Experimental access to the content of an olfactory memory trace in larval *Drosophila*

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Erklärung

gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität zu Würzburg vom 15. März 1999:

Die vorgelegte Dissertation besteht aus zwei Publikationen, einem Manuskript und einer zusätzlichen „Allgemeinen Einleitung und Diskussion“. Die Mitwirkung der Co-Autoren jeder Publikation wird auf den folgenden Seiten herausgearbeitet.

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

Chen, Yi-chun Prof. Dr. Bertram Gerber

Structure of the thesis

This thesis consists of two parts, which deal with into the behavioural, cellular and molecular account in the context of associative olfactory learning of larval *Drosophila melanogaster*. Initially, I present a short summary in English and German, and a "General introduction & discussion" to give an overview of the background and implications of this thesis.

Part I deals with how two defining aspects- quality and intensity- of an odour play a role in the content of behaviourally defined olfactory memory and perception. This part contains one publication and one manuscript prepared for publication. Part II comprises one publication concerning the cellular site and molecular mode of Synapsin action for associative plasticity of larval *Drosophila*.

Part I.1.

Chen Y-c, Mishra D, Schmitt L, Gerber B (2011) A behavioural odour-similarity 'space' in larval *Drosophila*. *Chemical Senses* 36(3): 237-49.

Part I.2.

Mishra D*****, **Chen Y-c***, Yarali A, Gerber B Olfactory memories are intensity-specific in larval *Drosophila*.

Part II.1.

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

***** authors with equal contribution

Finally, a Curriculum vitae, List of publications and Acknowledgements at the end complete this thesis.

This work would not have been possible without the effort of many people, and the supervision of my mentor Bertram Gerber. I take the opportunity to express my joy in this collaborative work and sincerely acknowledge the co-authors of each manuscript, whose contributions are explicated below.

Dissertation unter Einschluss mehrerer Manuskripte

Erklärung zu Eigenanteilen an Publikationen und Zweitpublikationsrechten

Publikation (Vollständiges Zitat): **Chen Y-c**, Mishra D, Schmitt L, Gerber B

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

Chemical Senses 2011; 36(3): 237-49

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	**CYc**	MD	GB		
Datenerhebung	**CYc**	MD			
Daten-Analyse und Interpretation	**CYc**	MD	GB		
Schreiben des Manuskripts	GB	**CYc**	MD		

ggf. Erläuterung:

Manuskript (Vollständiges Zitat): Mishra D*****, **Chen Y-c***, Yarali A, Gerber B

Olfactory memories are intensity-specific in larval *Drosophila*.

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	MD	**CYc**	YA	GB	
Datenerhebung	MD	**CYc**	YA		
Daten-Analyse und Interpretation	MD	**CYc**	YA	GB	
Schreiben des Manuskripts	MD	GB	YA	**CYc**	

ggf. Erläuterung:

Publikation (Vollständiges Zitat): Michels B, **Chen Y-c**, Mishra D, Saumweber T, Engmann O, Tanimoto H, Gerber B

Cellular site and molecular mode of Synapsin action in associative learning.

Learning and Memory 2011; 18(5): 332-44

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	MB	GB	TH	**CYc**	ST
Datenerhebung	MB	**CYc**	MD	ST	EA
Daten-Analyse und Interpretation	MB	**CYc**	ST	SB	GB
Schreiben des Manuskripts	MB	GB	ST	**CYc**	

ggf. Erläuterung:

Für alle in dieser „Dissertation unter Einschluss mehrerer Manuskripte“ verwendeten Manuskripte liegen die notwendigen Genehmigungen der Verlage und Co-Autoren für die Zweitpublikation vor.

Mit meiner Unterschrift bestätige ich die Kenntnisnahme und das Einverständnis meines direkten Betreuers.

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Datum Unterschrift

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Summary

Animals need to evaluate their experiences in order to cope with new situations they encounter. This requires the ability of learning and memory. *Drosophila melanogaster* lends itself as an animal model for such research because elaborate genetic techniques are available. *Drosphila* larva even saves cellular redundancy in parts of its nervous system. My Thesis has two parts dealing with associative olfactory learning in larval *Drosophila*. Firstly, I tackle the question of odour processing in respect to odour quality and intensity. Secondly, by focusing on the evolutionarily conserved presynaptic protein Synapsin, olfactory learning on the cellular and molecular level is investigated.

Part I.1. provides a behaviour-based estimate of odour similarity in larval *Drosophila* by using four recognition-type experiments to result in a combined, task-independent estimate of perceived difference between odour-pairs. A further comparison of these combined perceived differences to published calculations of physico-chemical difference reveals a weak correlation between perceptual and physico-chemical similarity.

Part I.2. focuses on how odour intensity is interpreted in the process of olfactory learning in larval *Drosophila*. First, the dose-effect curves of learnability across odour intensities are described in order to choose odour intensities such that larvae are trained at intermediate odour intensity, but tested for retention either with that trained intermediate odour intensity, or with respectively HIGHer or LOWer intensities. A specificity of retention for the trained intensity is observed for all the odours used. 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 associative olfactory memory trace formation.

In part II.1. of the thesis, the cellular site and molecular mode of Synapsin function is investigated- an evolutionarily conserved, presynaptic vesicular phosphoprotein. On the cellular level, the study shows a Synapsin-dependent memory trace in the mushroom bodies, a third-order “cortical” brain region of the insects; on the molecular level, Synapsin engages as a downstream element of the AC-cAMP-PKA signalling cascade.

Zusammenfassung

Tiere müssen ihre eigenen Erfahrungen heranziehen, damit sie neue Situationen meistern können. Dies setzt die Fähigkeit zum Lernen und ein Gedächtnis voraus. *Drosophila melanogaster* eignet sich dank der Vielzahl verfügbarer genetischer Methoden als ein Modellorganismus für solche Forschung. Die *Drosophila* Larve kommt zudem in Teilen ihres Nervensystems ohne zelluläre Redundanz aus. Meine Doktorarbeit gliedert sich in zwei Teile, die das assoziative olfaktorische Lernen der *Drosophila* Larven zum zentralen Gegenstand haben. Erstens bearbeite ich den Prozess der Geruchswahrnehmung hinsichtlich der Duftqualität und Duftintensität. Im zweiten Teil meiner Arbeit erforschen wir das olfaktorische Lernen auf zellulärer und molekularer Ebene und konzentrieren uns dabei auf das hochkonservierte präsynaptische Protein Synapsin.

Teil I.1. handelt von der Ähnlichkeit zwischen Duftpaaren in der Wahrnehmung von *Drosophila* Larven anhand vier verschiedener Typen von Lernexperiment. Mit diesen Experimenten ließ sich eine Abschätzung der vom Tier wahrgenommenen Ähnlichkeiten zwischen Paaren von Duftstoffen erreichen. Ein Vergleich dieser wahrgenommenen Ähnlichkeiten mit veröffentlichten physikalisch-chemischen Ähnlichkeiten ergibt eine schwache Korrelation.

Teil I.2. befasst sich damit, wie die Intensität eines Duftes in die olfaktorische Wahrnehmung und das Gedächtnis der *Drosophila* Larven integriert sein könnte. Zunächst wird die Lernbarkeit verschiedener Duftstoffe abhängig von ihren Intensitäten beschrieben; anhand dieser Dosis-Wirkungskurven werden dann Duftintensitäten so ausgewählt, dass die Larven mit der mittleren Duftintensität trainiert werden, aber mit einer höheren, oder mit einer niedrigeren Duftintensität getestet werden. Es zeigt sich eine Spezifität des Gedächtnisabrufs für die trainierte Intensität, und zwar für alle verwendeten Duftstoffe. Eine solche Spezifität für Intensität bereichert das Bild des ‚Inhalts' von olfaktorischen Gedächtnisspuren und damit die Anforderungen an Computermodelle über Riechen und Geruchslernen.

Im Teil II.1. habe ich in Zusammenarbeit mit Birgit Michels auf zelluläre Ebene die Funktion von Synapsin beim assoziativen Lernen von *Drosophila* Larven untersucht- ein evolutionär konserviertes, präsynaptisches, vesikel-assoziiertes Phosphoprotein. Auf zellulärer Ebene zeigt die Studie eine Synapsin-abhängige Gedächtnisspur im Pilzkörper, einer dem olfaktorischen Cortex der Vertebraten womöglich homologen Struktur. Auf

molekularer Ebene wurde nachgewiesen, dass Synapsin als ein Zielprotein in der AC-cAMP-PKA Kaskade am Lernvorgang beteiligt ist.

General introduction & discussion

Our personal identity has more than one source: It relies on the DNA "blueprint" we inherit, the past experience we garnered and the current environment we face. The brain integrates all these aspects in order to generate fitting behaviour, starting from the interplay between environment and brain in early embryogenesis. Different environments allow different experiences; each experience is unique because of social context and especially in human beings, culture gives behaviour meaning. The brain records, transmits and recalls events in their proper sequence with attendant emotion, and organizes behaviour accordingly. An understanding of these processes is required to understand how the brain and mind work (Bruhn 2005).

Indeed, during the past 10 years of my residence in Germany, I realized how deeply my socially acquired Taiwanese background and in general, my past experience influence my daily behaviour towards different German occasions. For this reason, I was interested to better understand learning and memory processes, especially focussing on olfactory learning since smell provides essential, and emotionally "charged" information about our environment in daily life; whenever I smell roast almond redolence, I can "predict" German Christmas is approaching. During my PhD, I tackled this issue using a well-tractable animal system, namely odour-sugar associative conditioning behaviour in larval *Drosophila* (Scherer et al. 2003; Neuser et al. 2005; Saumweber et al. 2011a, b; for review Gerber and Stocker 2007; Gerber et al. 2009) focussing on the following aspects in particular:

Part I presents aspects of odour quality and odour intensity processing in describing the content of olfactory memory in *Drosophila* larvae- from the experiential and environmental point of view.

Part II focuses on an evolutionarily conserved, presynaptic vesicular phosphoprotein, Synapsin, which acts in larval associative learning- from the cellular and molecular point of view.

Why *Drosophila* larva? The *Drosophila* larva provides an attractive system for analyzing odour coding from both these mentioned points of view, given that it shares the same design of the olfactory circuits with adult flies and mammals, though it exhibits a lack of cellular redundancy across the initial steps of olfactory processing. This makes it relatively promising to understand the sense of smell from odour reception to behaviour (reviewed in Gerber and Stocker 2007; Stocker 2008; Gerber et al. 2009): Odour perception begins within

the external dorsal organ which represent the “nose” of larva. In a triplet manner, the merely 21 olfactory sensory neurons are organized in the dorsal organ, each expresses one single type of olfactory receptor from the *Or*-gene family (Hallem et al. 2004) (along with the co-receptor *Orco*, synonymous with previous *Or83b*: Larsson et al. 2004; Neuhaus et al. 2005; Benton et al. 2006) with its accordant ligand profile. Another class of chemosensory receptors also exists known as the ionotropic receptors from the *Ir*-gene family (Benton et al. 2009), but their contribution to odour coding in the larva remains unknown. The olfactory sensory neurons expressing the same receptor type converge into the same glomerulus (Gerber et al. 2009) in the antennal lobe (the functional analogue of the olfactory bulb in mammals). Within the antennal lobe, glomeruli are connected by local interneurons (Pauls and Selcho et al. 2010; Thum et al. 2011); furthermore, projection neurons (the functional analogue of the mitral cells) which obtain input from mostly one single glomerulus of the antennal lobe (Ramaekers et al. 2005) convey the olfactory information to two other brain regions: mushroom body and lateral horn (Marin et al. 2002; Wong et al. 2002). On the one hand, they target the calyx of the mushroom body (Ramaekers et al. 2005; Masuda-Nakagawa et al. 2005, 2009) which is arranged into approximately 40 calyx glomeruli (Marin et al. 2005; Masuda-Nakagawa et al. 2005; Ramaekers et al. 2005), so that one or exceptionally two projection neurons provide input to one single calyx glomerulus. About 600 Kenyon cells are located in the mushroom body calyx of each hemisphere (Technau and Heisenberg 1982), and most of the Kenyon cells receive information from typically six randomly selected calyx glomeruli (Masuda-Nakagawa et al. 2005; Murthy et al. 2008). Thus, not until up to the mushroom body, the larval olfactory pathway initiates divergence-convergence connectivity. As for the reward information, the gustatory sensory neurons in external dorsal, terminal and ventral organs, as well as three internal pharyngeal organs (dorsal, ventral and posterior pharyngeal sense organs) then receive reward inputs which are transmitted subsequently into distinct areas of the suboesophageal ganglion (reviewed in Gerber and Stocker 2007; Gerber et al. 2009). A group of nearly 20 *hugin*-expressing neurons of the suboesophageal ganglion in consideration of second-order gustatory neurons which seems to obtain this information and send it to the ring gland, the protocerebrum near mushroom body calyces, pharyngeal muscles, and ventral nerve cord (Melcher and Pankratz 2005). Furthermore, from the subesophageal ganglion dopaminergic and octopaminergic neurons arise which likely mediate respective appetitive and aversive reinforcement information impinging onto the Kenyon cells (Schroll et al. 2006; Selcho et al. 2009), and further mushroom body output neurons are modulated afterwards. Thus mushroom body output is thought to connect to premotor circuits

to organize learned olfactory behaviour. On the other hand, the projection neurons proceed through another route to the lateral horn which is involved in innate olfactory behaviour and continues to pre-motor circuitry (reviewed in Stocker 1994; Keene and Waddel 2007; Gerber et al. 2009).

By relying on such a network I ask how larvae actually perceive odours. Paramount to our first approach is *not* to directly ask how the larvae perceive a given odour, but rather to ask whether the larvae perceive two given odours as *different from each other*. Further topics concern how odour intensity is integrated into the odour perception and how the presynaptic protein Synapsin affects odour-sugar associative learning.

I.1. Describing the content of olfactory memory in Drosophila larvae: Odour quality

Using four kinds of recognition task, we seek to come up with one behaviour-based estimate of odour similarity:

(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 results in a combined, task-independent estimate of perceived difference between odour-pairs. A comparison of these combined perceived differences to published calculations of physico-chemical difference (Schmuker et al. 2007; Haddad et al. 2008) reveals a weak correlation. One exception is the odour-pair 3-octanol and benzaldehyde, which is distinct in physico-chemical aspect and in sensory representation (Kreher et al. 2005), but is regarded as the most similar odour-pair in *Drosophila* larval behaviour. This implies olfactory perception be “determined” not at the level of sensory neurons, but at the higher-order neurons after transmitting along the olfactory circuit. A corresponding conclusion is also reached by Niewalda et al. (Niewalda et al. 2011) in adult *Drosophila*, a study including calcium imaging of odour-evoked activity.

One noticeable feature of our approach (as compared to Cobb and Domain 2000; Boyle and Cobb 2005; Guerrieri et al. 2005; Kreher et al. 2008) is that we choose odour dilutions on a behavioural, rather than a physico-chemical or formal basis. That is, we were adjusting odour dilutions based on the dose-response curves for equal learnability, rather than

using the same dilution or based on the physical properties for all odours. This consideration yielded “symmetrical” measurements of odour perception which is comparable to physico-chemical based measurements of odour molecules.

I.2. Describing the content of olfactory memory in Drosophila larvae: Odour intensity

The olfactory system may allow animals to discriminate odours relying upon odour quality, odour intensity or a combination of both. The coding of odour quality is often proposed to be combinatorial regarding the composition of activated OSNs along the olfactory pathway. However, it is less obvious how odour intensity is processed.

Here we investigate whether olfactory memory traces include information about the intensity of the learnt odour. Firstly, we describe the dose-effect curves of learnability across odour intensities for four different odours (*n*-amyl acetate, 3-octanol, 1-octene-3-ol, benzaldehyde) in order to adjust odour dilutions for equal learnability based on this dose-response curve. 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 experimental strategy had been advocated by Yarali et al. (Yarali et al. 2009), who showed in odour-shock learning of adult *Drosophila* that three of the four odours (*n*-amyl acetate, 3-octanol and 4-methylcyclohexanol) showed a specificity of retention for the trained intensity, while for benzaldehyde, this was not the case. Indeed, in adult *Drosophila* the genetic and neuronal basis for benzaldehyde responsiveness seems to differ from those of other odours (Ayer and Carlson 1992; Helfand and Carlson 1989; Keene et al. 2004; see discussion in Yarali et al. 2009) while this is not apparently so in larval *Drosophila* (Part I.2 Fig. 4Dii).

Furthermore, even though many investigators have found that 4-methylcyclohexanol can be well learned in adults (e.g. Yarali et al. 2009), larvae seem behaviourally little responsive to 4-methylcyclohexanol (Part I.2 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 regarding benzaldehyde and 4-methylcyclohexanol between larvae and adults may be based on different receptor repertoires of the two life stages (Hallem et al. 2006; Kreher et al. 2008).

II.1. Cellular site and molecular mode of Synapsin action in associative learning

Synapsin is an evolutionarily conserved phosphoprotein associated with synaptic vesicles (Hilfiker et al. 1999; Sudhof 2004), which in *Drosophila* is dispensable for basic synaptic transmission (Godenschwege et al. 2004); however, it is required for larval odour-sugar associative learning (Michels et al. 2005). Synapsin binds 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. Upon phosphorylation, it 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).

On the molecular level, since *Aplysia* Synapsin was considered to be an excellent substrate for cAMP-dependent protein kinase (Fiumara et al. 2004), one may suspect that one or both of the PKA consensus sites of Synapsin is required for reserve-pool vesicle recruitment. My contribution here was to generate transgenic fly lines which express a Synapsin protein that cannot be phosphorylated at either of the two predicted PKA sites because of a replacement of the serine with alanine under the control of an upstream activating sequence (UAS). We generated these flies successfully and provided evidence that expressing such a mutated Synapsin transgene cannot rescue the deficit of the syn^{97CS} mutant in associative learning, thus assigning Synapsin as a downstream effector of the AC-cAMP-PKA cascade. Whether both or which of these two sites are necessary or whether further phosphorylation sites play also a role is under investigation at the moment, including the ADAR-dependent RNA editing at one of these sites (Diegelmann et al. 2006) as well.

On the cellular level, a Synapsin-dependent memory could be pinpointed to only a handful of cells using *D52H*-Gal4 (6- 12 cells) in the mushroom body. In any event, it would be interesting to generate single-cell Gal4 strains e.g. by MARCM (Lee and Luo 1999), or the recently published Brainbow technique (Hampel et al. 2011) out of *D52H*-Gal4 pattern, in order to perform learning experiments combined with physiology during memory acquisition and/ or memory retrieval.

Taken together this thesis provides different aspects of larval associative learning on the behavioural, cellular and molecular level. It allows to appreciate the content of olfactory memory in *Drosophila* larvae in respect of odour quality and intensity. Further on the cellular and molecular level, it investigated the role of Synapsin- a presynaptic phosphoprotein- in the larval odour-sugar associative learning.

