCT); the second regimen involved reciprocal training (AM/ OCT+). Then, animals were tested for their preference between AM *versus* OCT. Associative learning is indicated by a relatively higher preference for AM after AM+/ OCT training as compared to the reciprocal AM/ OCT+ training (behavioural paradigms not using such a reciprocal design [Honjo and Furukubo-Tokunaga 2005; Honjo and Furukubo-Tokunaga 2009] can be confounded by non-associative effects [Gerber and Stocker 2007] and are therefore not discussed throughout this paper). These differences in preference were quantified by the associative performance index (PI; see below).

Petridishes (Sarstedt, Nümbrecht, Germany) with 85 mm inner diameter were filled with 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany) allowed to solidify, covered with their lids, and, at room temperature, left untreated until the following day. As reward we used 2 mol fructose (FRU, purity: 99 %; Roth, Karlsruhe, Germany) added to 1 l of agarose.

Experiments were performed in red light under a fume hood at 21- 24° C. Before experiments, we replaced the regular lids of the petridishes with lids perforated in the center by 15 1-mm holes to improve aeration. A spoonful of food medium containing

larvae was taken from the food bottle and transferred to a glass vial. Thirty animals were collected, washed in tap water and transferred to the assay plates. Immediately before a trial, two containers loaded both with the same odour had been placed onto the assay plate on opposite sides of the plate. Within each reciprocal training condition, for half of the cases we started with AM, for the other with OCT. Thus, for half of the cases we started with a reward- substrate, for the other with a plate without reward. After 5 min, the larvae were transferred to a fresh plate with the alternative odour and the respective other substrate for 5 min. This cycle was repeated three times.

For testing, the larvae were placed in the middle of a fresh assay plate which did not contain the reward. One container of AM was placed on one side and one container of OCT on the other side. After 3 min, the number of animals on the "AM" or "OCT" side was counted. Then, the next group of animals was trained reciprocally. For both reciprocally trained groups, we then calculate an odour preference ranging from –1 to 1 as the number of animals observed on the AM side minus the number of animals observed on the OCT side, divided by the total number of animals:

(1) $$PREF = (\#_{AM} - \#_{OCT}) / \#_{TOTAL}$$

For all learning experiments, these PREF values are documented in the Supplementary material (Fig. S3).

To determine whether these preferences are different depending on training regimen, we calculated an associative performance index ranging from –1 to 1 as:

(2) $$PI = (PREF_{AM+/OCT} - PREF_{AM/OCT+}) / 2$$

After data for one such index for one genotype was collected, data for the next genotype of the respective experiment were gathered; that is, all genotypes to be compared statistically were run side by side (in temporal "parallelity").

Statistical analyses

We displayed the PI scores as box plots (middle line: median; box boundaries and whiskers: 25/ 75 % and 10/ 90 % quantiles, respectively). For statistical comparisons, we used non-parametric analyses throughout (multiple-genotype comparisons: Kruskal-Wallis [KW] tests; two-genotype comparisons: Mann-Whitney U-tests [MW]). To retain an experiment-wide error of 5 % in cases of multiple tests, the significance level was adjusted by a Bonferroni correction, i.e. by dividing 0.05 by the number of the respective tests. All calculations were performed with Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA) on a PC.

