MECHANISM OF LEARNING AND PLASTICITY

IN LARVAL *DROSOPHILA*

Lern- und Plastizitätsmechanismen in *Drosophila* Larven

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submitted by

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AFFIDAVIT

I hereby confirm that my thesis entitled

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is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, _____ _____

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation

"LERN- UND PLASTIZITÄTSMECHANISMEN IN *DROSOPHILA* LARVEN"

eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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STRUCTURE OF THE THESIS

This thesis consists of two chapters, both of which deal with learning and memory in the fruit fly *Drosophila melanogaster*. Initially, I present a short summary in English and German and a 'General Introduction' to give an overview of the thesis.

CHAPTER I Olfactory associative learning in *Drosophila* larvae

Firstly, together with Jana Husse and Bertram Gerber we established an one-odour-version of the associative learning paradigm for *Drosophila* larva established by Neuser et al 2005 and investigated a possible dissociation between innate attraction and learnability of odours.

Secondly, in cooperation with Thomas Niewalda and colleagues we focussed on salt processing, choice, feeding and learning showing a shift from appetitive to aversive in a concentration-dependent way.

Thirdly, together with Michael Schleyer and Bertram Gerber we studied the neurogenetics underlying this kind of learning and developed a behavior-based circuit-model of how outcome expectations organize learned behavior in larval *Drosophila.*

In cooperation with Birgit Michels and cooleagues we analyzed the cellular site and molecular mode of Synapsin action.

Mainly I focussed on the role of the Synapse Associated Protein of 47 kDa (SAP47) in behavioral and synaptic plasticity.

These five aspects correspond to five publications concerning

- Innate attractiveness and associative learnability of odours can be dissociated in larval *Drosophila.*
- Salt processing in larval *Drosophila*: Choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way.
- A Behavior-based circuit-model of how outcome expectations organize learned behavior in larval *Drosophila.*
- Cellular site and molecular mode of Synapsin action in associative learning
- Behavioral and synaptic plasticity are impaired upon lack of the synaptic protein SAP47

CHAPTER II Associative learning in *Drosophila*

Together with Ayse Yarali we studied predictive learning of pain-relief in adult fruit flies. It contains one manuscript:

- Genetic distortion of the balance between punishment and relief learning in *Drosophila.*

Finally, I present a General Discussion to summarize the findings and give an outlook of the presented projects.

A complete list of References, Curriculum Vitae and Acknowledgements complete this thesis.

This work had not been possible without the effort of many people, and the supervision of Bertram Gerber. I take the opportunity to express my joy in this collaborative work and sincerely acknowledge all co-authors of each manuscript, whose contributions are explicated below. Special thanks also to Miriam Koblowsky for the nice picture of Walltraut, the nice number one girl (Cover).

„Dissertation unter Einschluss mehrerer Manuskripte“ in der GSLS

Erklärung zu Eigenanteilen an Publikationen und Zweitpublikationsrechten

Publikation (Vollständiges Zitat): **T Saumweber***, J Husse*, and B Gerber.

Innate attractiveness and associative learnability of odours can be dissociated in larval *Drosophila*.

Chemical Senses 2011; 36(3):223-35

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

ggf. Erläuterung:

Publikation (Vollständiges Zitat): T Niewalda, N Singhal, A Fiala, **T Saumweber**, S Wegener and B Gerber.

Salt Processing in Larval *Drosophila*: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way.

Chemical Senses 2008; 33: 685–692

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
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Datenerhebung	TN	NS	**TS**	SW	
Daten-Analyse und Interpretation	TN	NS	BG	**TS**	SW
Schreiben des Manuskripts	BG	AF	TN	NS	**TS**

ggf. Erläuterung:

Publikation (Vollständiges Zitat): M Schleyer, **T Saumweber**, W Nahrendorf, B Fischer, D von Alpen, D Pauls, A Thum and B Gerber.