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I.1. Describing the content of olfactory memory in *Drosophila* larvae: Odour quality

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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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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

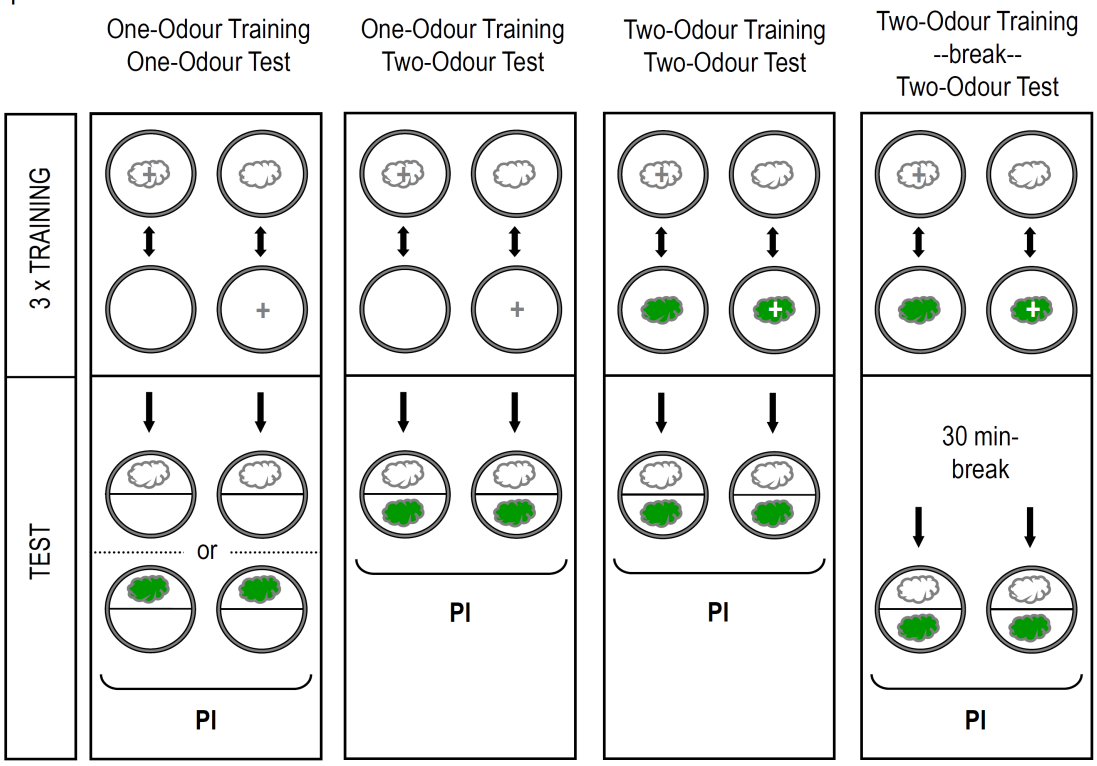


Figure 1 The rationale of the learning tasks

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

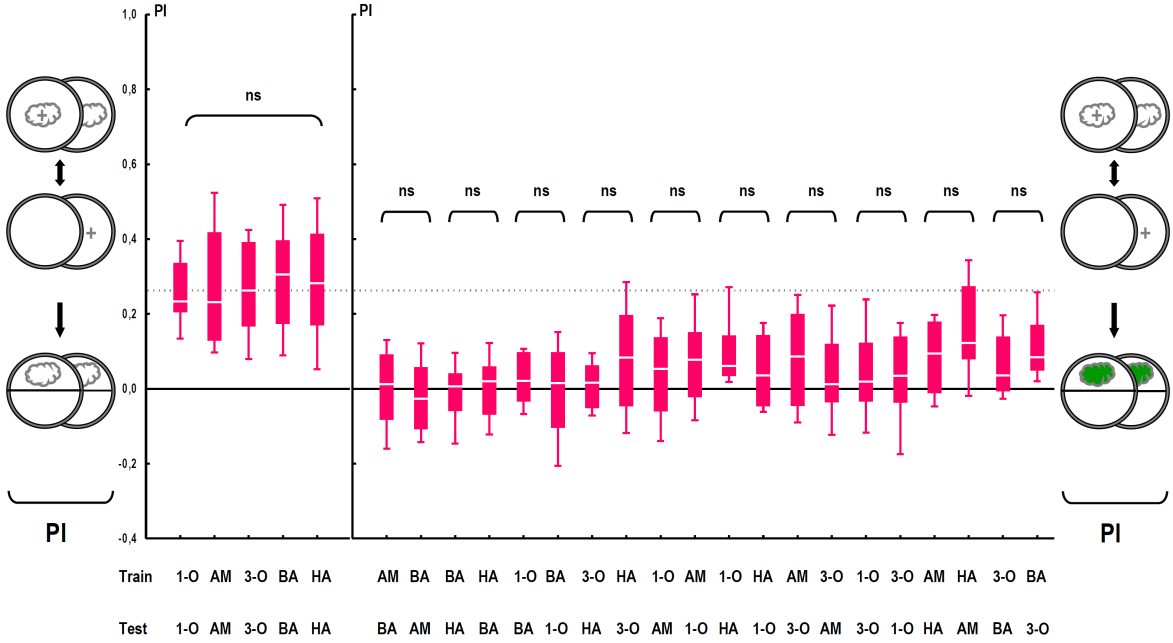


Figure 2 Symmetry of perceived distances

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:

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

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:

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

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

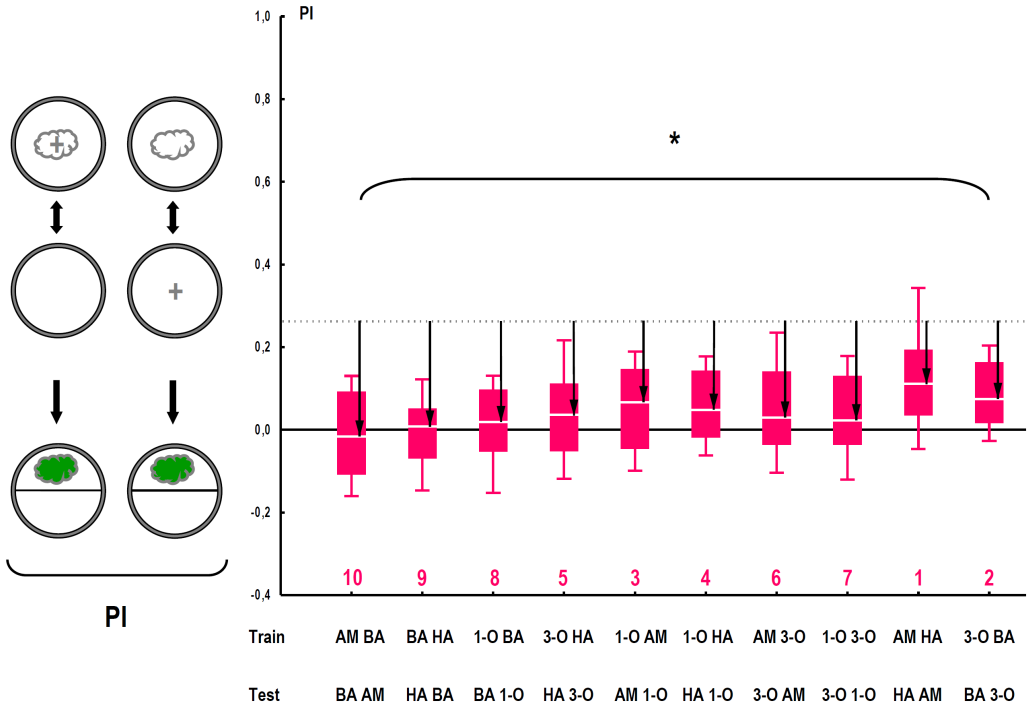


Figure 3B

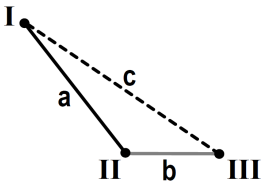


Figure 3C

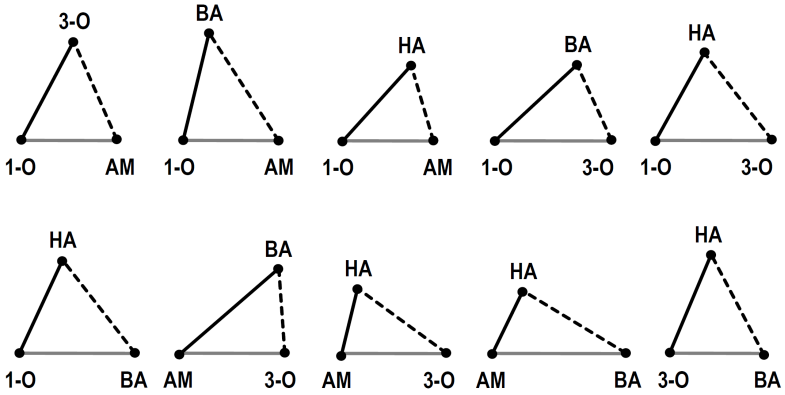


Figure 3 *Odour pairs differ in perceived distances in an one-odour training, one-odour test task*

(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

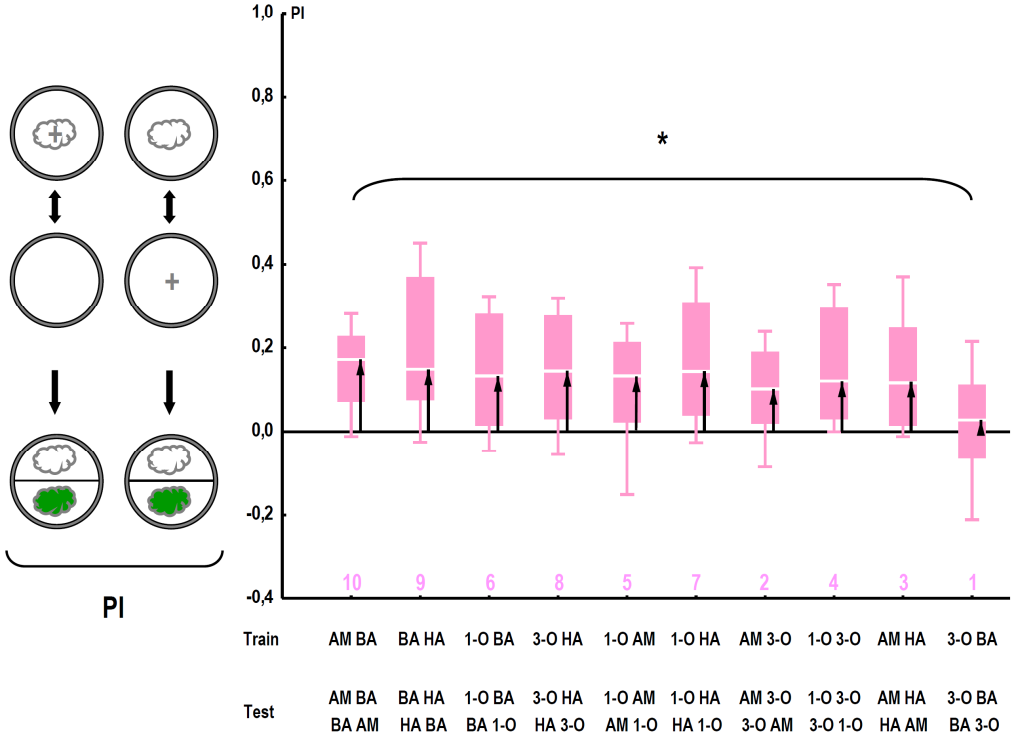


Figure 4 Odour pairs differ in perceived distances in an one-odour training, two-odour test task

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

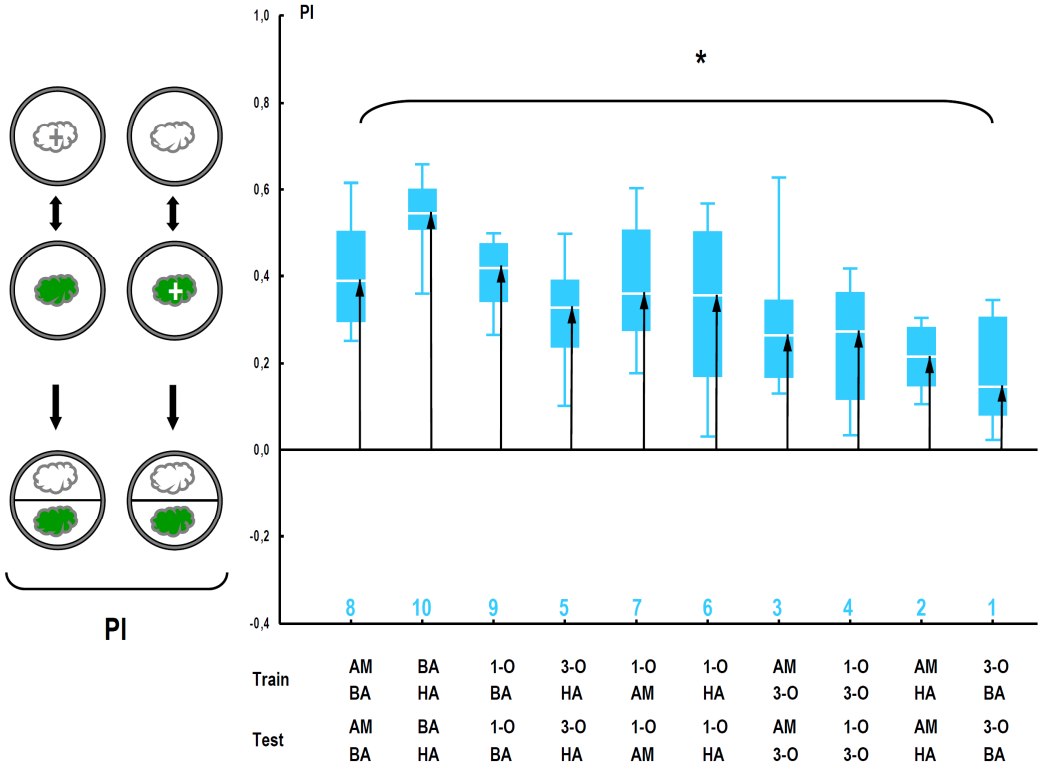


Figure 5 Odour pairs differ in perceived distances in a two-odour training, two-odour test task

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

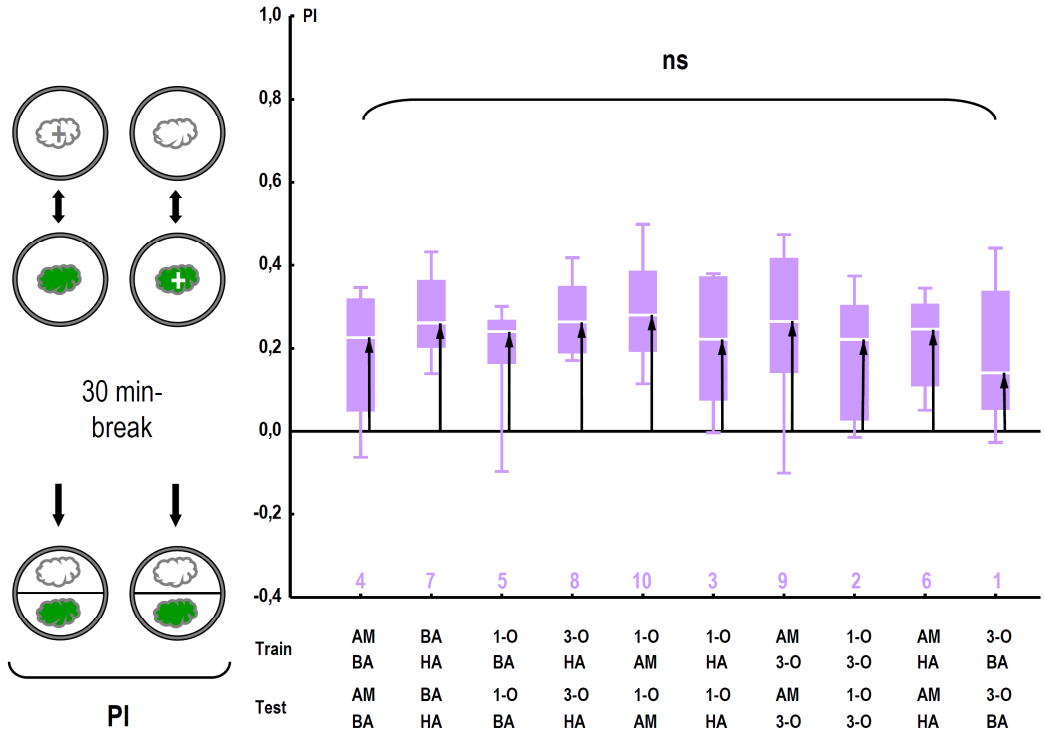


Figure 6 Perceived distances after a 30-min retention period

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

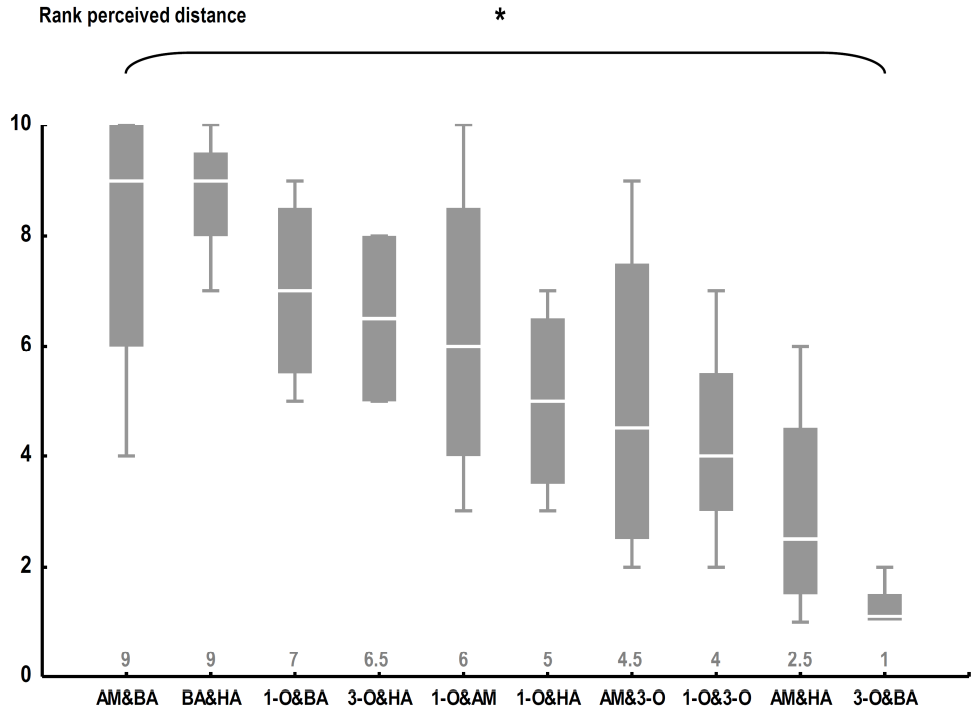


Figure 7 Estimating task-independent perceptual distances among odours

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.