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SUPPLEMENTARY MATERIAL

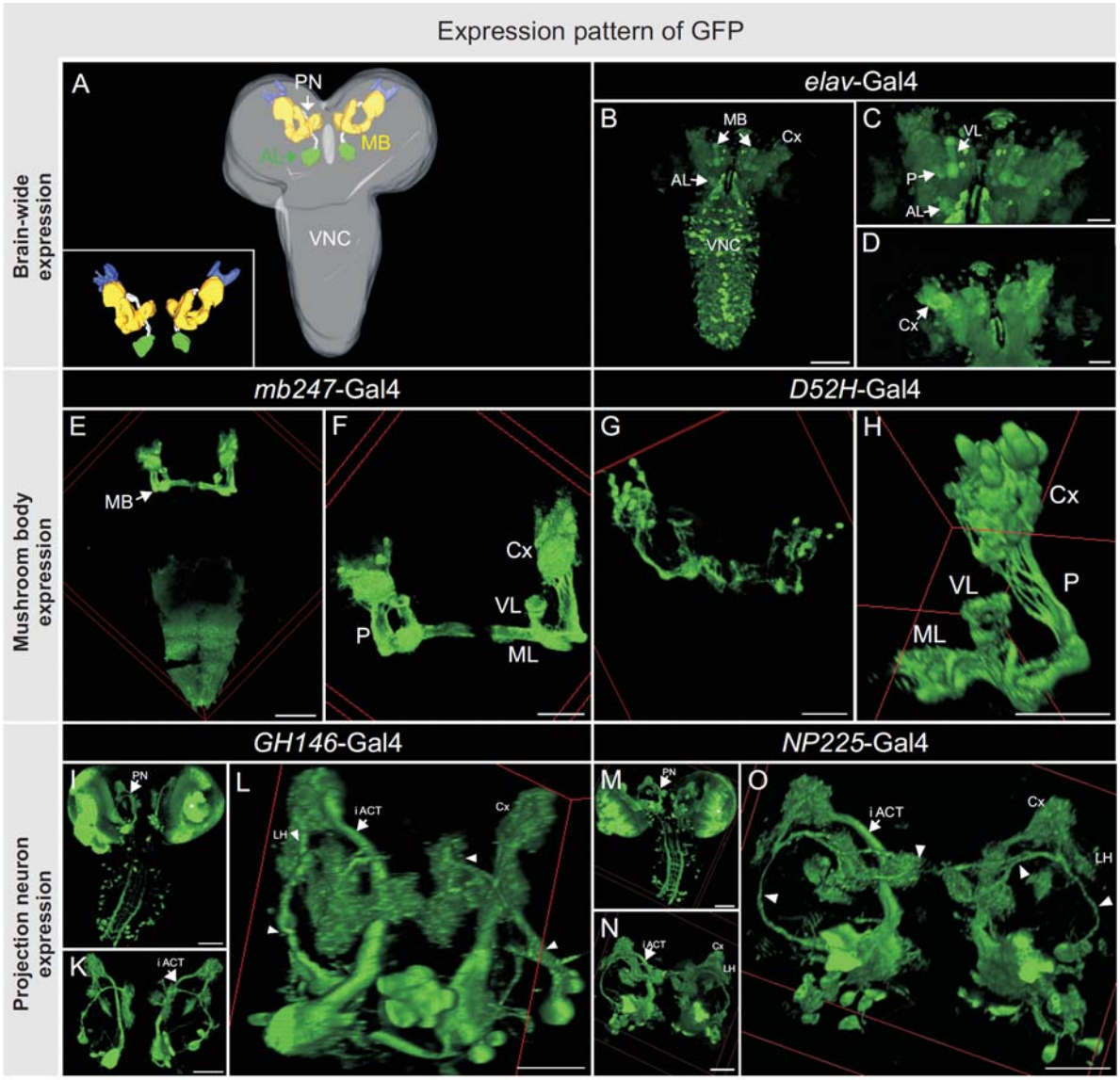

Fig. S1

Figure S1: Expression pattern of the various Gal4-strains used for behavioural experiments. Three-dimensional reconstructions of anti-GFP immunoreactivity (green) of whole-mount larval third-instar brains using the ImageJ 3D Viewer. (A) Dorsal view with the major brain regions reconstructed. The inset shows a magnified view of the MB. (B-D) Brain-wide expression of GFP using e*lav*-Gal4. (B) Whole brain. (C, D) Details of the brain seen in B. (E-H) Mushroom body expression of GFP using *mb247*-Gal4 (E, F) with whole brain (E) and a magnified view of the mushroom body (F). (G, H) Mushroom body expression of GFP using *D52H*-Gal4 showing (G) both mushroom bodies and (H) a magnified view of a single mushroom body. (I-O) Projection neuron expression of GFP in whole mounts using (I-L) *GH146*-Gal4 or (M-O) NP225-Gal4 as drivers for GFP expression. Additionally to projection neuron staining, a mushroom body extrinsic neuron (►) shows strong GFP immunoreactivity as well. (I, M) Whole brain. (K, L and N, O) Magnification of projection neurons and extrinsic mushroom body neurons. Optic lobe Anlagen (*), antennal lobe (AL), inner antennocerebral tract (iACT), projection neuron (PN), mushroom body (MB), calyx (Cx), peduncle (P), medial lobe (ML)vertical lobe (VL), lateral horn (LH), ventral nerve cord (VNC). Scale bars: 50µm in B, E, I, M; 25µm in C, D, F-H, K, L, N, O.