A behaviour-based circuit-model of how outcome expectations organize learned behaviour in larval *drosophila*.

Learning and Memory, accepted July, 2011

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	MS	BG	**TS**	DP	AT
Datenerhebung	MS	WN	BF	**TS**	DP, DvA
Daten-Analyse und Interpretation	MS	BG	**TS**	DP, DvA	AT
Schreiben des Manuskripts	MS	BG	**TS**	AT	DP

ggf. Erläuterung:

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

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

Learning and Memory 2011; 18: 332-344

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
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Daten-Analyse und Interpretation	BM	YCC	**TS**	BS	BG
Schreiben des Manuskripts	BM	BG	**TS**	HT	

ggf. Erläuterung:

Publikation (Vollständiges Zitat): **T Saumweber**, A Weyhersmüller, S Hallermann, S Diegelmann, B Michels, D Bucher, N Funk, D Reisch, G Krohne, S Wegener, E Buchner and B Gerber.

Behavioral and synaptic plasticity are impaired upon lack of the synaptic protein SAP47.

J. Neuroscience 2011; 31(9): 3508-3518

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	**TS**	BG	EB	SH	BM
Datenerhebung	**TS**	AW	NF, BM	DR, DB	SW, SD
Daten-Analyse und Interpretation	**TS**	BG	AW, SH	DB, EB	DR, GK
Schreiben des Manuskripts	**TS**	BG	AW	SH	BM, SD

ggf. Erläuterung:

Publikation (Vollständiges Zitat): A Yarali, M Krischke, B Michels, **T Saumweber**, MJ Mueller and B Gerber.

Genetic Distortion of the Balance between Punishment and Relief Learning in *Drosophila*.

J. Neurogenetics 2009; 23(1): 235-247

Beteiligt an	**Autoren-Initialen,** Verantwortlichkeit abnehmend von links nach rechts				
Planung der Untersuchungen	AY	BG	MK	BM	**TS**
Datenerhebung	AY	MK	BM	**TS**	MJM
Daten-Analyse und Interpretation	AY	BG	MJM	BM	**TS**
Schreiben des Manuskripts	AY	BG	MJM	BM	**TS**

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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CHAPTER II Associative learning in *Drosophila*

SUMMARY

According to a changing environment it is crucial for animals to make experience and learn about it. Sensing, integrating and learning to associate different kinds of modalities enables animals to expect future events and to adjust behavior in the way, expected as the most profitable. Complex processes as memory formation and storage make it necessary to investigate learning and memory on different levels. In this context *Drosophila melanogaster* represents a powerful model organism. As the adult brain of the fly is still quite complex, I chose the third instar larva as model - the more simple the system, the easier to isolate single, fundamental principles of learning. In this thesis I addressed several kinds of questions on different mechanism of olfactory associative and synaptic plasiticity in *Drosophila* larvae. I focused on short-term memory throughout my thesis.

First, investigating larval learning on behavioral level, I developed a one-odor paradigm for olfactory associative conditioning. This enables to estimate the learnability of single odors, reduces the complexity of the task and simplify analyses of ‘‘learning mutants’’. It further allows to balance learnability of odors for generalization-type experiments to describe the olfactory ‘‘coding space’’. Furthermore I could show that innate attractiveness and learnability can be dissociated and found finally that paired presentation of a given odor with reward increase performance, whereas unpaired presentations of these two stimuli decrease performance, indicating that larva are able to learn about the presence as well as about the absence of a reward.

Second, on behavioral level, together with Thomas Niewalda and colleagues we focussed on salt processing in the context of choice, feeding and learning. Salt is required in several physiological processes, but can neither be synthesized nor stored. Various salt concentrations shift the valence from attraction to repulsion in reflexive behaviour. Interestingly, the reinforcing effect of salt in learning is shifted by more than one order of magnitude toward higher concentrations. Thus, the input pathways for gustatory behavior appear to be more sensitive than the ones supporting gustatory reinforcement, which is may be due to the dissociation of the reflexive and the reinforcing signalling pathways of salt.