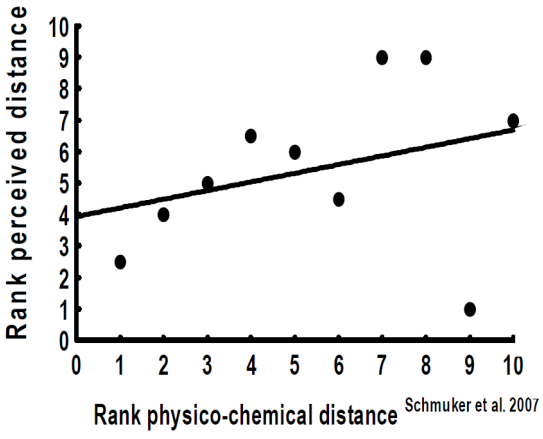
Table 2

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

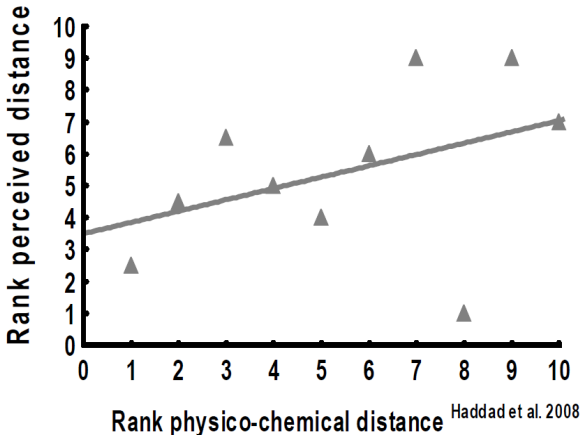
Table 2 Physico-chemical distances between odours

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 8A



B



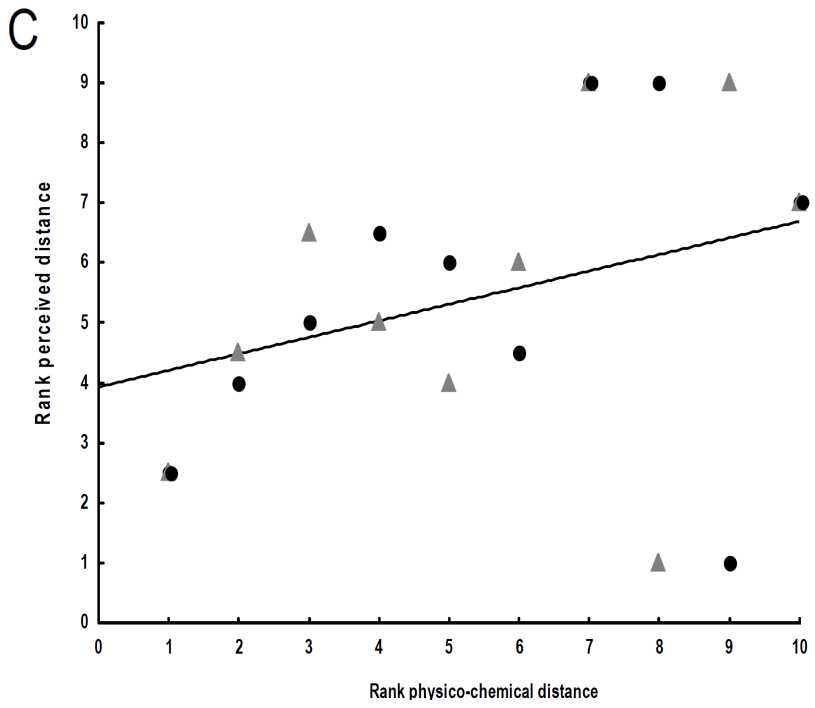


Figure 8 Comparing perceived distances to physico-chemical distances

(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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I.2. Describing the content of olfactory memory in *Drosophila* larvae: Odour intensity

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.

Fig: 1

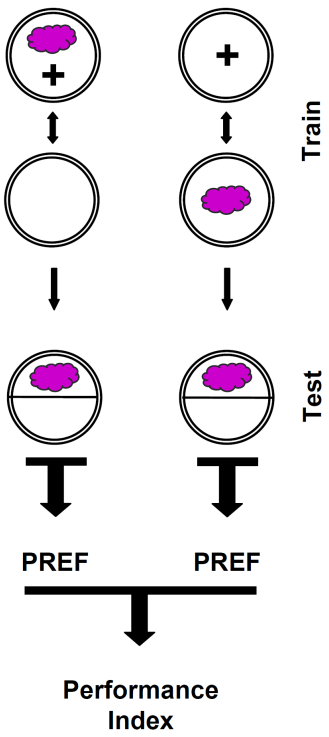


Figure 1

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

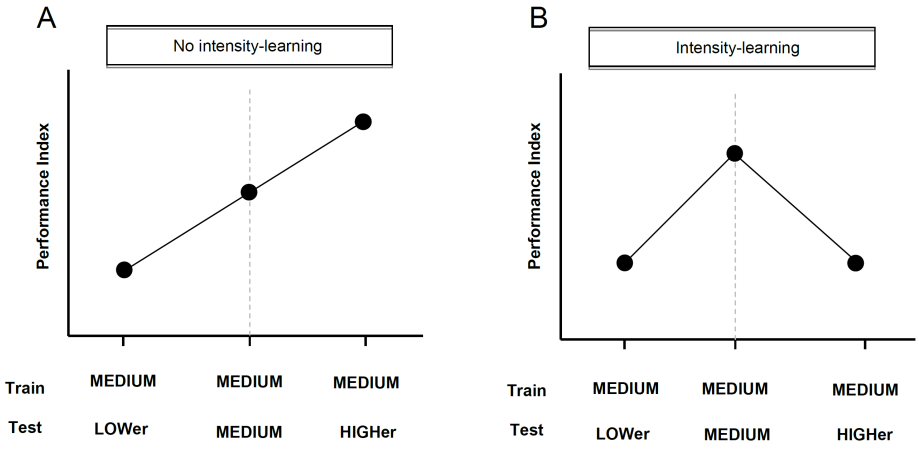


Figure 2

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)

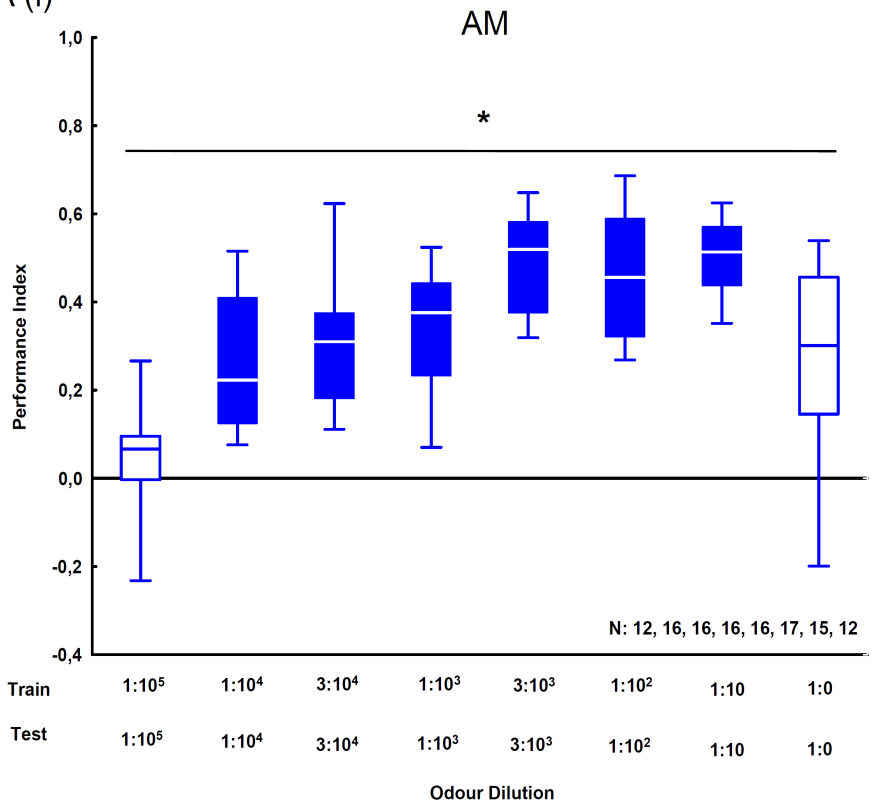


Fig: 3A (ii)

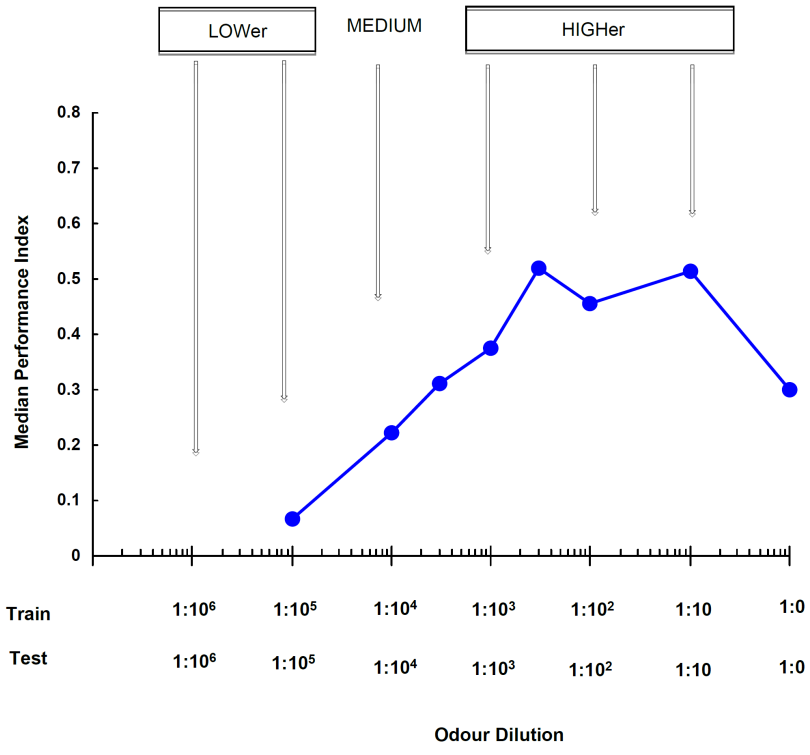
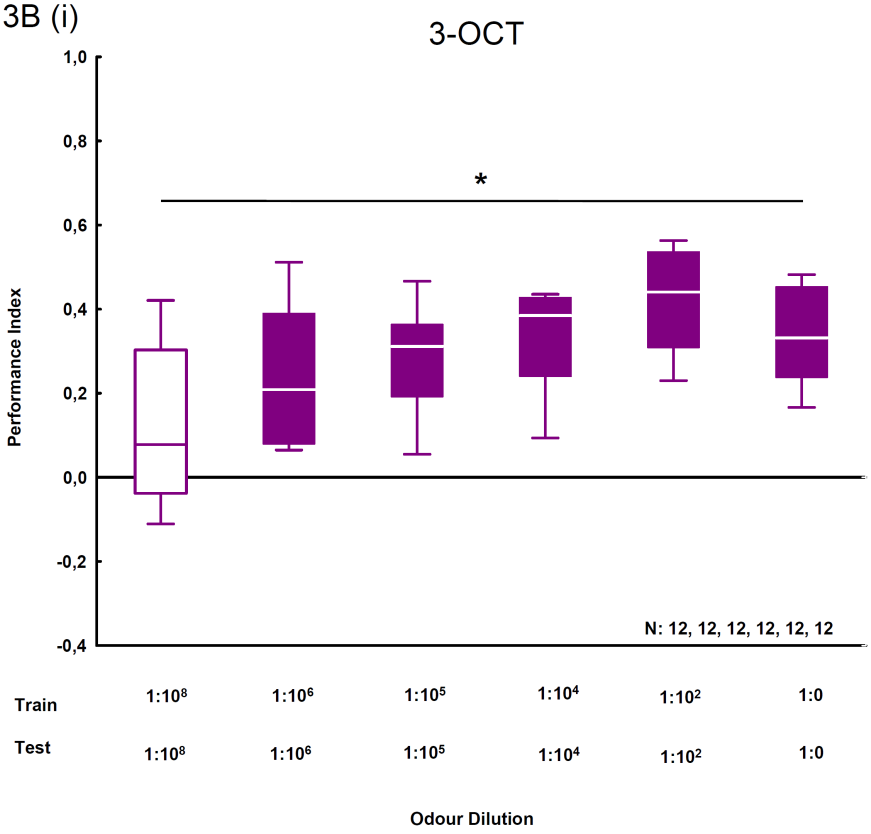


Fig: 3B (i)



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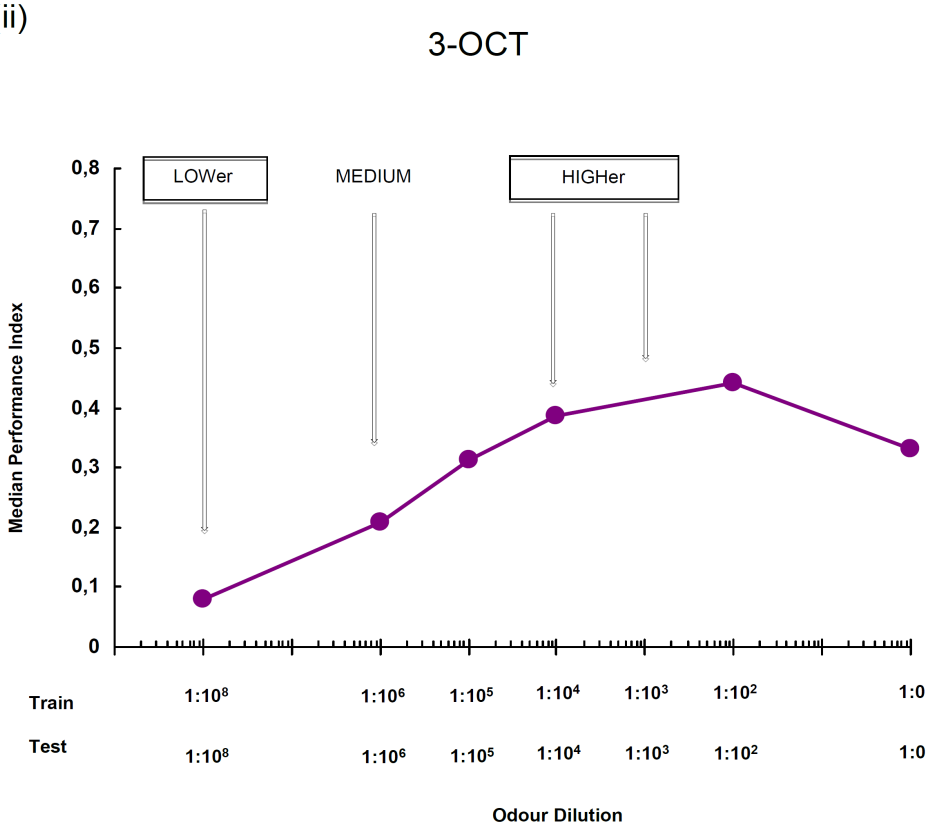

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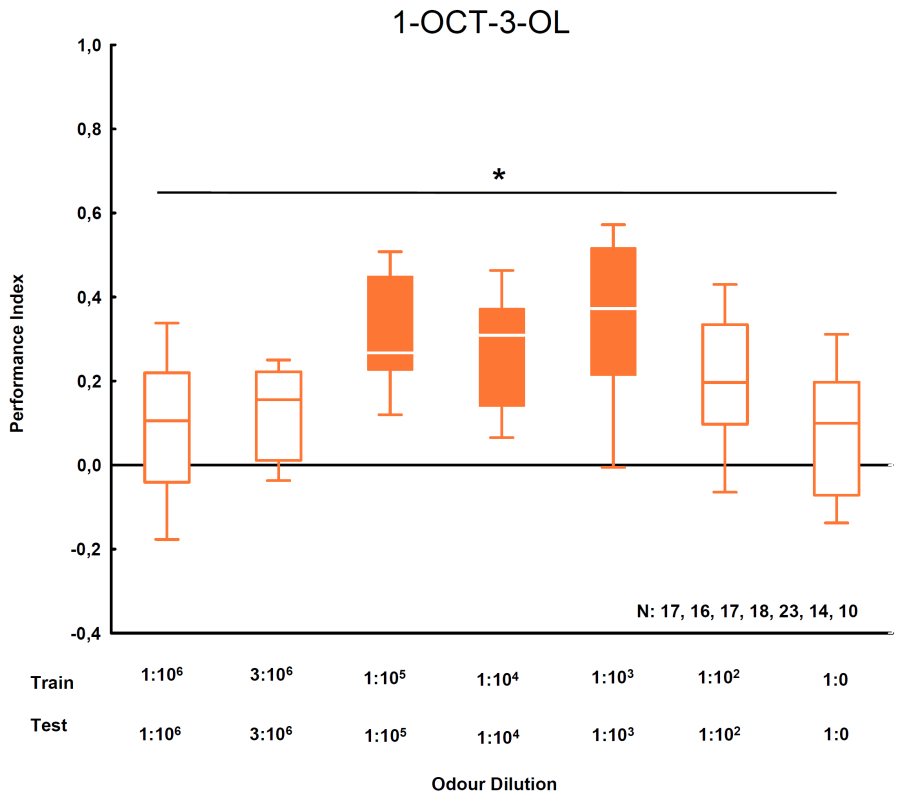

Fig: 3C (ii)

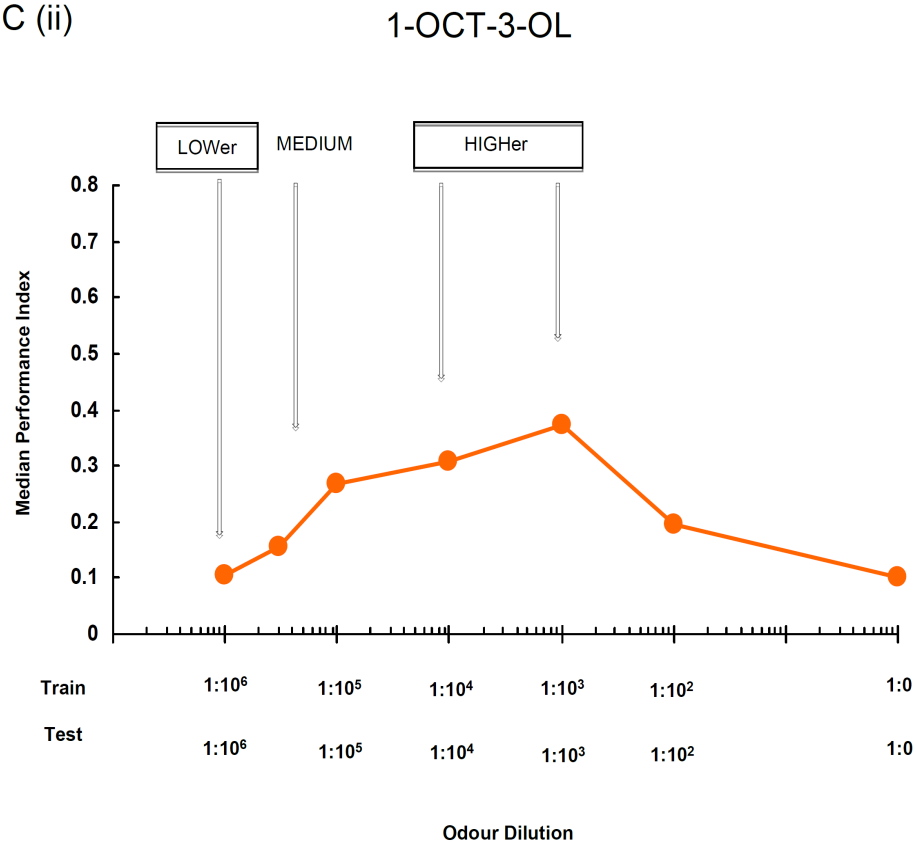

Fig: 3D (i)

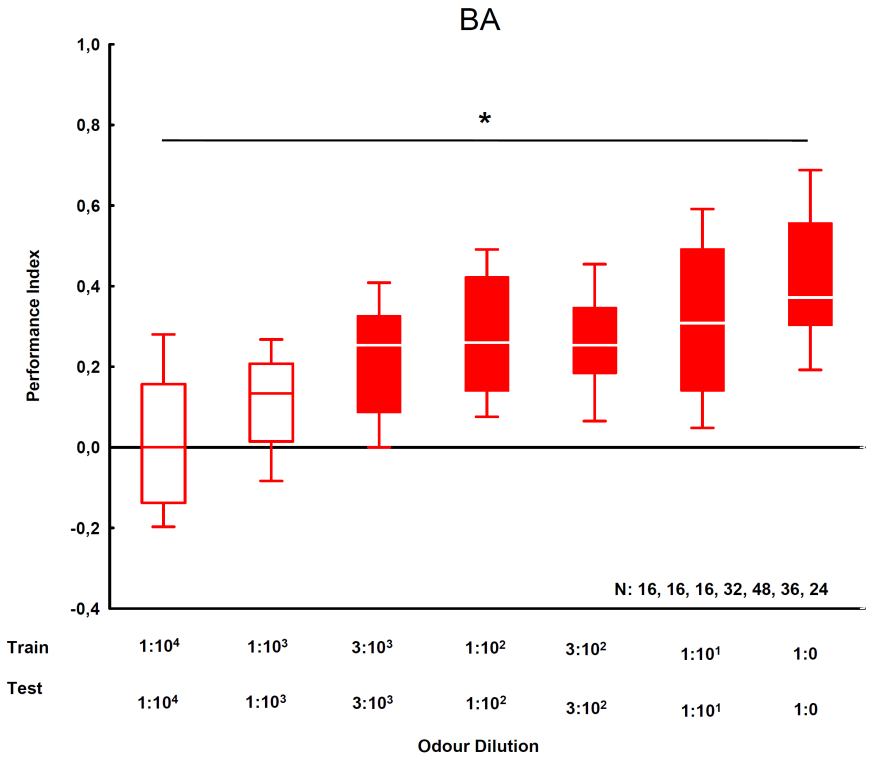

Fig: 3D (ii)