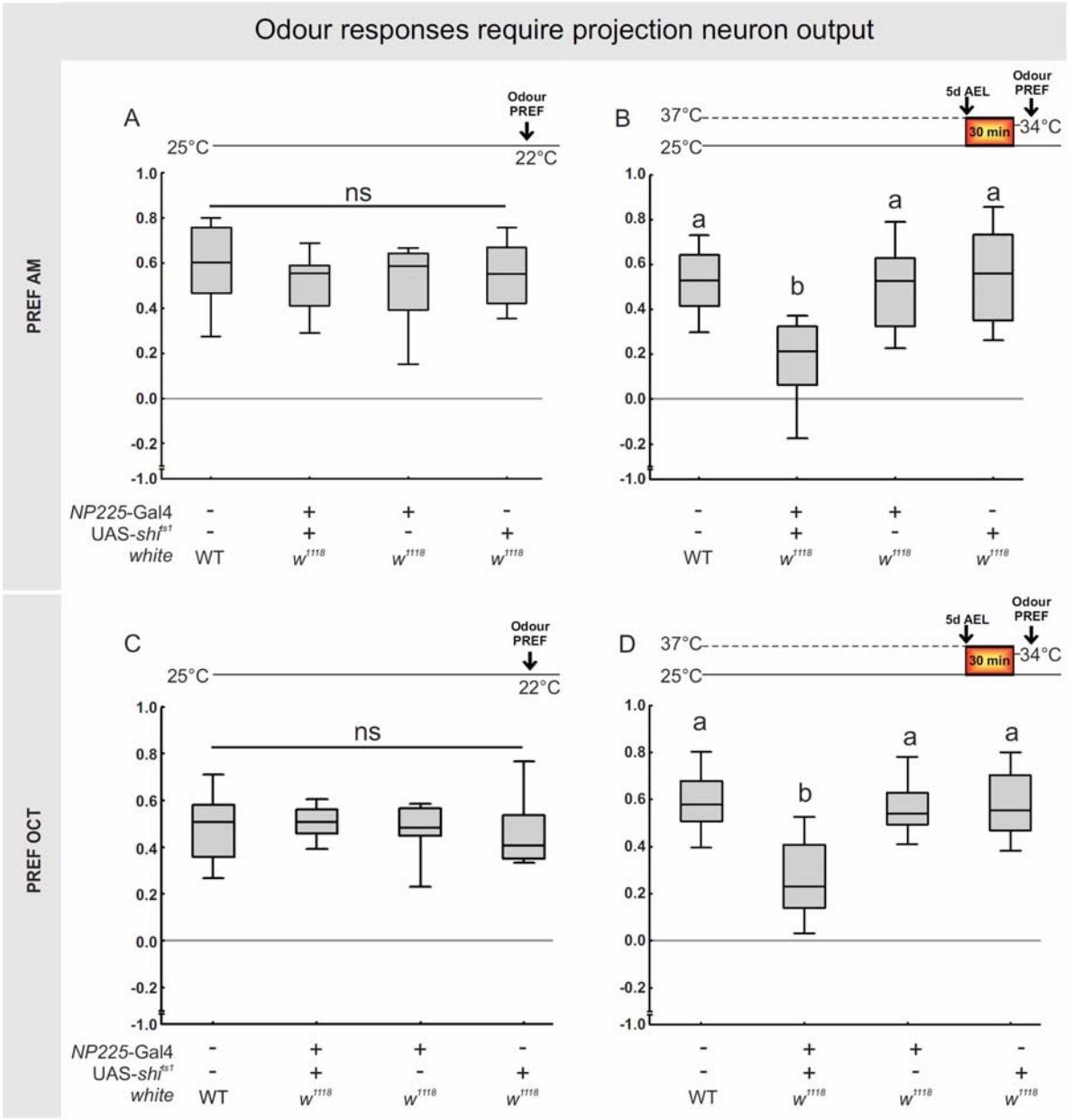

Fig. S2

Figure S2: Blocking synaptic output from projection neurons massively reduces odour preferences. The following genotypes were generated: for the experimental group we crossed *NP225*-Gal4 to UAS-*shi*^{ts1}, yielding double heterozygous larvae (; *NP225*-Gal4/ +; UAS-*shi*^{ts1}/ +); for the driver control we crossed *NP225*-Gal4 to no-transgene carrying flies yielding single-heterozygous (; *NP225*-Gal4 / + ;); for the effector control we crossed UAS-*shi*^{ts1} to no-transgene carrying flies yielding ; ; UAS-*shi*^{ts1}/ + animals. Experimentally naive larvae were incubated in their food vials for 30 min on 37 °C in a water bath. To test their ability to detect odours, we determined their PREF values when given a choice between either paraffin-diluted AM versus paraffin, or between undiluted OCT versus an empty container. These odour preference tests were performed either at 34 °C (restrictive temperature) or at room temperature (22 °C). *NP225*-Gal4/ UAS-*shi*^{ts1} larvae do not differ from controls when AM Preference (A; KW-test: P= 0.58; H= 1.94; df= 3; N= 16 for all genotypes) or OCT Preference (C; KW-test: P= 0.57; H= 2.00; df= 3; N= 16 for all genotypes) was measured at 22 °C. However, when synaptic output of projection neurons is blocked at restrictive temperature, odour preferences of *NP225*-Gal4/ UAS-*shi*^{ts1} are significantly lower than of control larvae, both for AM (B; KW-test: P< 0.05; H= 28.36; df= 3; N= 20 for all genotypes; *NP225*-Gal4/ UAS-*shi*^{ts1} versus wild-type CS: MW: P< 0.05/ 3; U= 29; N= sample size as above ; *NP225*-Gal4/ UAS-*shi*^{ts1} versus projection-neuron DRIVER control: MW: P< 0.05/ 3; U= 53; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus EFFECTOR

control: MW: P< 0.05/ 3; U= 45; sample sizes as above) and for OCT (D; KW-test: P< 0.05; H= 27.45; df= 3; N= 20 for all genotypes; *NP225*-Gal4/ UAS-*shi*^{ts1} versus wild-type CS: MW: P< 0.05/ 3; U= 37; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus projection-neuron DRIVER control: MW: P< 0.05/ 3; U= 50; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus EFFECTOR control: MW: P< 0.05/ 3; U= 46; sample sizes as above).