Third, in cooperation with Michael Schleyer we performed a series of behavioral gustatory, olfactory preference tests and larval learning experiments. Based on the available neuroanatomical and behavioral data we propose a model regarding chemosensory processing, odor-tastant memory trace formation and the ‘decision’ like process. It incorporates putative sites of interaction between olfactory and gustatory pathways during the

establishment as well as behavioral expression of odor-tastant memory. We claim that innate olfactory behavior is responsive in nature and suggest that associative conditioned behavior is not a simple substitution like process, but driven more likely by the expectation of its outcome.

Fourth, together with Birgit Michels and colleagues we investigated the cellular site and molecular mode of Synapsin, an evolutionarily conserved, presynaptic vesicular phosphoprotein and its action in larval learning. We confirmed a previously described learning impairment upon loss of Synapsin. We localized this Synapsin dependent memory trace in the mushroom bodies, a third-order "cortical" brain region, and could further show on molecular level, that Synapsin is as a downstream element of the AC-cAMP-PKA signalling cascade. This study provides a comprehensive chain of explanation from the molecular level to an associative behavioral change.

Fifth, in the main part of my thesis I focused on molecular level on another synaptic protein, the Synapse associated protein of 47kDa (Sap47) and its role in larval behavior. As a member of a phylogenetically conserved gene family of hitherto unknown function. It is localized throughout the whole neuropil of larval brains and associated with presynaptic vesicles. Upon loss of Sap47 larvae exhibit normal sensory detection of the to-be-associated stimuli as well as normal motor performance and basic synaptic transmission. Interestingly, short-term plasticity is distorted and odorant–tastant associative learning ability is reduced. This defect in associative function could be rescued by restoring Sap47 expression. Therefore, this report is the first to suggest a function for Sap47 and specifically argues that Sap47 is required for synaptic as well as for behavioral plasticity in *Drosophila* larva. This prompts the question whether its homologs are required for synaptic and behavioral plasticity also in other species.

Further in the last part of my thesis I contributed to the study of Ayse Yarali. Her central topic was the role of the White protein in punishment and relief learning in adult flies. Whereas stimuli that precede shock during training are subsequently avoided as predictors for punishment, stimuli that follow shock during training are later on approached, as they predict relief. Concerning the loss of White we report that pain-relief learning as well as punishment learning is changed. My contribution was a comparison between wild type and the $white^{1118}$ mutant larvae in odor-reward learning. It turned out that a loss of White has no effect on larval odorant-tastant learning. This study, regarding painrelief learning provides the very first hints concerning the genetic determinants of this form of learning.

ZUSAMMENFASSUNG

In einer belebten, sich stetig wandelnden Umwelt ist es essenziell für Lebewesen, Informationen wahrzunehmen und Erfahrungen zu sammeln, um ihr Verhalten entsprechend zu modifizieren. Verschiedene Arten von Reizen werden wahrgenommen, integriert und gespeichert. Dies ermöglicht Tieren künftige Ereignisse vorherzusehen und ihr Verhalten entsprechend ihren Erwartungen anzupassen. Die Komplexität von Lernprozessen und Gedächtnisspeicherung macht es notwendig, diese Prozesse auf unterschiedlichen Ebenen zu untersuchen. In diesem Zusammenhang hat sich *Drosophila melanogaster* als besonders geeigneter Modellorganismus herauskristallisiert. Trotz einer relativ geringen neuronalen Komplexität im Vergleich zu höheren Organismen, zeigt sie ein reichhaltiges Verhaltensrepertoire. Dennoch ist das Gehirn von adulten Furchtfliegen ein hoch komplexes System. Je einfacher ein System ist, umso vielversprechender ist es scheinbar, einzelne fundamentale Aspekte dieses Systems zu isolieren und zu untersuchen. In meiner Arbeit nutzte ich daher als Modelorganismus das dritte Larvenstadium der Fliege und untersuchte auf verschiedenen Ebenen unterschiedliche Mechanismen olfaktorischer, assoziativer und synaptischer Plastizität. Dabei fokussierte ich mich stets auf Kurzzeitgedächtnis.