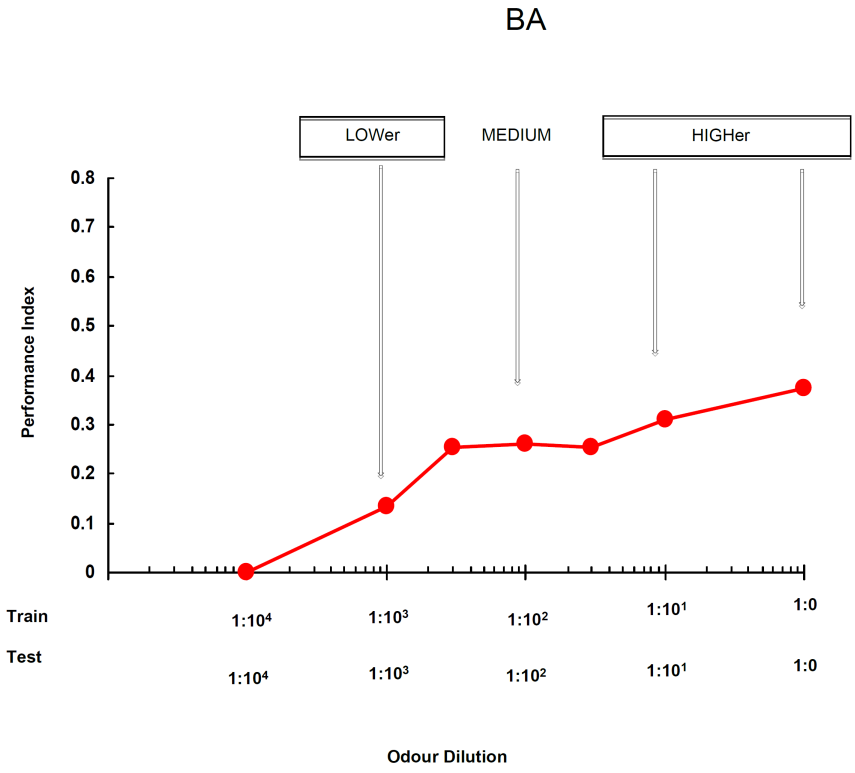

Figure 3

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

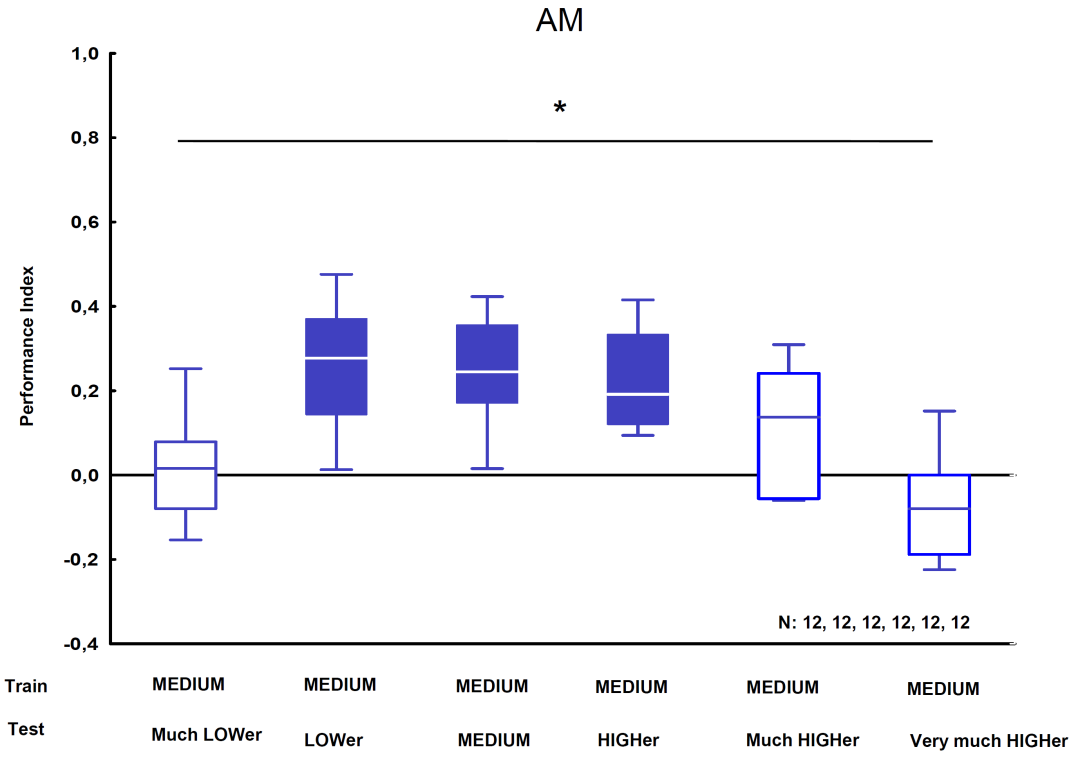

Fig: 4B

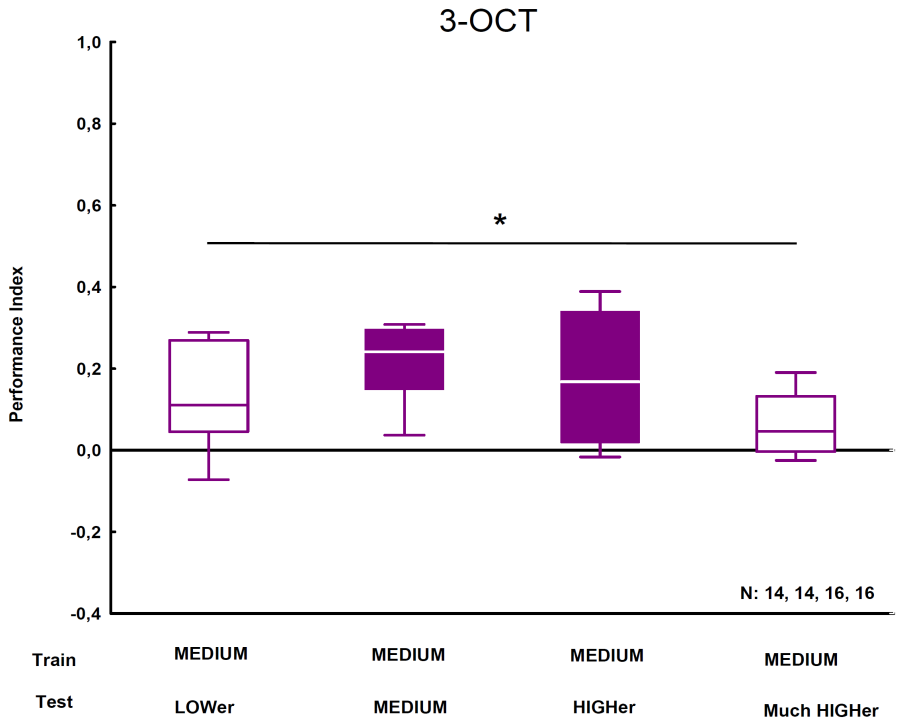

Fig: 4C

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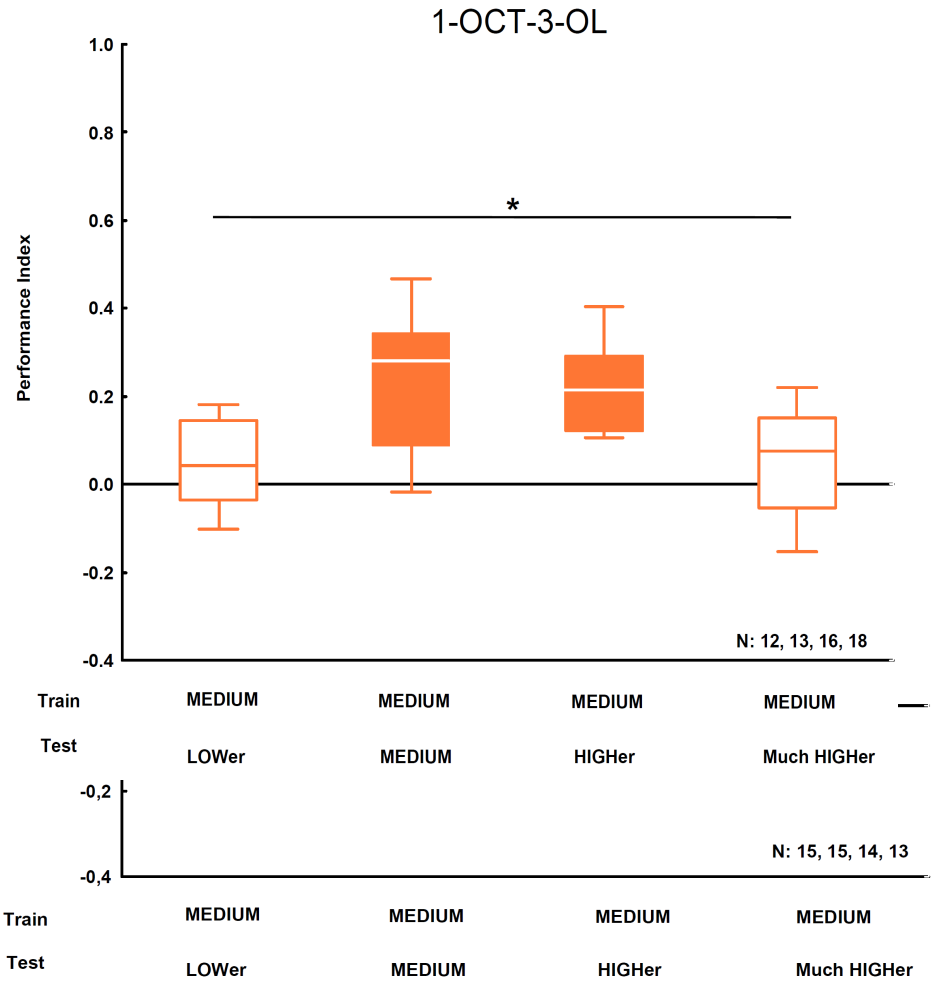


Fig: 4D (ii)

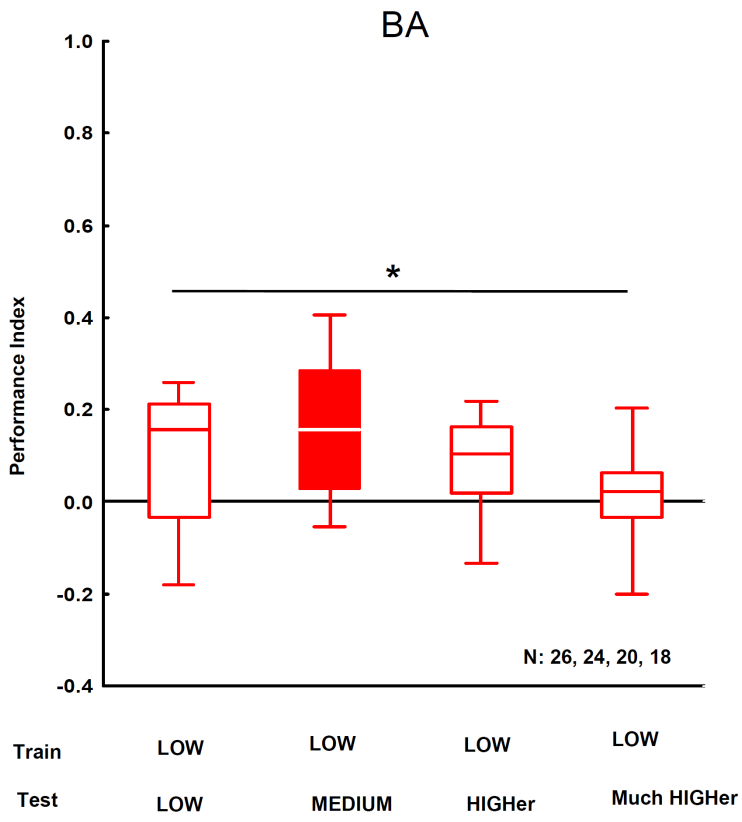


Fig: 4E

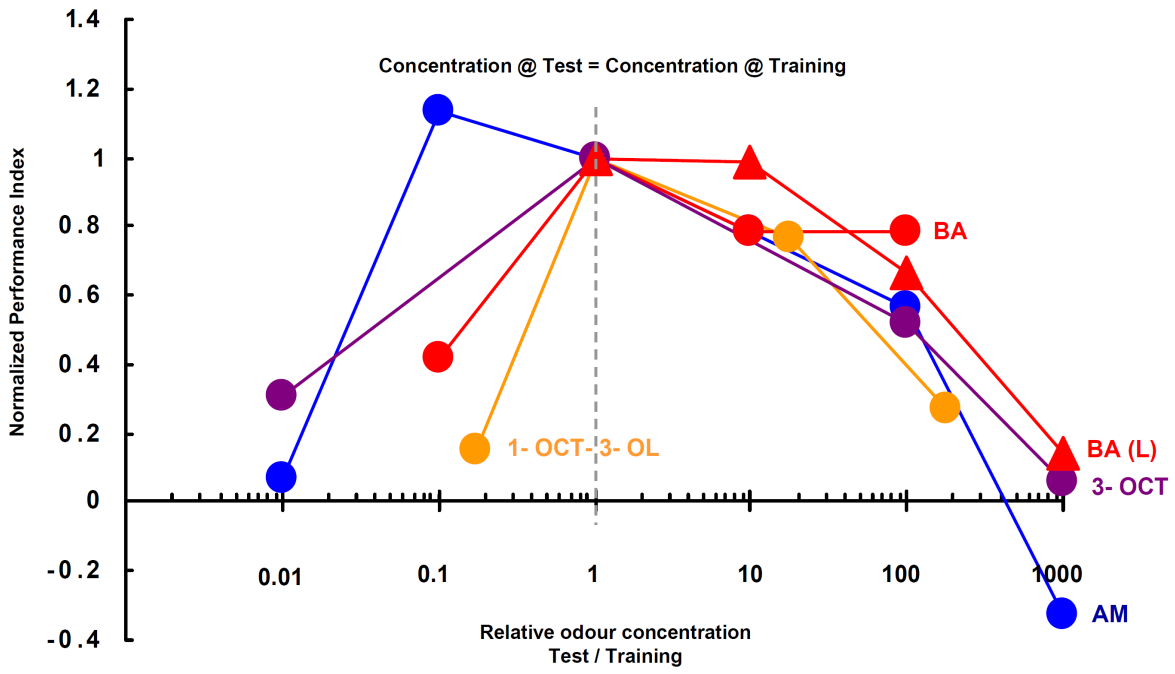


Figure 4

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

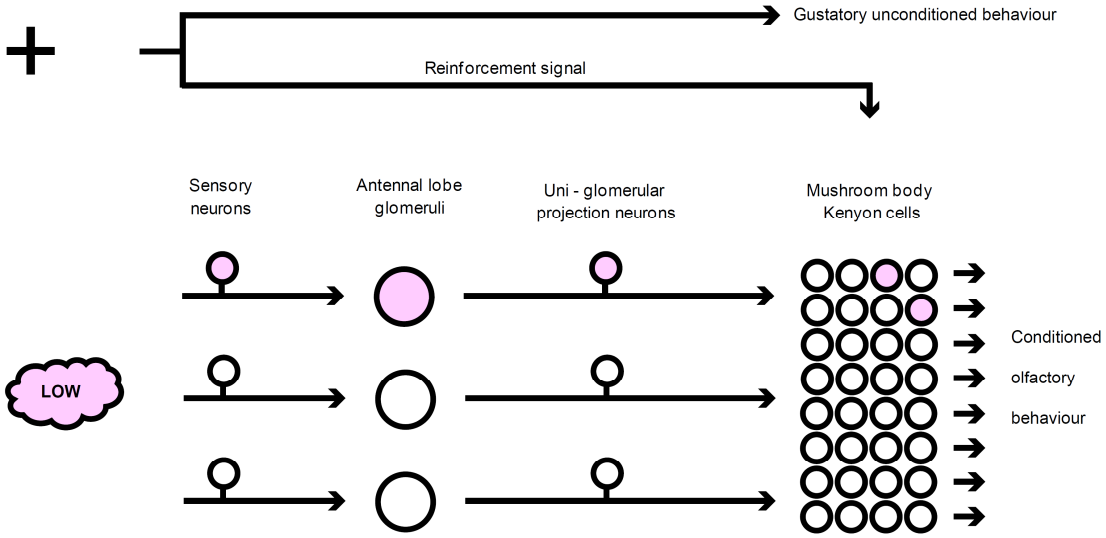
activity pattern evoked by a low intensity of an odour is not always nested within that evoked by a higher intensity of the same odour (e.g. for ethyl acetate, see Wang et al, 2004, loc. cit. Fig. 3). It remains unclear what kind of a connectivity scheme could transform nested representations at the 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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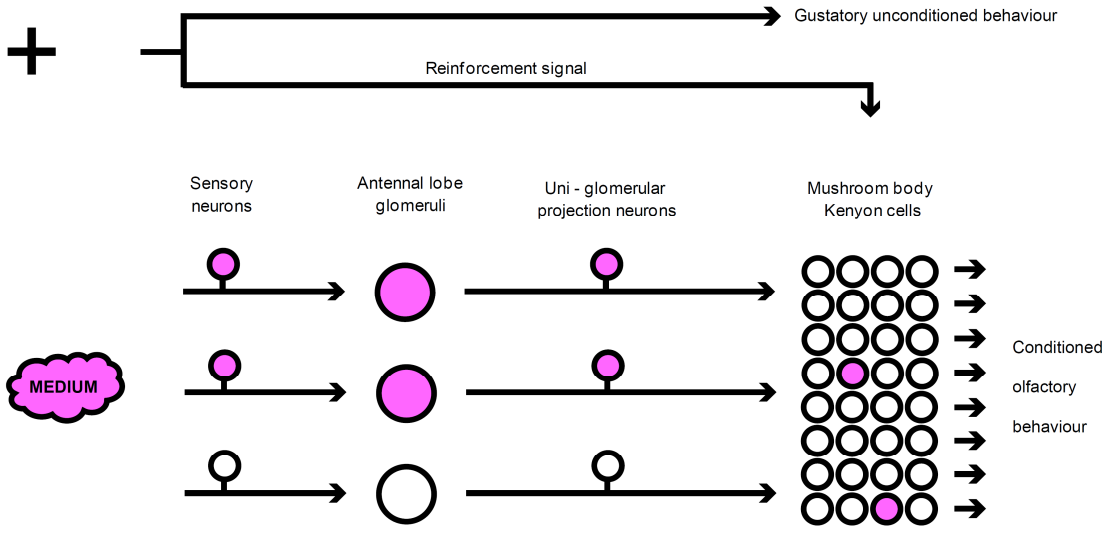
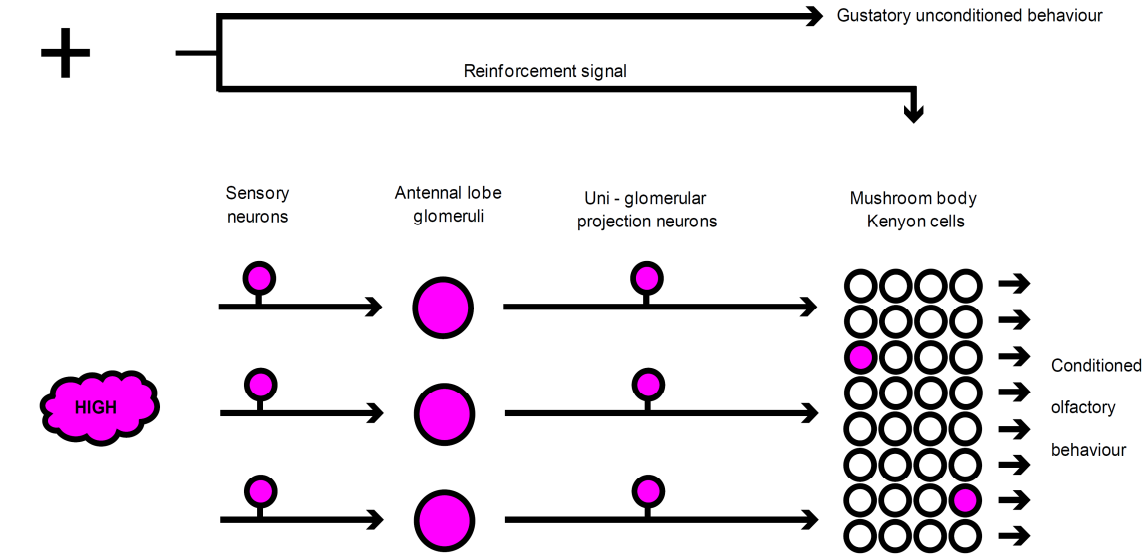