All other details as in the legend of Fig. 1.

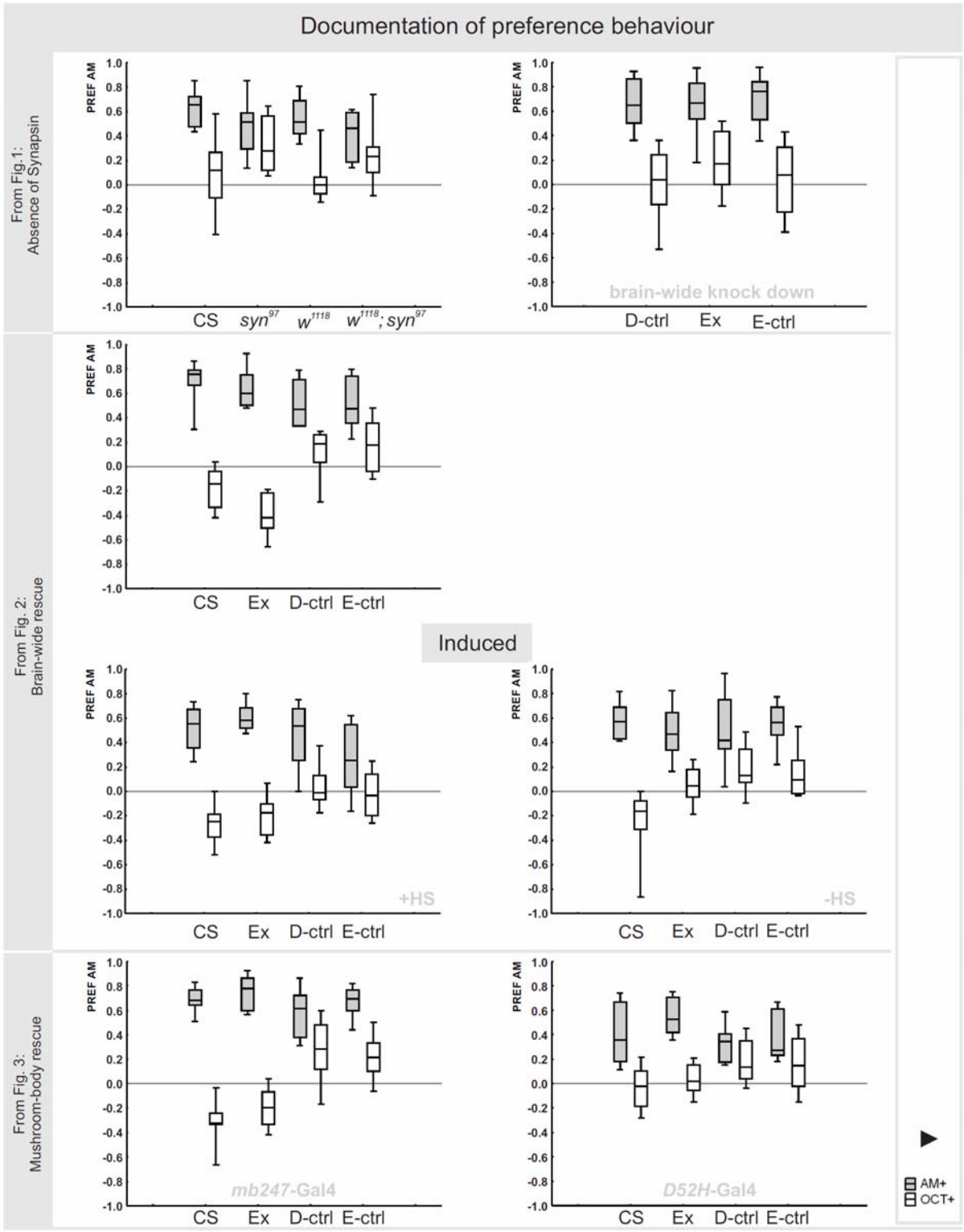
Documentation of preference behaviour
From Fig.1: Absence of Synapsin
From Fig. 2: Brain-wide rescue
From Fig. 3: Mushroom-body rescue
PREF AM
CS
syn97
w1118
w1118; syn97
brain-wide knock down
D-ctrl
Ex
E-ctrl
Induced
+HS
-HS
mb247-Gal4
D52H-Gal4
AM+
OCT+