Zunächst untersuchte ich assoziatives Lernen auf Verhaltensebene. Hierfür entwickelte ich ein Ein-Duft-Lernparadigma für olfaktorische klassische Konditionierung von *Drosophila* Larven. Dies ermöglicht, die Lernbarkeit von einzelnen Düften zu untersuchen, reduziert die Komplexität der Aufgabenstellung für die Larven und vereinfacht die Analyse von Lernmutanten. Weiterhin erlaubt es die Lernbarkeit von Düften für Generalisierungs-experimente zu balancieren, um zu beschreiben, wie Duftidentitäten im Nervensystem kodiert werden. Ich konnte zeigen, dass die Lernbarkeit von Düften nicht unmittelbar mit der naiven Duftpräferenz korreliert. Ferner konnte in dieser Studie nachgewiesen werden, dass durch gepaarte Präsentation von Duft und Zuckerbelohnung die Präferenz im Bezug auf diesen Duft zunimmt, wohingegen ungepaarte Präsentation dieser beiden Reize zu einer Abnahme der Duftpräferenz führt. Dies weist darauf hin, dass es Larven auch möglich ist etwas über die Abwesenheit der Belohnung zu lernen.

In einer zweiten Studie befasste ich mich, in Zusammenarbeit mit Thomas Niewalda, mit der Verarbeitung von Salz im Bezug auf das Wahl-, Fress- und Lernverhalten von *Drosophila* Larven. Salze spielen in mehreren physiologischen Prozessen eine bedeutende Rolle, können von Larven aber weder synthetisiert noch gespeichert werden. Unterschiedliche Salzkonzentrationen haben unterschiedliche Auswirkungen auf das Larvenverhalten.

Während niedrige Konzentrationen von Larven bevorzugt werden, werden hohe Salzkonzentrationen vermieden. Lernexperimente zeigten, dass Salz ebenfalls dosisabhängig als positiver oder negativer Verstärker wirkt. Interessanterweise zeigt sich im Vergleich zum Wahl- und Fressverhalten, dass der Punkt, an dem Salz von einem appetitiven zu einem aversiven Stimulus wird, um mehr als eine Größenordnung in Richtung höherer Konzentrationen verschoben ist. Die Sensitivität der gustatorischen Transduktion ist somit höher als die Transduktion des Verstärkersignals. Möglicherweise liegt dies an der Dissoziation dieser beiden Transduktionswege.

In der dritten Studie dieser Arbeit wurden, in Kooperation mit Michael Schleyer, eine Vielzahl an olfaktorischen und gustatorischen Präferenztests, sowie eine Reihe an Lernexperimenten durchgeführt. Basierend auf bekannten Neuroanatomiestudien und unseren Verhaltensdaten, propagieren wir ein Model für Duft- und Geschmacksprozessierung, die Etablierung von Gedächtnisspuren, sowie Entscheidungsprozessen. Sowohl mögliche Interaktionen zwischen olfaktorischen und gustatorischen Transduktionswegen, sowie der Abruf von Gedächtnisinhalten werden berücksichtigt. Wir schlagen vor, dass naives olfaktorisches Verhalten natürlicherweise reflexiv ist. Assoziativ konditioniertes Verhalten kann allerdings nicht als reiner Substitutionsprozess betrachtet werden, sondern wird besser interpretiert im Hinblick auf die Erwartung, die er auslöst, woraufhin ein bestimmtes Verhaltensprogramm gestartet wird.