Fig: 5A (iii)



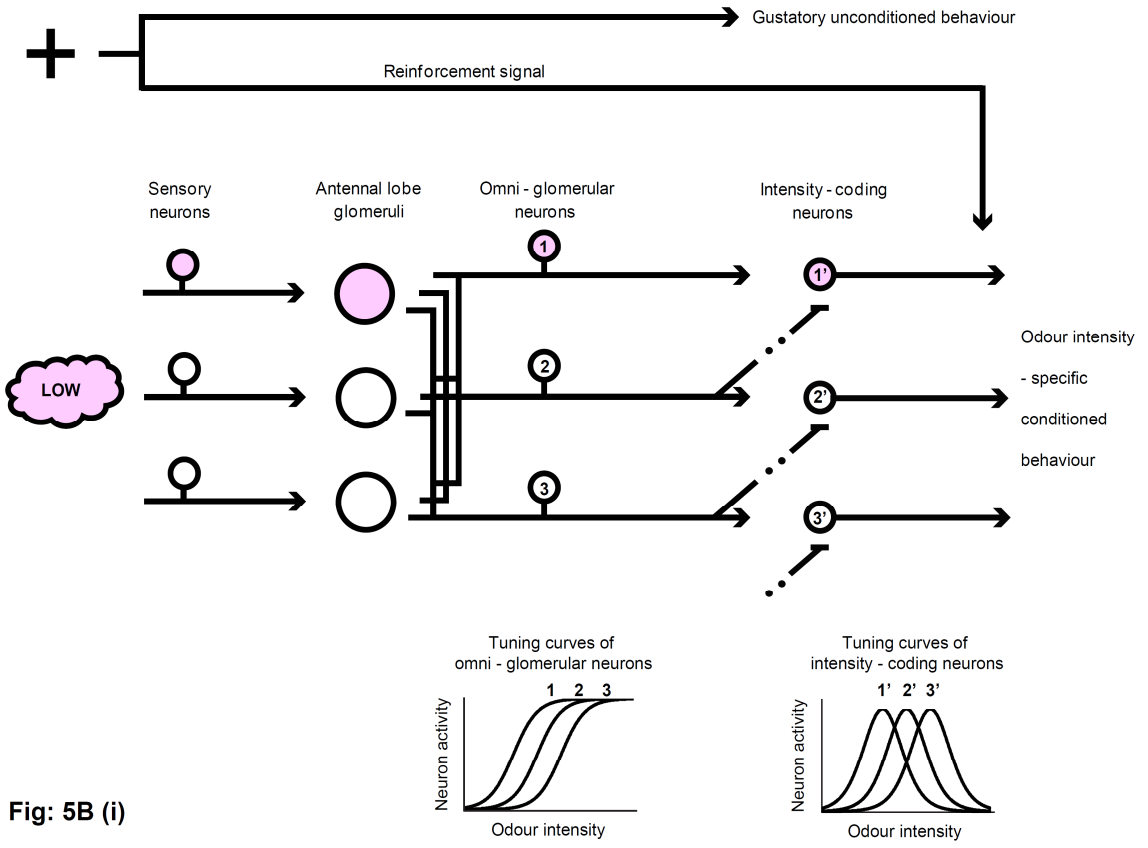


Fig: 5B (i)

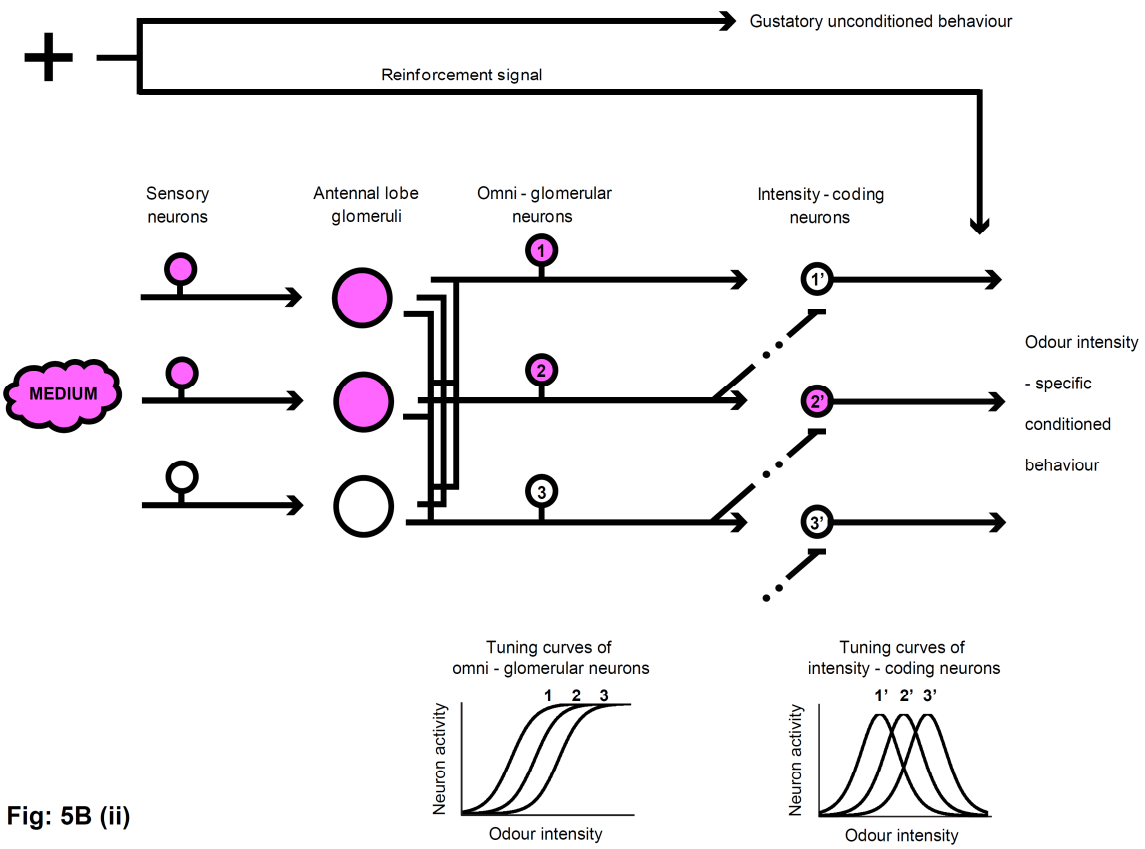


Fig: 5B (ii)

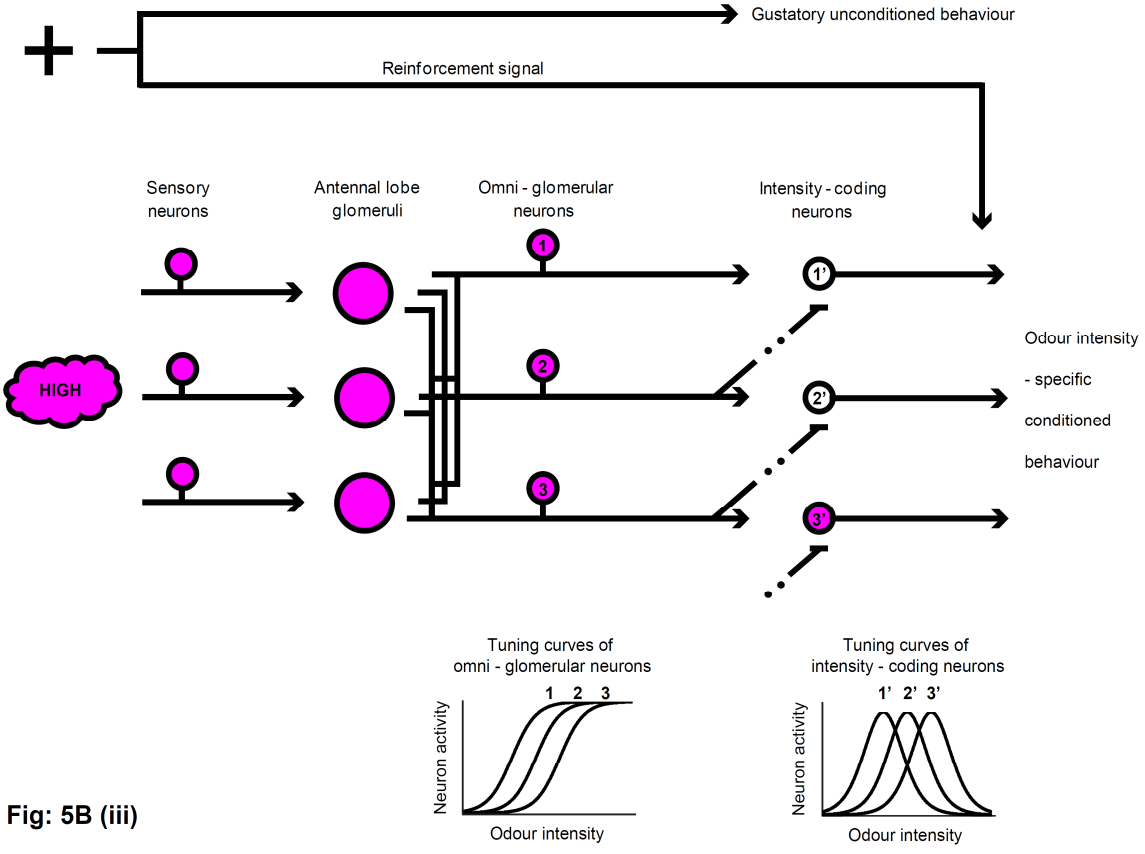


Fig: 5B (iii)

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.

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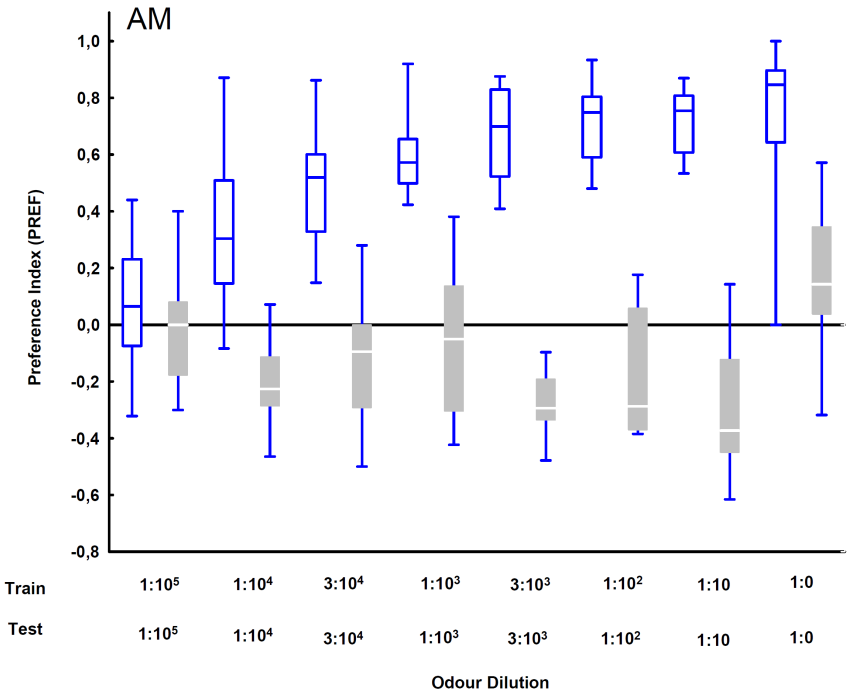
Supplementary material

Fig: S1A



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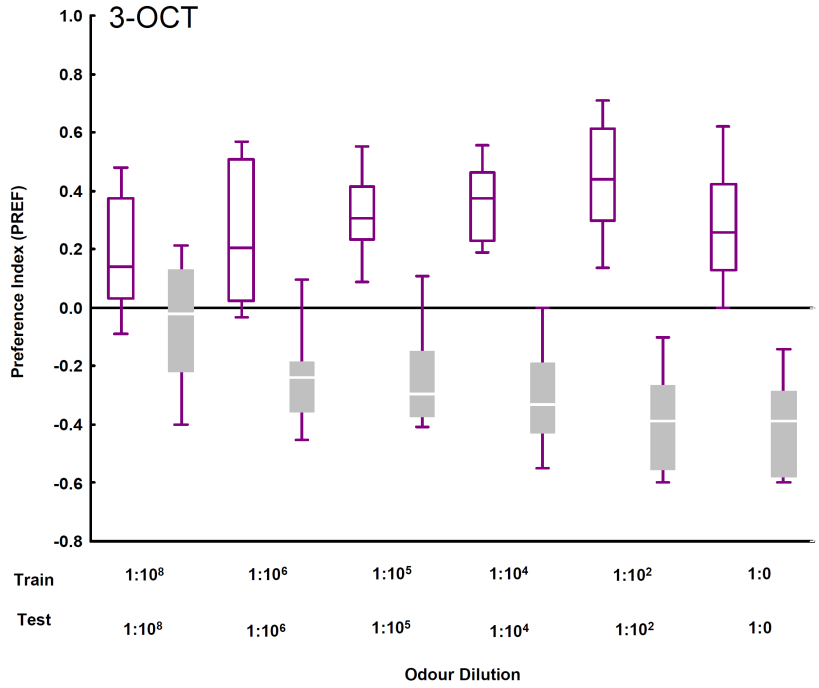

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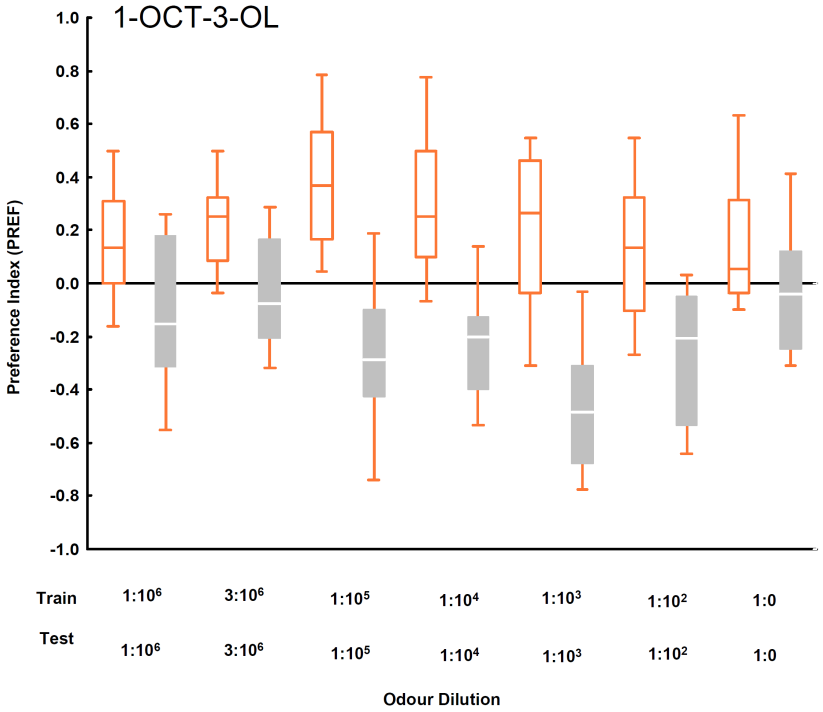

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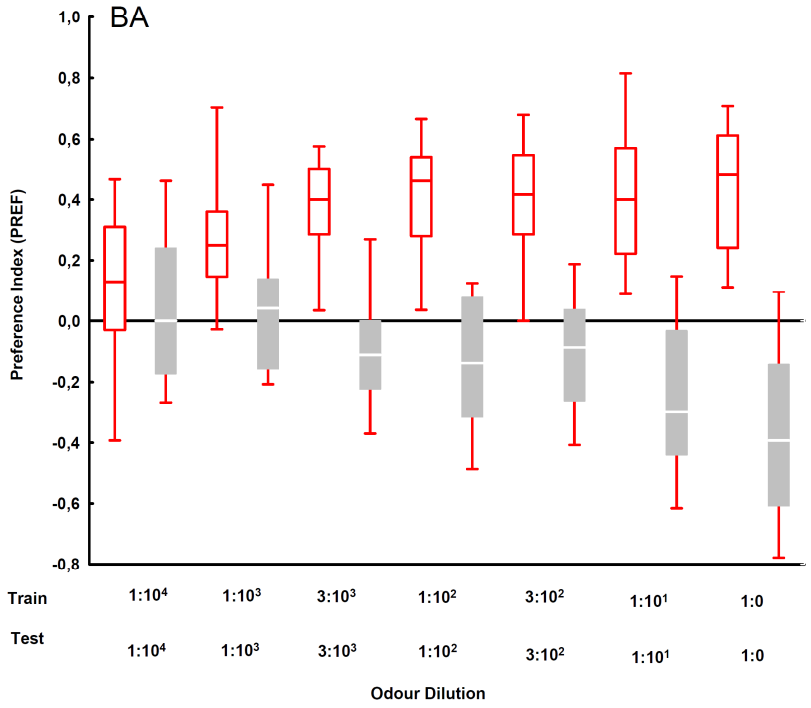

Figure S1

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.