Fig. S3 part 1

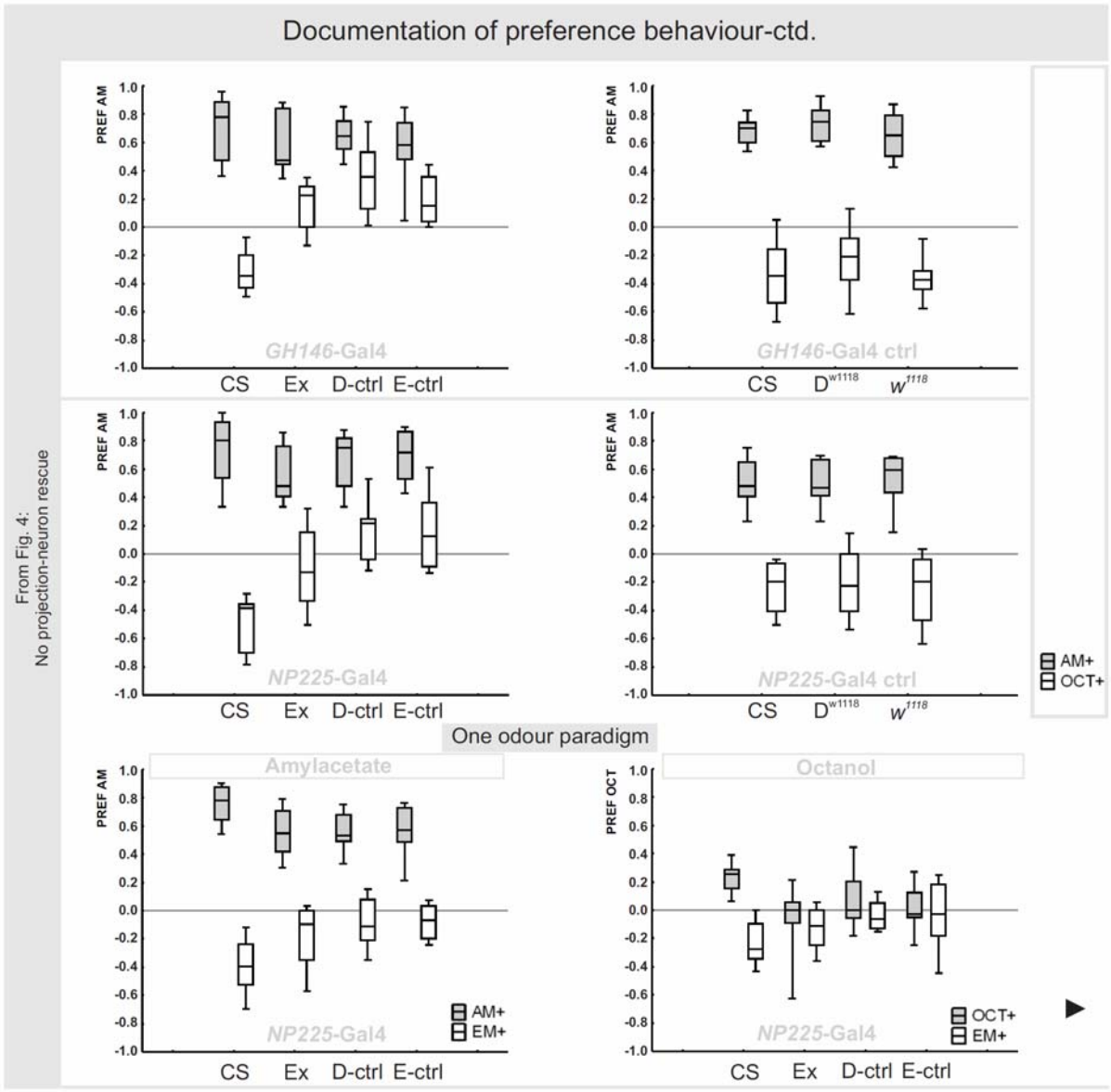
Documentation of preference behaviour-ctd.
From Fig. 4:
No projection-neuron rescue
PREF AM
GH146-Gal4
GH146-Gal4 ctrl
NP225-Gal4
NP225-Gal4 ctrl
CS
Ex
D-ctrl
E-ctrl
D^w1118
w^1118
AM+
OCT+
One odour paradigm
Amylacetate
Octanol
PREF OCT
EM+