In Zusammenarbeit mit Birgit Michels untersuchte ich auf zellulärer Ebene die molekulare Funktion von Synapsin im assoziativen Lernen von *Drosophila* Larven. Synapsin gehört zu den hochkonservierten, präsynaptischen, vesikulären Phosphoproteinen. Wir konnten einen früher bereits beschriebenen Lernphänotyp von Synapsin Mutanten Larven bestätigen. Die Synapsin abhängige Gedächtnisspur konnten wir auf wenige Zellen im Pilzkörper, einer dem olfaktorischen Cortex der Vertebraten homologen Struktur, lokalisieren. Auf molekularer Ebene wurde nachgewiesen, dass Synapsin ein Zielprotein in der bekannten AC-cAMP-PKA Lernkaskade ist. Diese Studie zeigt einen Zusammenhang zwischen molekularen Mechanismen assoziativer Plastizität und einer daraus resultierenden Verhaltensänderung der Tiere.

In meinem Hauptprojekt befasste ich mich auf molekularer Ebene mit einem weiteren synaptischen Protein, dem Synapsen assoziierten Protein von 47kDa (Sap47) und seiner Rolle im Verhalten von *Drosophila* Larven. Sap47 wird in allen neuropilen Bereichen expremiert und ist mit synaptischen Vesikeln assoziiert. Das Fehlen von Sap47 beeinflusst weder die Detektion der zu assoziierenden Reize, noch das Kriechverhalten der Larven. Auch die

synaptische Übertragung, ausgelöst durch einzelne Stimulationen an der neuromuskulären Synapse, ist nicht beeinträchtigt. Interessanterweise führt das Fehlen von Sap47 sowohl zu veränderter Kurzzeit-Plastizität an dieser Synapse, sowie zu einer Einschränkung in der Bildung von Duft-Zucker-Gedächtnis. Diese Studie liefert einen ersten Hinweis auf eine Funktion von Sap47 in synaptischer und assoziativer Plastizität. Es stellt sich die Frage, ob auch in anderen Organismen die zu *Drosophila* Sap47-homologen Proteine notwendig für synaptische und Lernplastizität sind.

Im letzten Teil meiner Dissertation war ich an einem Projekt von Ayse Yarali beteiligt. Die zentrale Fragestellung in dieser Studie war, ob eine Mutation im *white* Gen Bestrafungs- und/ oder Erleichterungslernen beeinflusst. Wird ein neutraler Reiz während einer Trainingsphase mit einem Elektroschock bestraft, wird dieser später konsequent vermieden, da er einen Elektroschock vorhersagt (Bestrafungslernen). Eine Umkehrung der Reihenfolge der Stimulipräsentation, sodass dem Schock stets ein neutraler Stimulus folgt, führt später, in der Testphase, zu einer positiven Reaktion auf diesen naiv neutralen Reiz (Erleichterungslernen). Ein Verlust des White Proteins in $white^{1118}$ Mutanten verändert beide Arten von Gedächtnissen in adulten Fliegen. Meine Beteiligung an dieser Arbeit war ein Vergleich zwischen wildtypischen Larven und $white^{1118}$ mutanten Larven in Duft-Zucker Assoziationsexperimenten. Es zeigte sich, dass der Verlust dieses Proteins auf larvale Duft-Zucker Konditionierung keinen Einfluss hat. Im Larvenlernen kann somit das Verhalten von transgenen Tieren, die zumeist eine Mutation im *white* Gen als Markergen tragen, interpretiert werden, ohne die Funktion des *white* Gens berücksichtigen zu müssen. Im Bezug auf Erleichterungslernen liefert diese Arbeit einen ersten Hinweis auf eine genetische Komponente, der entscheidend für diese Art des assoziativen Lernens ist.