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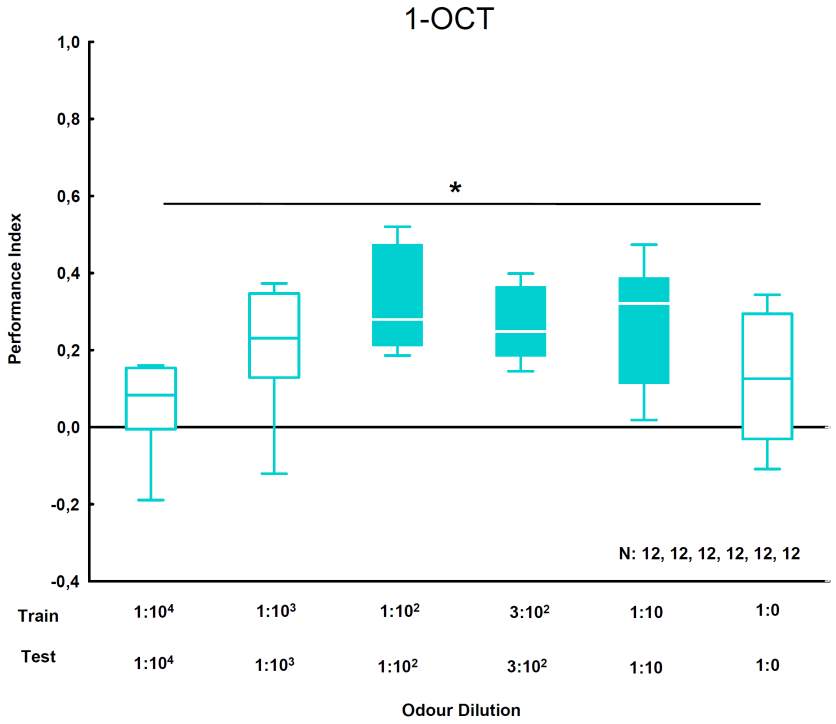

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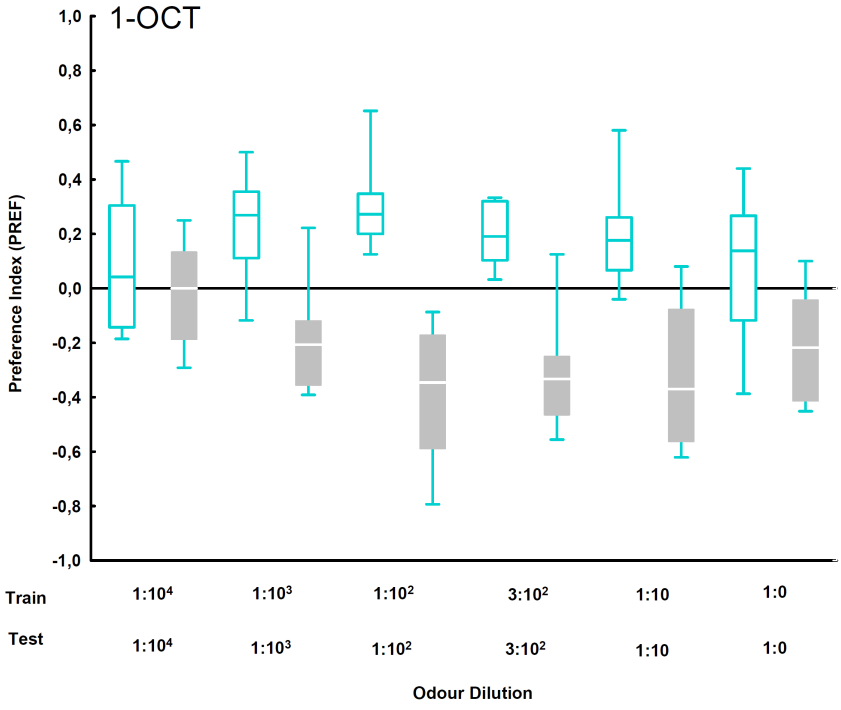

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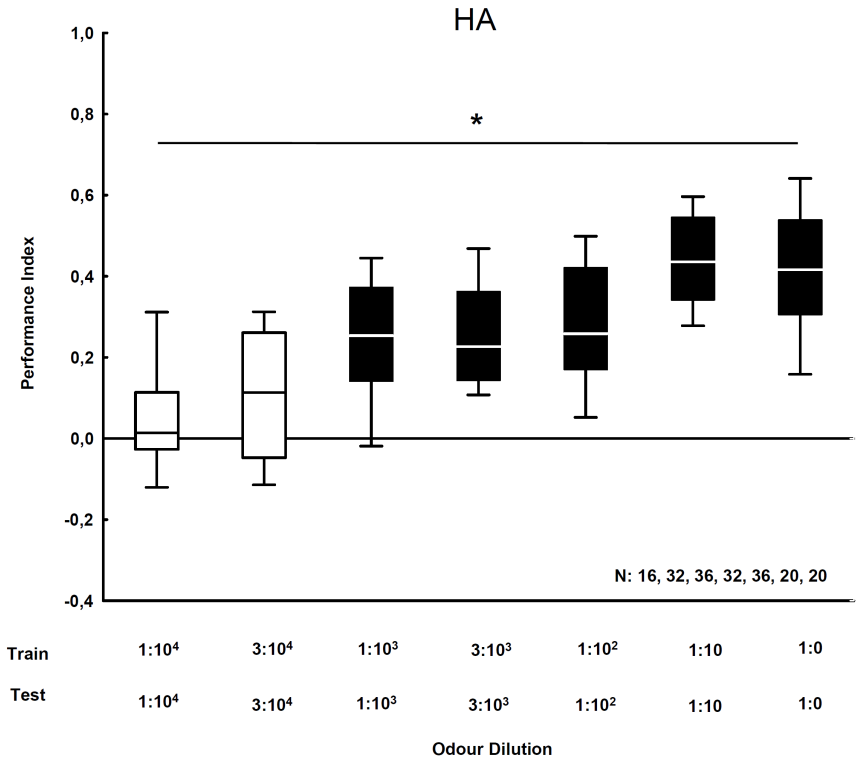

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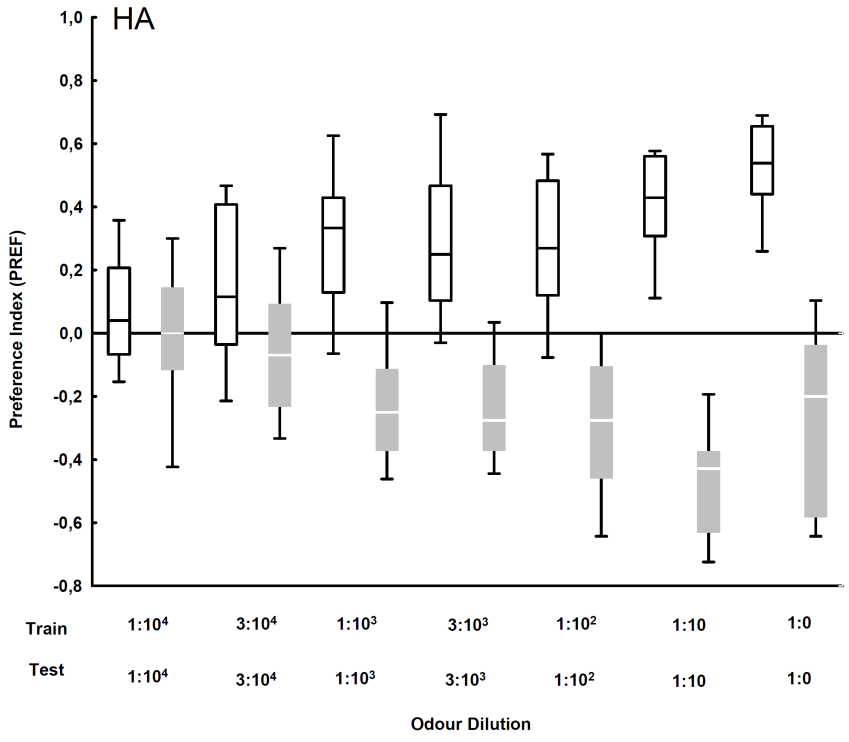

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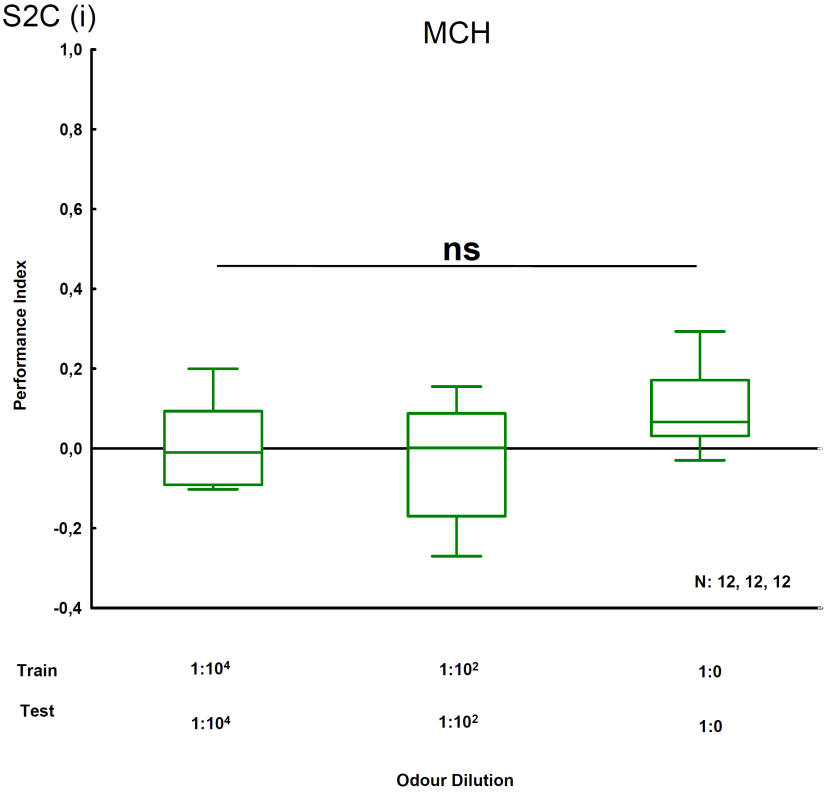

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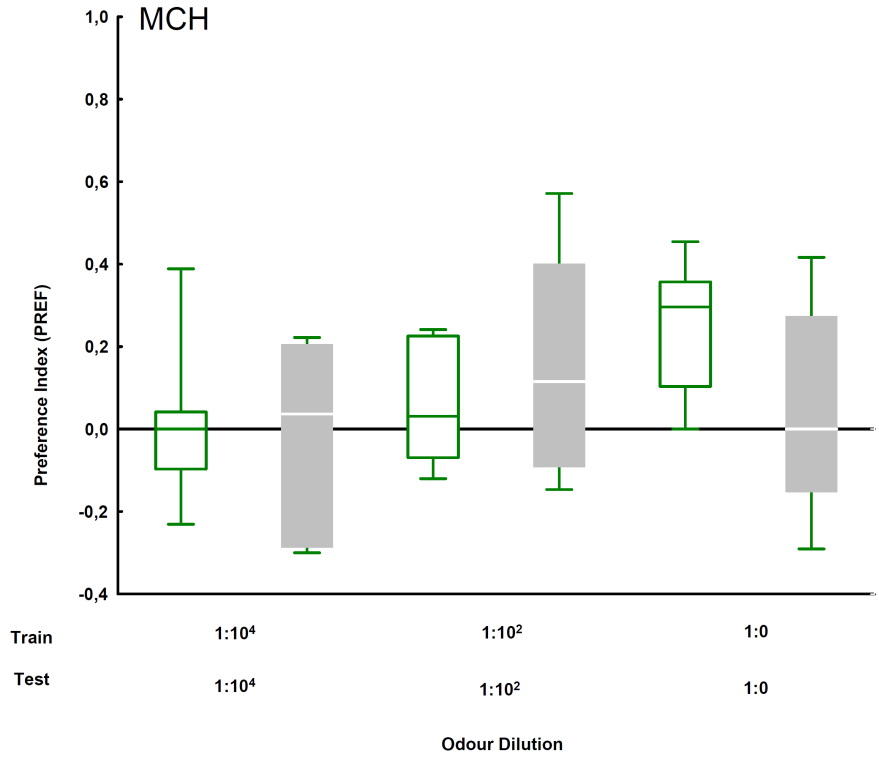

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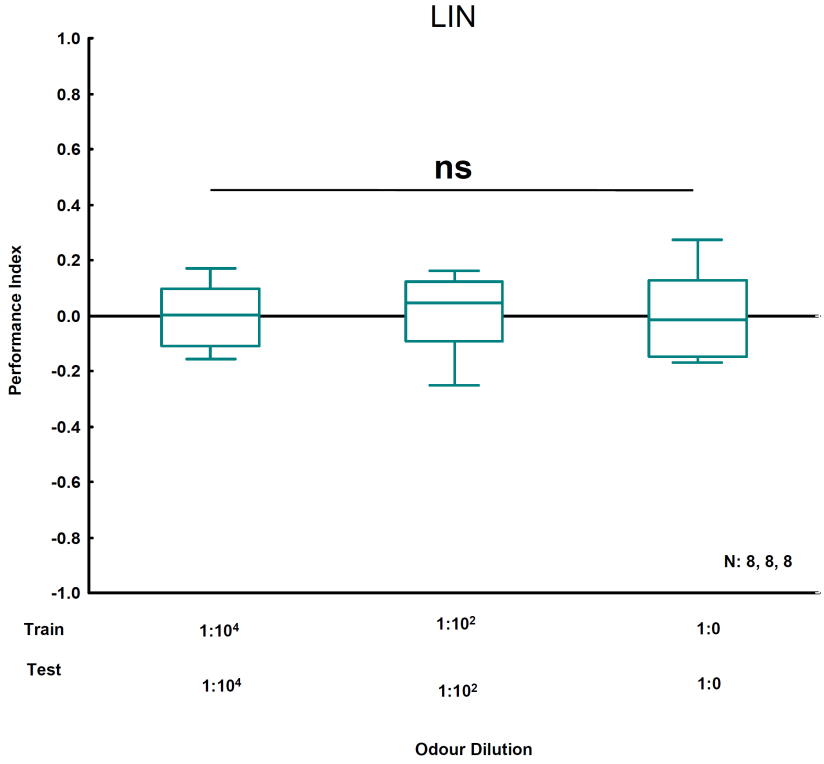

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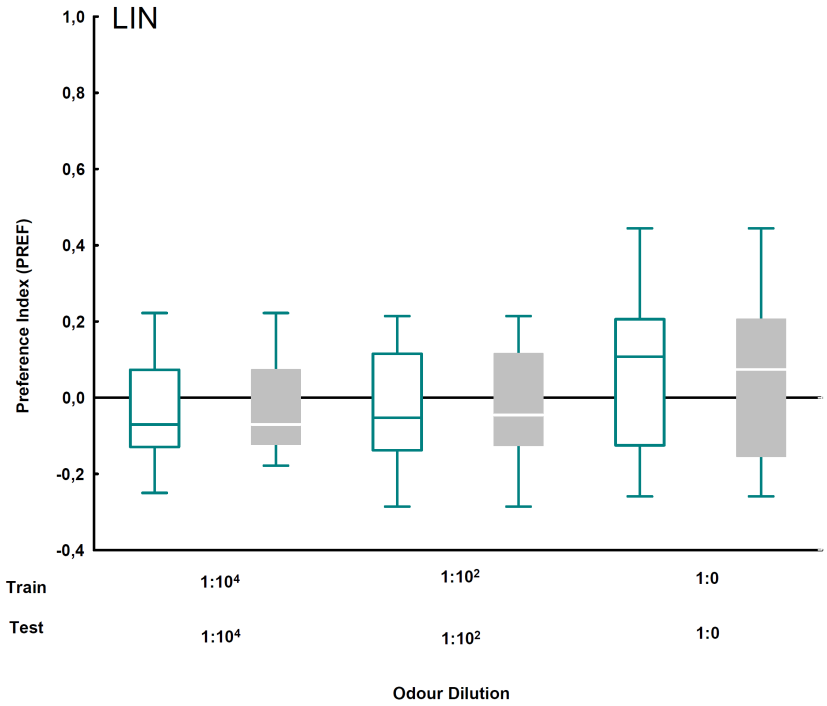

Figure S2

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)

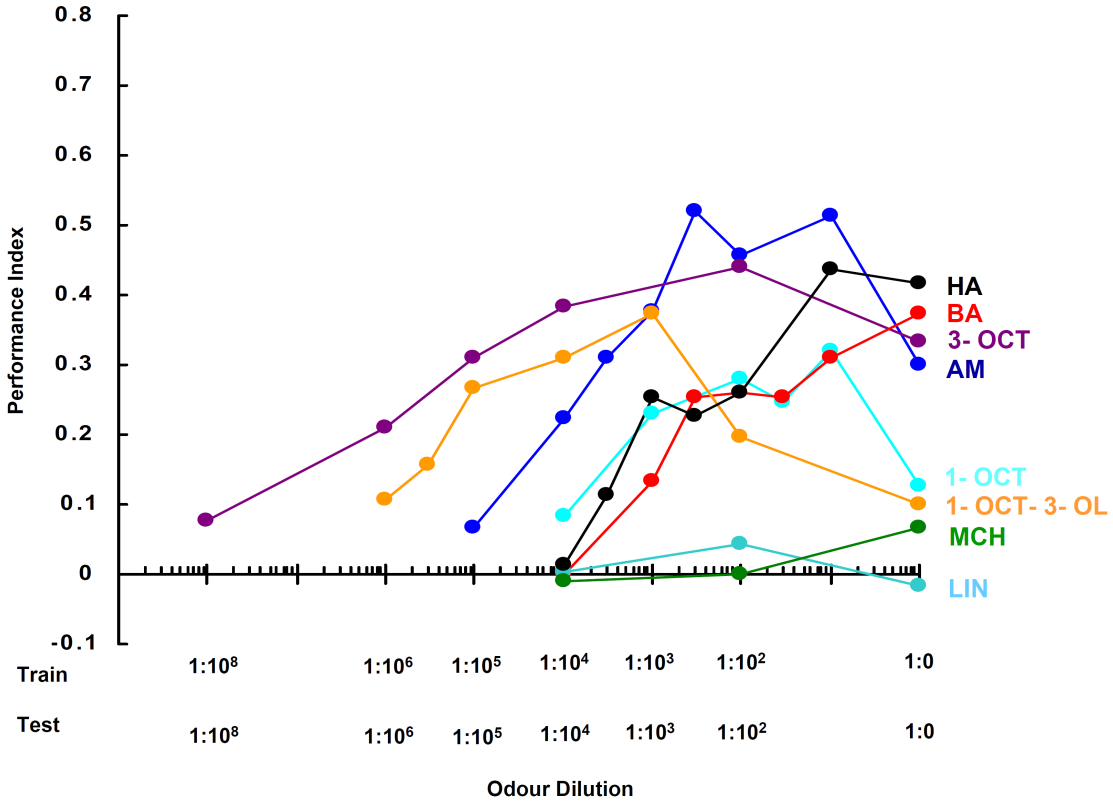
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
Performance Index
HA
BA
3- OCT
AM
1- OCT
1- OCT- 3- OL
MCH
LIN
Train
Test
1:10^8
1:10^6
1:10^5
1:10^4
1:10^3
1:10^2
1:0
Odour Dilution

Fig: S3 (B)

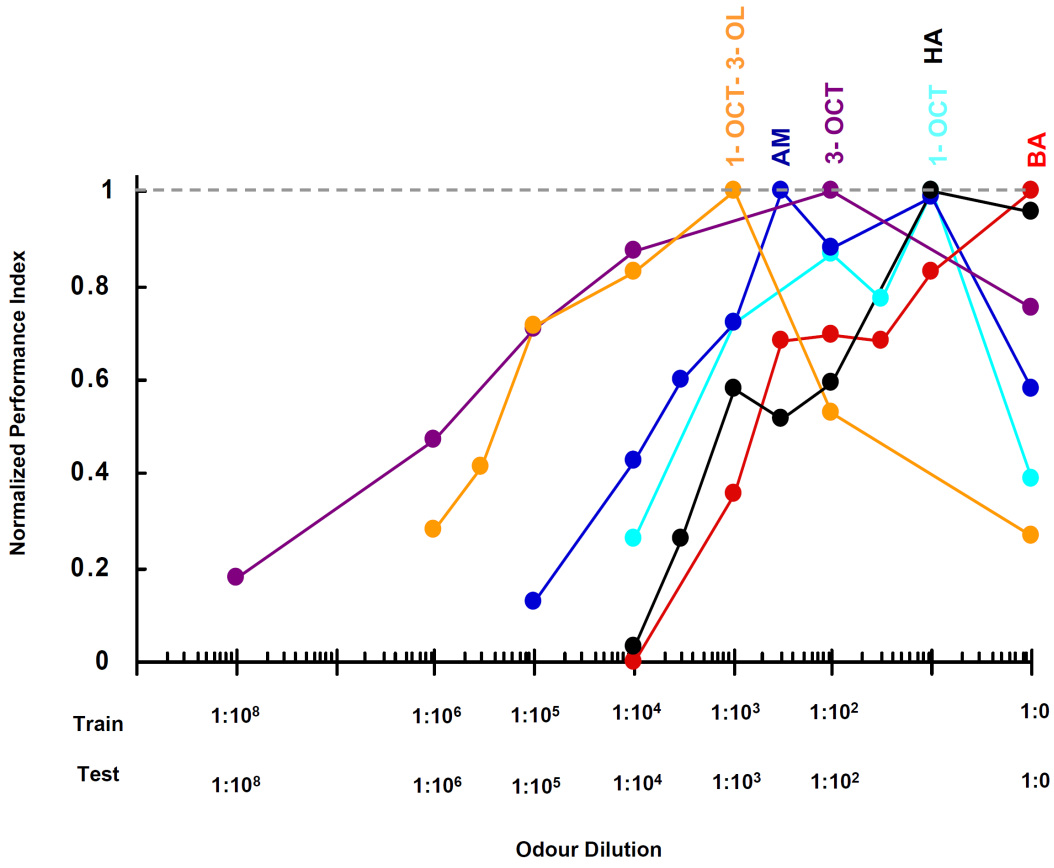

Figure S3

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.