Fig. S3 part 2

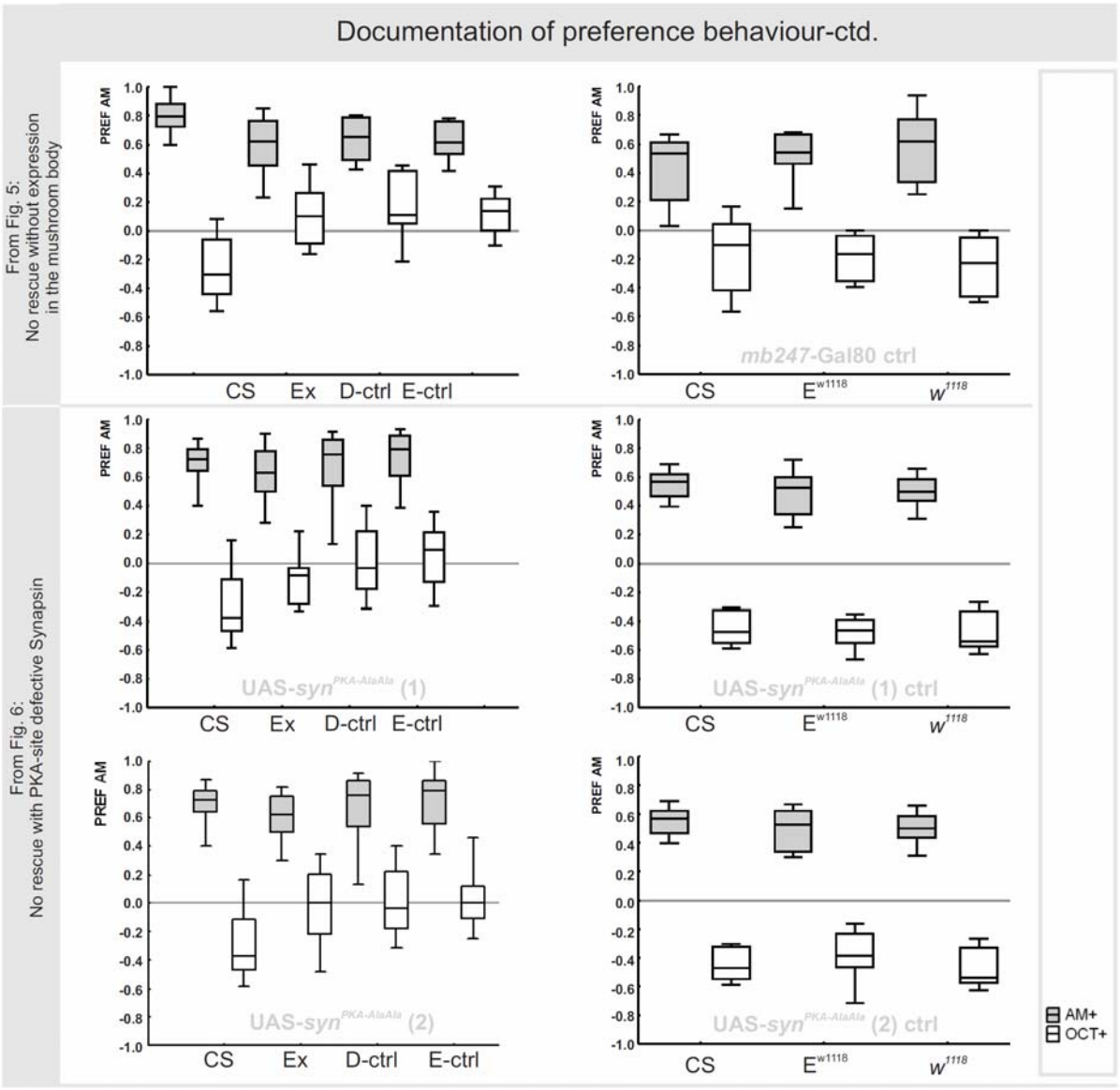

Fig. S3 part 3

Figure S3: Odour preferences, separated by training regimen. For documentation, we present the AM preferences from the reciprocally trained groups, i.e. the $PREF_{AM}$ scores after either AM had been rewarded during training (AM+, gray boxes) or after OCT had been rewarded during training (OCT+, white boxes) for all behavioural experiments reported in the body text. Overall, genotypes show a slant towards AM, independent of the rewarded odour. The effect of associative training consists in the observation that $PREF_{AM}$ scores are increased after AM+ training, and decreased after OCT+ training. In the one odour paradigm $PREF_{AM}$ scores or $PREF_{OCT}$ scores are presented after either AM or OCT, respectively, had been rewarded during training (AM+ or OCT+, gray boxes) or after EM had been rewarded during training (EM+, white boxes). D-ctrl: driver-control in the w^{1118}; syn^{97} background, E-ctrl: effector-control in the w^{1118}; syn^{97} background, Ex: experimental group in the w^{1118}; syn^{97} background, D^{w1118}: driver-cotrol in the w^{1118} background, E^{w1118}: effector-control in the w^{1118} background.

See the accompanying CD attached at the back of this thesis for supplementary Movies S1- S6

All movies are three-dimensional reconstructions of anti-GFP immunoreactivity of whole-mount larval third-instar brains.

Movie S1: *Drosophila* larval brain with the major brain regions reconstructed. Shown are antennal lobes (green), projection neurons (white), the mushroom bodies (yellow), and the Kennyon cell bodies (blue). The light grey shade sketches the rest of the larval brain. Based on a brain from a larva obtained by crossing *GH146*-Gal4; *mb247*-Gal4 to UAS-*GFP*. The 3D representation was obtained from 1 micron confocal serial sections using ImageJ software.

Movie S2: Gal4 expressing cells in e*lav*-Gal4 monitored by UAS-*GFP* (green). The larval brain shows GFP expression throughout all neuropil regions, with strong expression in the mushroom bodies.

Movie S3: Gal4 expressing cells in *mb247*-Gal4 monitored by UAS-*GFP* (green). View on the larval mushroom body. In terms of expression pattern, *mb247*-Gal4 leads to GFP-expression in all basic compartments of the larval mushroom body, i.e. in calyx, peduncle and lobes.

Movie S4: Gal4 expressing cells in *D52H*-Gal4 monitored by UAS-*GFP* (green). View on a single mushroom body. Expression is found in only very few mushroom body neurons (~7 mushroom body neurons per brain hemisphere). Notably, although only so few mushroom body neurons are covered, GFP expression reveals all basic compartments of the larval mushroom bodies; in particular the mushroom body input regions (the calyx) seems to be covered fairly well.

Movie S5: Gal4 expressing cells in *GH146*-Gal4 monitored by UAS-*GFP* (green). View on the projection neurons in the larval brain. When the *GH146*-Gal4 driver is used to express GFP, additionally to the expression in the projection neurons, a single mushroom body-extrinsic neuron per hemisphere is GFP-positive.

Movie S6: Gal4 expressing cells in *NP225*-Gal4 monitored by UAS-*GFP* (green). Same as Movie S5 but using *NP225*-Gal4 as another projection-neuron Gal4 strain.

Summary

An animal depends heavily on its sense of smell and its ability to form olfactory associations as this is crucial for its survival. This thesis studies in two parts about such associative olfactory learning in larval *Drosophila*. The first part deals with different aspects of odour processing while the second part is concerned with aspects related to memory and learning.

Chapter I.1 highlights how odour intensities could be integrated into the olfactory percept of larval *Drosophila*. I first describe the dose-effect curves of learnability across odour intensities for different odours and then choose odour intensities from these curves such that larvae are trained at intermediate odour intensity, but are tested for retention with either that trained intermediate odour intensity, or with respectively HIGHer or LOWer intensities. I observe a specificity of retention for the trained intensity for all the odours used. Further I compare these findings with the case of adult *Drosophila* and propose a circuit level model of how such intensity coding comes about. Such intensity specificity of learning adds to appreciate the richness in 'content' of olfactory memory traces, and to define the demands on computational models of olfaction and olfactory learning.

Chapter I.2 provides a behaviour-based estimate of odour similarity using four different types of experiments to yield a combined, task-independent estimate of perceived difference between odour-pairs. Further comparison of these perceived differences to published measures of physico- chemical difference reveals a weak correlation. Notable exceptions to this correlation are 3-octanol and benzaldehyde.

Chapter I.3 shows for two odours (3-octanol and 1-octene-3-ol) that perceptual differences between these odours can either be ignored after non-discriminative training (generalization), or accentuated by odour-specific reinforcement (discrimination). Anosmic $Or83b^{1}$ mutants have lost these faculties, indicating that this adaptive adjustment is taking place downstream of *Or83b* expressing sensory neurons.

Chapter II.1 of this thesis deals with food supplementation with dried roots of *Rhodiola rosea.* This dose-dependently improves odour- reward associative function in larval *Drosophila*. Supplementing fly food with commercially available tablets or extracts,

however, does not have a 'cognitive enhancing' effect, potentially enabling us to differentiate between the effective substances in the root versus these preparations. Thus *Drosophila* as a genetically tractable study case should now allow accelerated analyses of the molecular mechanism(s) that underlie this 'cognitive enhancement' conveyed by *Rhodiola rosea.*

Chapter II.2 describes the role of Synapsin, an evolutionarily conserved presynaptic phosphoprotein using a combined behavioural and genetic approach and asks where and how, this protein affects functions in associative plasticity of larval *Drosophila*. This study shows that a Synapsin-dependent memory trace can be pinpointed to the mushroom bodies, a 'cortical' brain region of the insects. On the molecular level, data in this study assign Synapsin as a behaviourally- relevant effector of the AC-cAMP-PKA cascade.

Zusammenfassung*

Das Überleben von Tieren ist in hohem Maße abhängig von ihrer Fähigkeit zu riechen und olfaktorische Gedächtnisse zu bilden. Meine Arbeit besteht aus zwei Abschnitten, in denen ich solche Prozesse anhand von *Drosophila* Larven untersuche. Im ersten Abschnitt beschreibe ich verschiedene Aspekte der Geruchsprozessierung, der zweite Abschnitt betrifft Gedächtnis- und Lernprozesse.