II.1. 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

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Cellular site and molecular mode of synapsin action in associative learning

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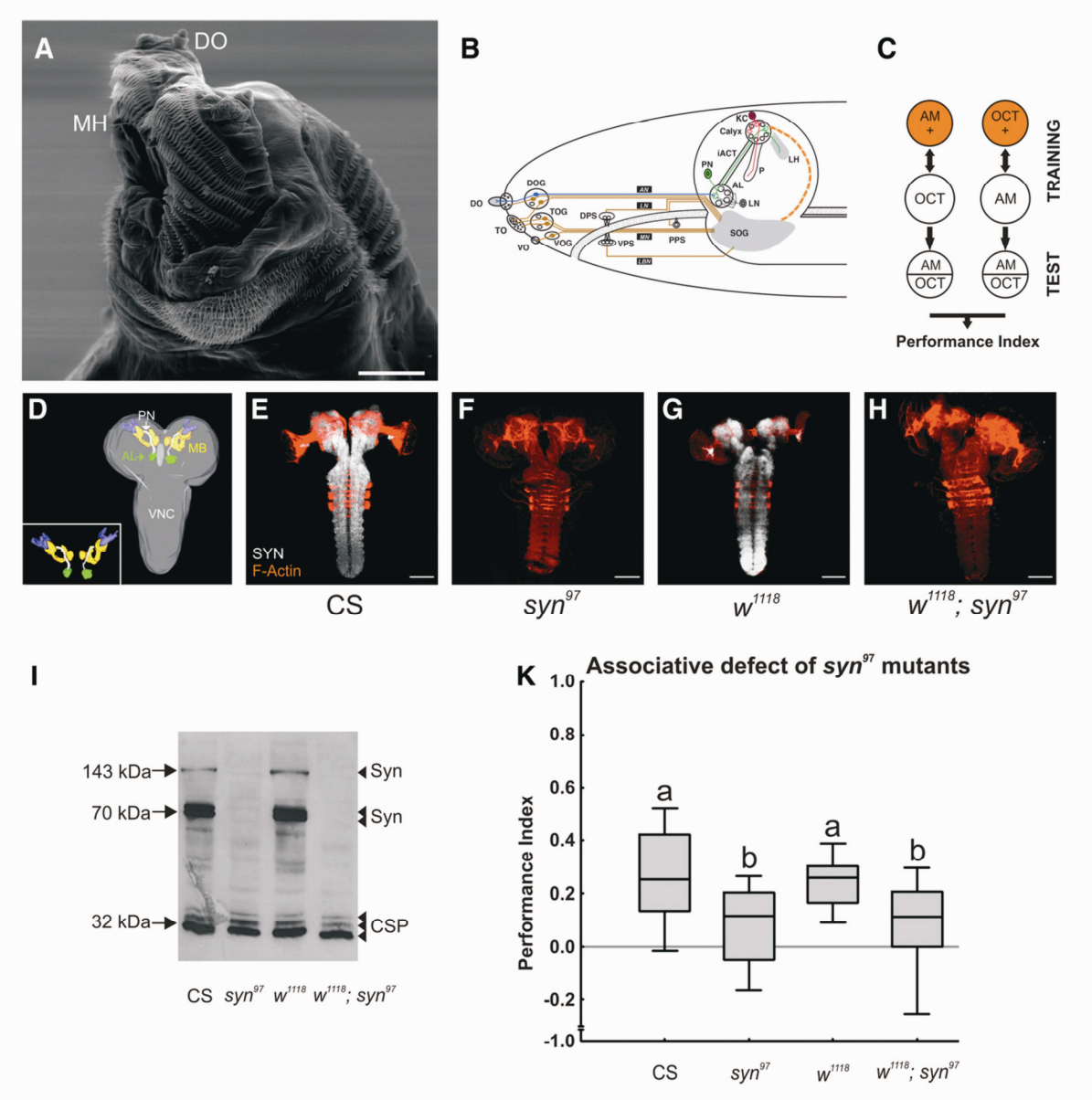
Abstract

Synapsin is an evolutionarily conserved, presynaptic vesicular phosphoprotein. Here, we ask where and how synapsin functions in associative behavioral plasticity. Upon loss or reduction of synapsin in a deletion mutant or via RNAi, respectively, *Drosophila* larvae are impaired in odor-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 behaviorally 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 behavioral 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 odor-food associative memory traces are established, and what their molecular nature is.



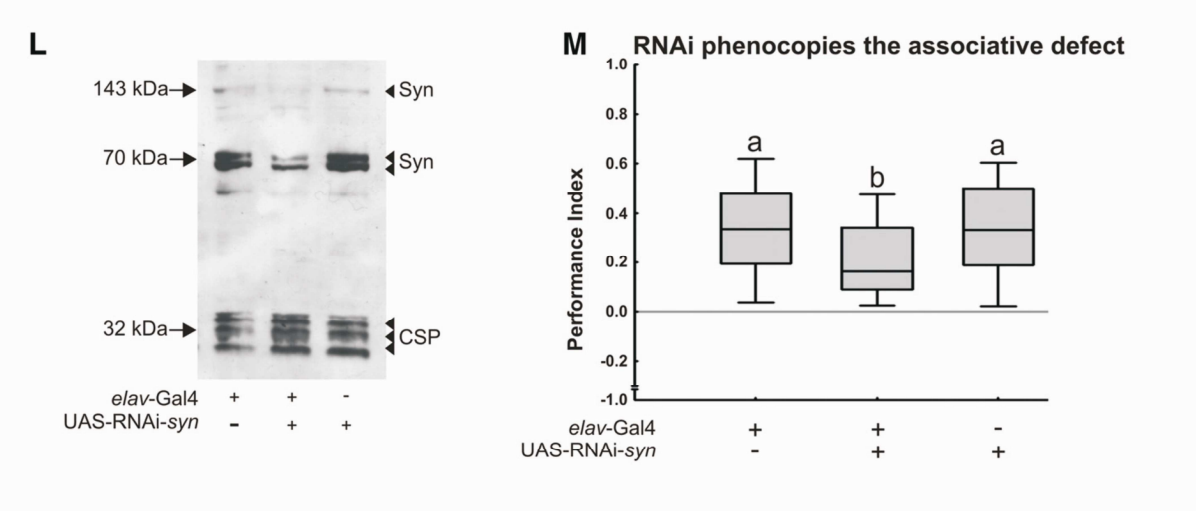


Figure 1:

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 Stocker 2008, with permission from Landes Bioscience and Springer Science+Business Media © 2008).

C The odor–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 then tested for choice between AM vs. 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 MB, PN, and AL (see also Supplemental 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 toward the mushroom bodies; the pharynx is shown stippled; VNC, ventral nerve cord. Scale bars: 50 µm.

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 in 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, odors 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 odor-sugar reward memory (Michels et al., 2005) (adult odor-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.

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 odor-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 odor-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.

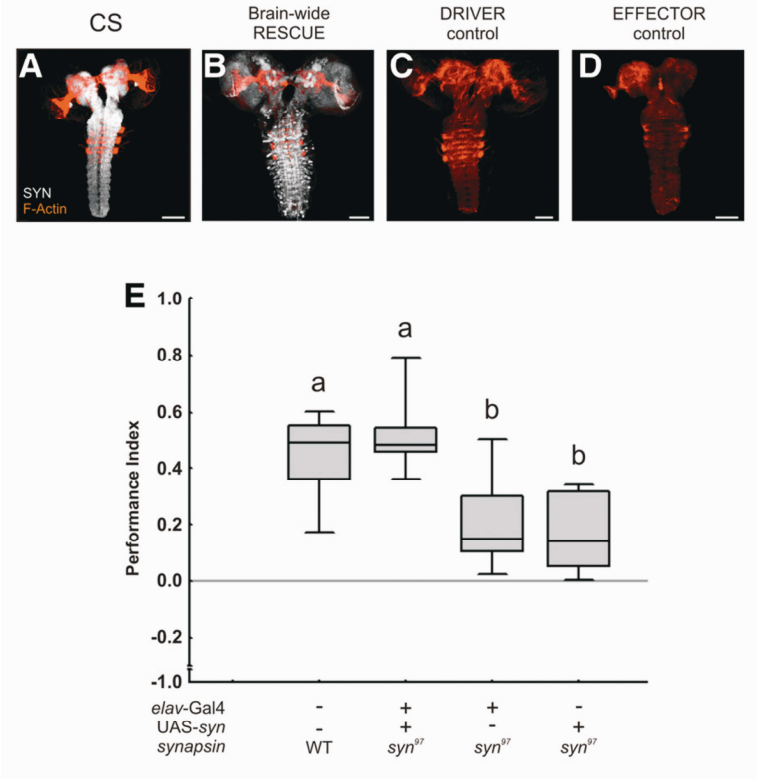
Brain-wide rescue

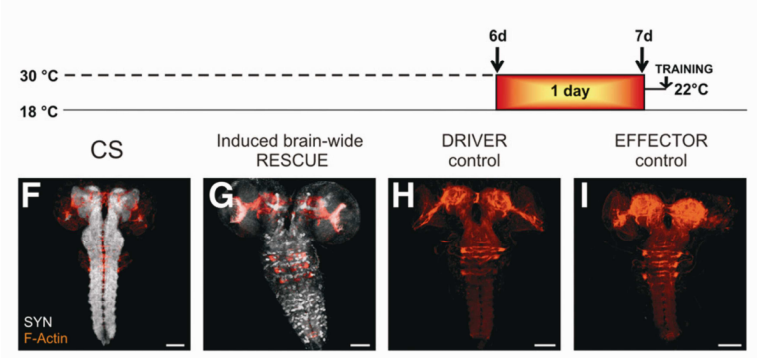
In brain-wide RESCUE larvae, synapsin expression is restored throughout the brain (Fig. 2B; S1B-D; Movie S2). Comparing performance scores between genotypes shows a difference in associative ability (Fig. 2E; KW: P< 0.05; H= 19.03; df= 3; N= 9, 7, 7, 10). Specifically, the brain-wide RESCUE larvae perform 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.





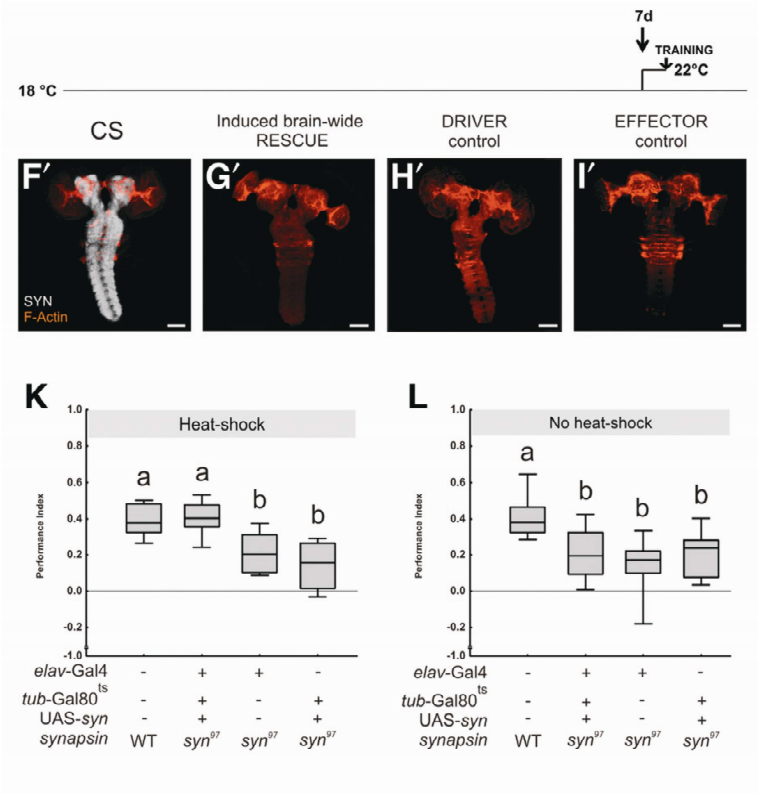


Figure 2:

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 50 µm.

All other details as in the legend of Fig. 1 (see also Fig. S1A-C, S3, Movie S2).

Local rescue at mushroom body

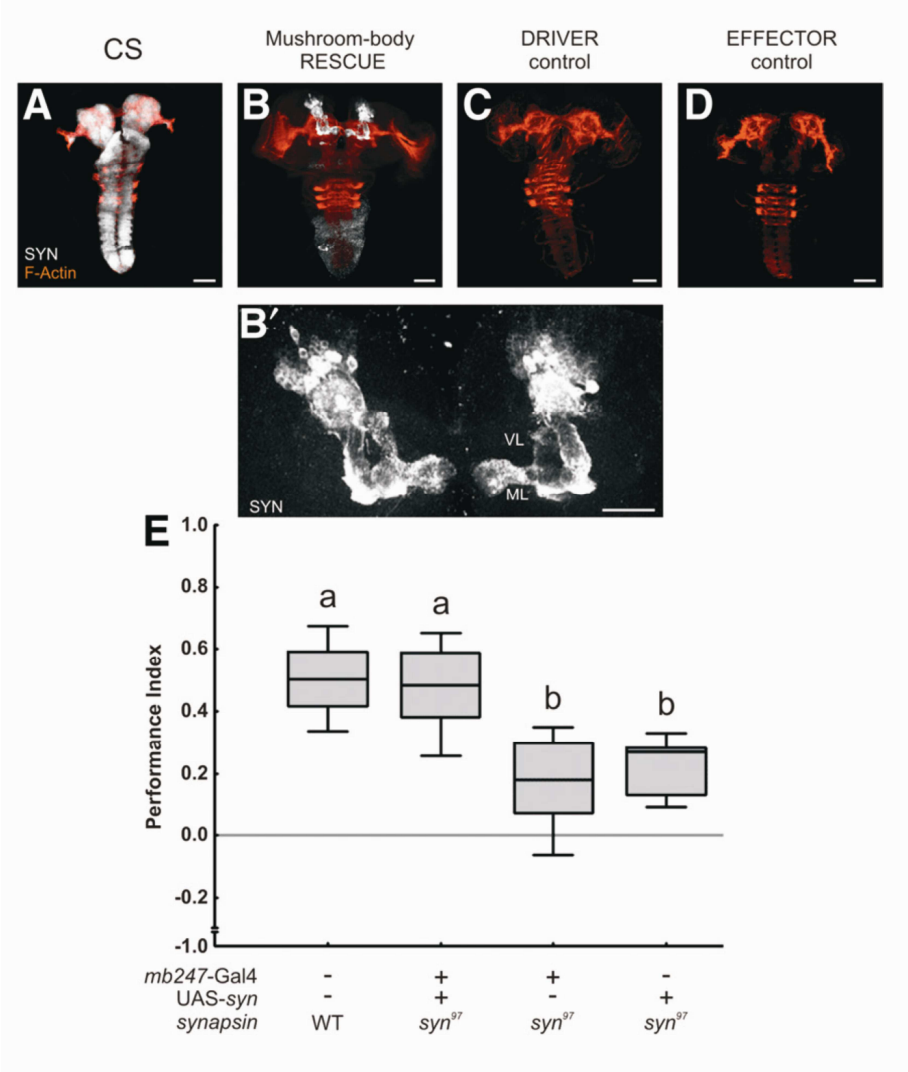
We next ask whether synapsin expression in only the mushroom bodies will restore the defect of the *syn*^{97} mutants in associative function. Associative performance scores differ between wild-type CS, mushroom-body RESCUE strain, DRIVER control, and EFFECTOR control (Fig. 3E; KW: P< 0.05; H= 21.39; 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 odor-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.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EFFECTOR control
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B
C
D
SYN
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B′
VL
ML
SYN
E
Performance Index
1.0
0.8
0.6
0.4
0.2
0.0
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mb247-Gal4 - + + -
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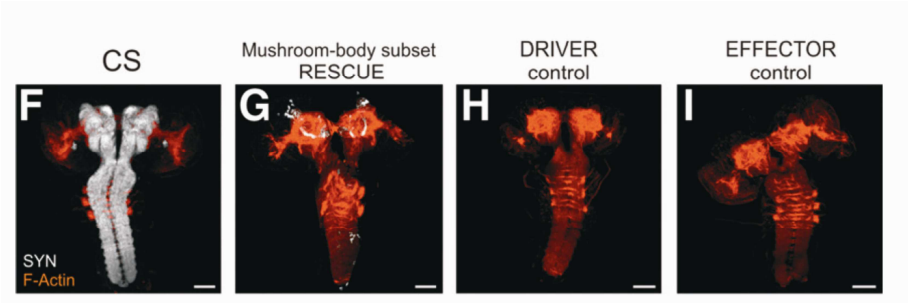
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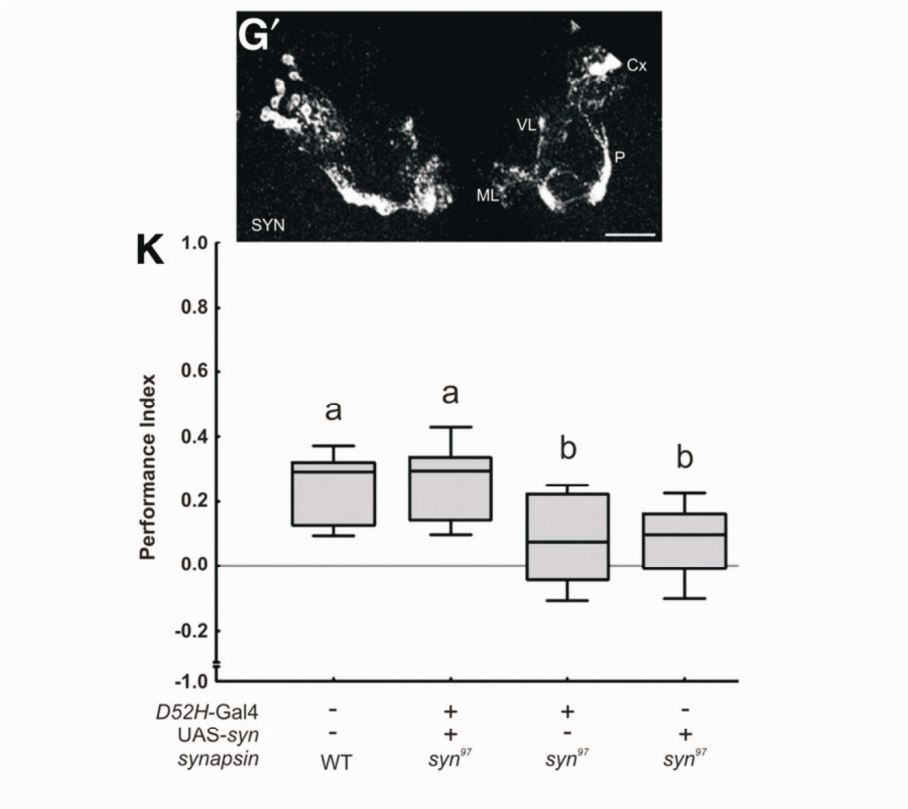


Figure 3:

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.