Kapitel I.1 handelt davon, wie Geruchsintensitäten in die olfaktorische Wahrnehmung von *Drosophila*-Larven integriert sein könnten. Zuerst beschreibe ich die Lernbarkeit verschiedener Duftstoffe abhängig von ihren Intensitäten. Anhand dieser Dosis-Wirkungs-Kurven wähle ich dann eine niedrige, eine mittlere, und eine hohe Duft-Intensität. Ich trainiere Larven mit der mittleren Duft-Intensität und teste sie entweder mit dieser mittleren Intensität, oder mit der höheren, oder mit der niedrigen Duft-Intensität. Ich beobachte, dass der Gedächtnisabruf mit der trainierten Intensität für alle verwendeten Duftstoffe am besten ist. Außerdem vergleiche ich diese Ergebnisse mit denen von adulten Fruchtfliegen und schlage ein Schaltkreis-Modell vor, das erklärt, wie eine solche Kodierung der Intensität zustande kommen kann. Eine solche Spezifität für Intensitäten beim Lernen erweitert die bisher bekannte Fülle des ‚Inhalts' von olfaktorischen Gedächtnisspuren und die Anforderungen an Computermodelle über Riechen und Geruchslernen.

In Kapitel I.2 untersuche ich Ähnlichkeitsbeziehungen zwischen Duftpaaren anhand der Wahrnehmung von Larven. Ich verwende dazu vier verschiedene Typen von Lernexperimenten. Durch Kombination der Ergebnisse dieser vier Experimente erhalte ich eine aufgabenunabhängige Abschätzung der vom Tier wahrgenommenen Ähnlichkeiten zwischen Paaren von Duftstoffen. Ein Vergleich dieser wahrgenommenen Ähnlichkeiten mit veröffentlichten Messungen von physikalischen und chemischen Ähnlichkeiten ergibt eine schwache Korrelation. Eine erwähnenswerte Ausnahme zu dieser Korrelation ist das Duftpaar 3-Octanol und Benzaldehyd.

Kapitel I.3 zeigt für zwei Duftstoffe (3-Octanol und 1-Octen-3-ol), dass die wahrgenommene Ähnlichkeit zwischen diesen beiden Duftstoffen abhängig ist von der Art des Trainings. Wenn die Tiere nicht-diskriminativ trainiert werden, werden die Düfte vom Tier generalisiert, während diskriminatives Training die wahrgenommene

Unterschiede zwischen den Düften erhöht. Anosmische *Or83b*^{1}-Mutanten haben diese Fähigkeiten verloren, was darauf hindeutet, das diese adaptive Anpassung in Nervenzellen stattfindet, die den *Or83b*-exprimierenden sensorischen Neuronen nachgeschaltet sind.

In Kapitel II.1 untersuche ich die Auswirkung von Zugabe getrockneter Wurzeln der Pflanze *Rhodiola rosea* zum Fliegenfutter. Ich finde heraus, dass *Rhodiola rosea* dosisabhängig die olfaktorische Konditionierung von *Drosophila*-Larven verbessert. Die Zugabe von kommerziell verfügbaren Tabletten oder Extrakten zum Fliegenfutter hat keinen positiven Effekt auf solche „kognitiven" Fähigkeiten, was uns möglicherweise erlaubt, zwischen den effektiven Substanzen der Wurzel und diesen Präparaten zu differenzieren. *Drosophila* als genetisch manipulierbarer Modellorganismus sollte uns nun weiterführende Analysen der molekularen Mechanismen erlauben, die dieser „kognitiven Verbesserung" durch *Rhodiola rosea* zugrunde liegen.

Kapitel II.2 beschreibe ich die Funktion von Synapsin, einem evolutionär konservierten präsynaptischen Phosphoprotein. Ich verwende dazu einen kombinierten verhaltensbasierten und genetischen Ansatz. Untersucht wird, wo und wie dieses Protein assoziative Plastizität im Gehirn von *Drosophila*-Larven beeinflusst. Diese Studie zeigt, dass eine Synapsin-abhängige Gedächtnisspur im Pilzkörper, einer „kortikalen" Gehirnregion der Insekten, lokalisiert werden kann. Auf der molekularen Ebene zeigen die Ergebnisse dieser Studie Synapsin als einen im Verhalten wichtigen Effektor der AC-cAMP-Kaskade.

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Publications:

a) Michels B., Chen Yi., Tanimoto H., Mishra D., Engmann O., Saumweber T., Gerber B., (2011): Site and mode of Synapsin action in associative learning, *Learning and Memory, 18(5), 332- 44.*

b) Chen Yi., Mishra D., Schmitt L., Gerber B., (2011): A behavioural odour- similarity ‚space' in larval *Drosophila*, *Chemical Senses, 36(3), 237- 249.*

c) Mishra D., Louis M., Gerber B., (2010): Adaptive adjustment of the generalization-discrimination balance in larval *Drosophila*, *J. Neurogenetics – 24(3), 168-175.*

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