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 odor-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: $P> 0.05/ 3$; $U= 46$).

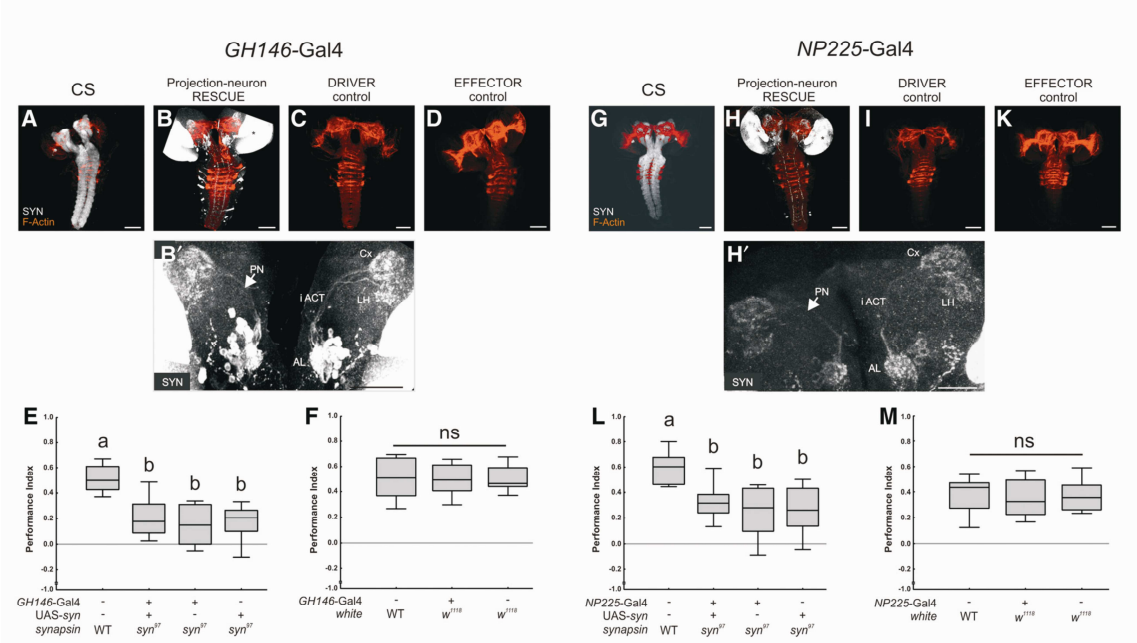
However, as is the case for any lack-of-rescue, the insertion of the driver construct may produce haploinsufficiency in the gene(s) neighbouring it, and this haploinsufficiency may lead to a learning defect masking an actually successful rescue. Therefore, we compare larvae heterozygous for the used 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 odor-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 odors is the same, whereas those projection neurons that allow making a difference between both odors could be spared from Gal4 expression. We therefore tested the projection neuron rescue larvae in a one-odor paradigm (Saumweber et al., 2011a), such that one of the

two odors is omitted. That is, larvae receive either paired or unpaired presentations of odor and reward, and then are assayed for their preference for the trained odor (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.



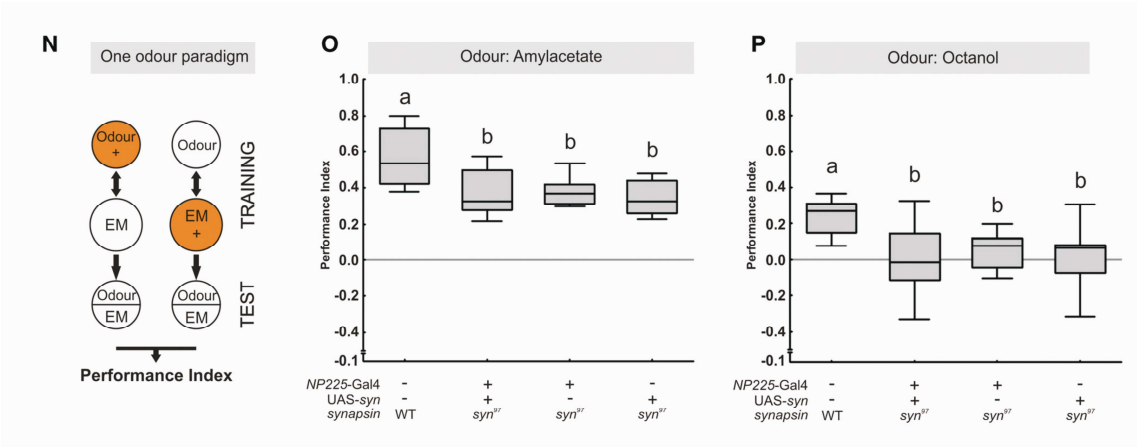


Figure 4:

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 *GH146*-Gal4 driver construct.

G-M Also another projection neuron driver (*NP225*-Gal4) is not sufficient to restore associative ability.

L, and does also does not entail haploinsufficiency (M).

N Schematic of the one-odor learning paradigm. Larvae receive either paired or unpaired presentations of odor and reward (orange label, +), and then are assayed for their preference for the trained odor.

O, P No rescue of associative function by synapsin expression (driver *NP225*-Gal4) in projection neurons in the one-odor paradigm using either AM (O) or OCT (P).

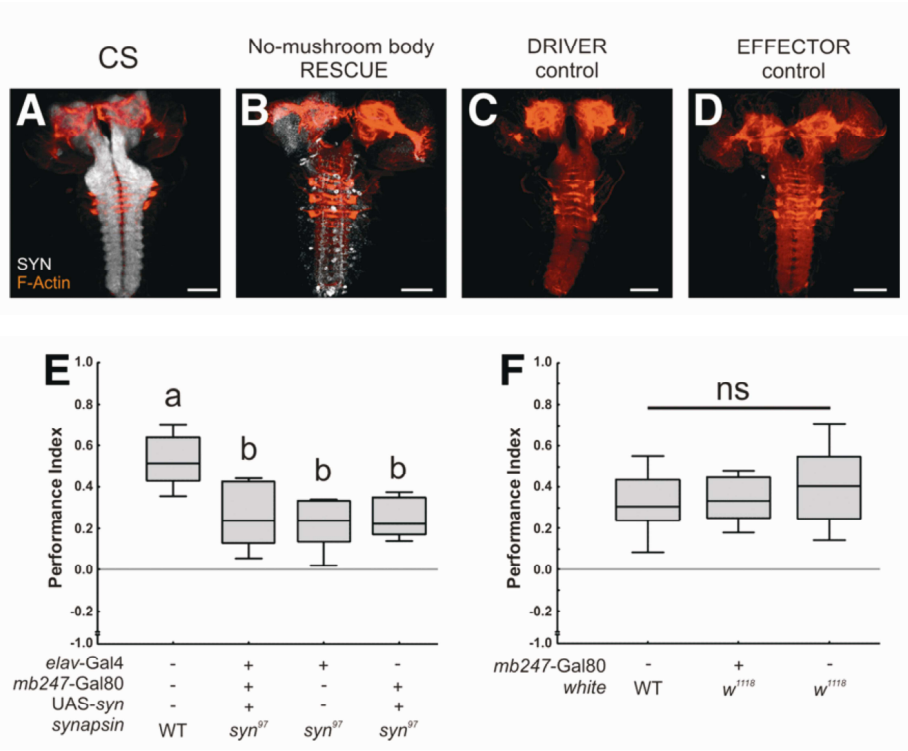
Optic lobe Anlagen (*), projection neuron (PN), antennal lobe (AL), inner antennocerebral tract (iACT), calyx (Cx), lateral horn (LH). Scale bars: 50 µm in A–D and G–K, 25 µm in B′ and H′. All other details as in the legend of Fig. 1 (see also Fig. S1H-M, S2, S3, Movies S5, S6).

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.



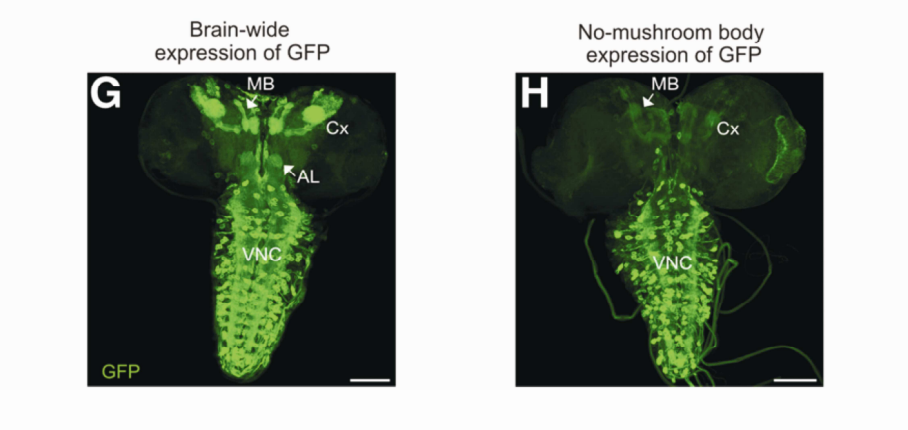


Figure 5:

No rescue by synapsin expression outside of the mushroom bodies.

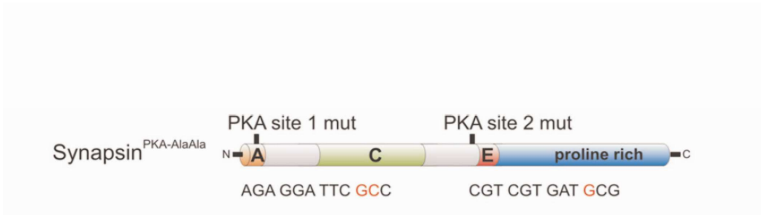
A-D Anti-synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes.
G, H Expression of GFP in *elav*-Gal4 flies (G) and *elav*-Gal4, *mb247*-Gal80 flies (H), each crossed to UAS-*GFP*. Antennal lobe (AL), mushroom body (MB), calyx (Cx) ventral nerve cord (VNC).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

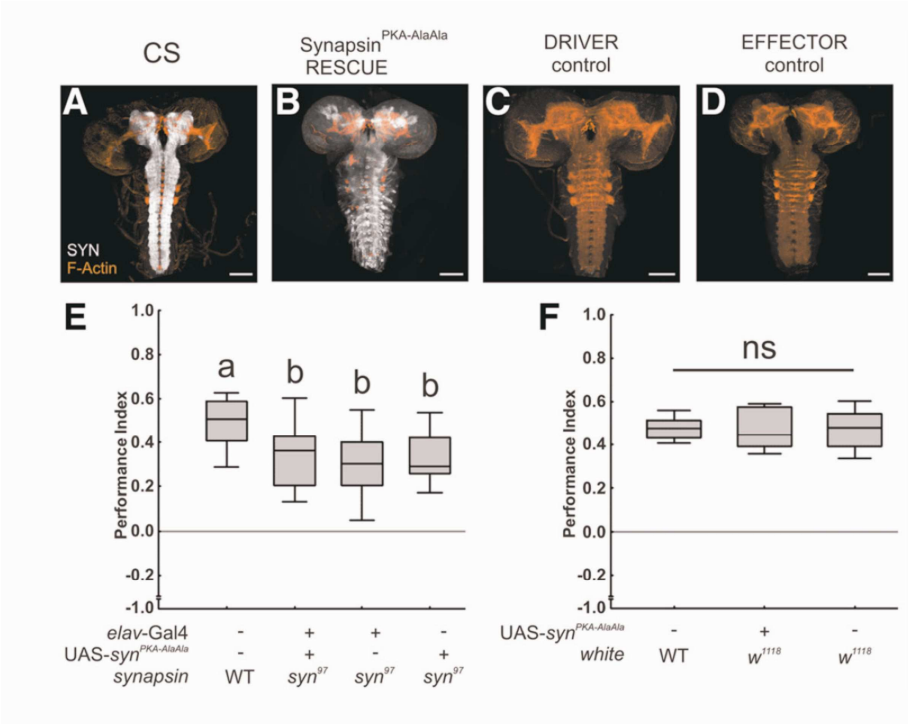
Scale bars: 50µm. All other details as in the legend of Fig. 1 (see also Fig. S3).

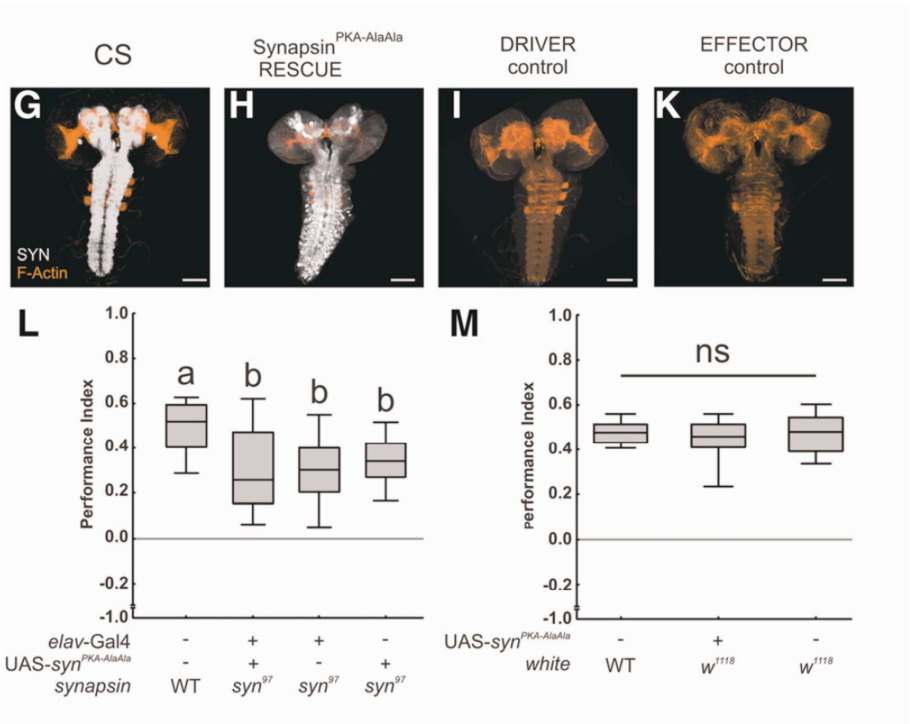
No rescue with PKA-site defective synapsin

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 <u>not</u> 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.







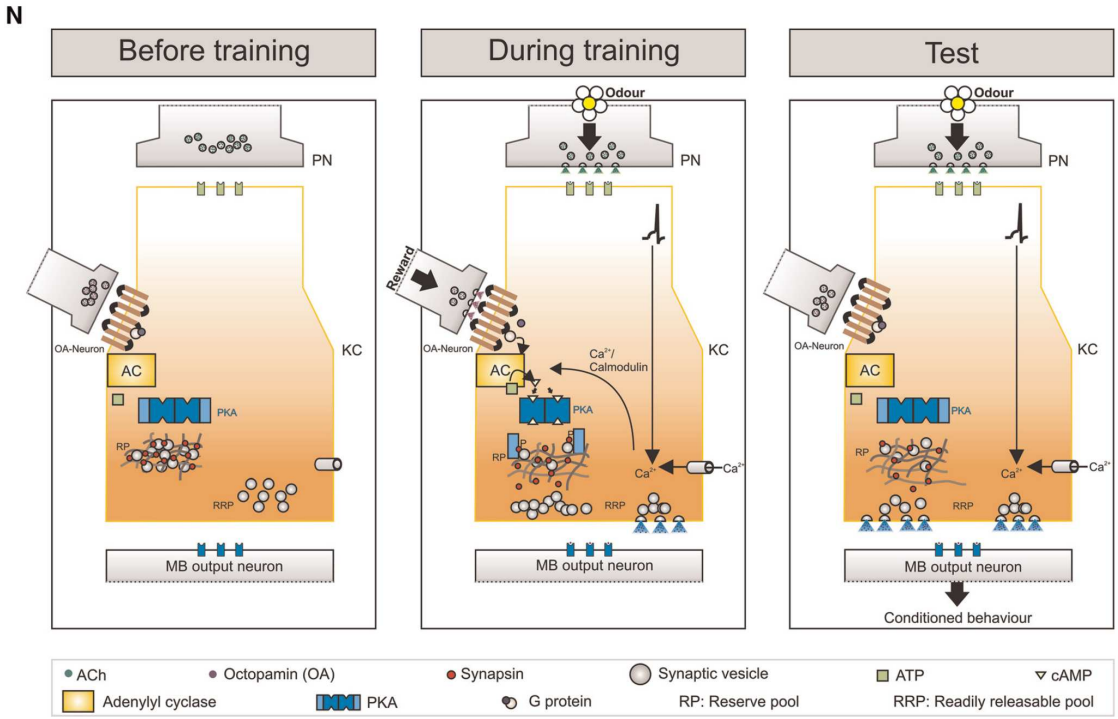


Figure 6:

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 of 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: 50 µm. 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 odor 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 odor, more transmitter can be released (Hilfiker et al., 1999). This strengthened output is proposed to mediate conditioned behavior towards the odor at test.

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 odor-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 odor-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 odor 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 odor-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 relatively 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., 2006b) 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 <u>associative</u> 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 odor-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 odors may be processed only outside the covered projection neurons. Thus, blocking synaptic output from these neurons should leave olfactory behavior unaffected - we find, however, that odor 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 odors may actually be the same. Discrimination between them may rely on between-odor differences outside of covered projection neuron subset. However, even in a one-odor 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 odor-electric shock associative paradigm. Twenty-five years later, Pauls et al., (2010a) reported that blocking synaptic output of mushroom body neurons by means of *shibire*^{ts} throughout training and testing reduces odor-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., (2010a) 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 odor-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 odor-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 behavior. Given the homology of many of the molecular determinants for synaptic and behavioral 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 behavioral 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., 2009b).

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-odor, 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 (behavioral 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 odor 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 odor 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 odor 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:

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

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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Curriculum vitae

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Manuscripts in preparation

Chen Y-c***, Mishra D*****, Gerber B Morphing of olfactory perception in larval *Drosophila*.

Mishra D*****, **Chen Y-c*****, Yarali A, Gerber B Olfactory memories are intensity-specific in larval *Drosophila*.

Chen Y-c, Mishra D, Götz V, Gerber B The perceptual relation between odours and their binary mixtures in larval *Drosophila*.

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真的很謝謝你們,當然還有怡穎!