GENERAL INTRODUCTION

"*We are who we are because of what we have learnt and what we remember*"
(*In Search of Memory*; Eric Kandel, 2006)

One major goal in neuroscience is to understand how brains enable organisms to control and modify behavior. From an animals perspective brain function is to sense and extract biologically relevant different modalities of information, process and integrate them and further generate an adjusted behavioral output according to a permanently changing environment. For accomplishing daily challenge of survival animals need the ability to remember the past, learn about the present and compare these experiences to predict future events. Then they adjust their behavior in the way expected as the most profitable.
A big advantage of using animals as subject in general is that one easily can control their environment to figure out which variables are important for the task of interest. Further the simpler the system, the easier it is to isolate single fundamental principles. As it might be more promising to study brain function in a simple organism instead of a much more complex organism like man, I choose the fruit fly *Drosophila melanogaster* larvae as model organism to investigate fundamental mechanisms of learning, especially olfactory associative learning.

Associative learning and memory

Brains organize behavior - this is what brains evolved for (Gerber et al., 2009). The interesting question is now how can brains modify behavior according to a changing environment? Brains enable animals to form, store and retrieve memories by making experience, a considerable advantage, e.g. for predicting food. Most experience contributes to behavior organization mainly via associative learning processes.
Conceptually, these mnemonic processes can be separated according to the kind and the source of information that is the basis for the learned behavior. From the very end of the 19th century two seminal pioneers started investigating associative learning.

First, the American psychologist Edward Lee Thorndike (1874-1949) may mark the beginning of experimental analysis of behavior in general and was probably also the first to study the process of learning in a truly systematic way (nicely summarized by Chance, 1999). He introduced several "Puzzle-Boxes" investigating the escape behavior of hungry cats. Cats could escape from such boxes by simple acts e.g. by pulling on a wire or press a button. After

repeatedly exposition to such a box, Thorndike noted that cats improved escape performance, which he interpreted as consequence of the association of sensations by the cat with its own behavior. Thorndike's proposals formed the basis for a number of subsequent theories of associative learning, all of which shared the assumption that learning is based on the growth of stimulus-response connections (Pearce and Bouton, 2001). The idea of operant conditioning was born and later taken up by Burrhus Frederic Skinner (1904-1990) creating the so called "Skinner Box". It is even nowadays used as a common paradigm to investigate operant conditioning in many model organisms like insects, rodents, birds and even primates. Until the mid 1960s findings result in three fundamental principles underlying the formation of associations. First, contiguity, determining that an association is formed between events occurring together, second frequency the more often events occur together the stronger the associative strength and third intensity, the higher the intensity of a positive or negative reinforcer, the stronger the association would be.

The second pioneer in the field was the Russian physiologist Ivan Petrovich Pavlov (1849-1936). His main area of research was on correlation between the nervous system and the autonomic functions of the body. He investigated digestive processes studying the relationship between salivation and digestion in dogs. He observed that food induces salivation in a reflexive manner. In a broad series of experiments he applied different kinds of stimuli like auditory, visual, and tactile stimulations to dogs. His discovery then was that if e.g. the sound of a bell repeatedly preceded food, dogs subsequently salivate upon presentation of the bell alone. He described this phenomenon as "conditioned reflex" (Pavlov, 1906). He suggested after pairing the sound (conditioned stimulus) by a couple of training cycles with food reward (unconditioned stimulus), the bell was associated with reward and later evokes the conditioned response – salivation. Based on the principles of stimulus-stimulus connections by pavlovian associative conditioning a new field of associative learning research emerged. In contrast to Thorndike´s instrumental conditioning experiments, where the animal, not the experimenter determines, when the punishment or reward will be delivered by its own behavior - what is difficult to control - in pavlovian conditioning the experimenter controls the application of both to be associated stimuli.

In the middle of the 20th century two publications changed the traditional view of learning processes. A fundamental improvement of the traditional view in associative learning research comes into play, when Rescorla (Rescorla, 1966) suggested that contiguity of two stimuli is not sufficient for conditioning. He developed the concept of contingency, a measure for the probability that the to-be-associated stimuli occur together. An unconditioned stimulus

can occur in the presence or the absence of the conditioned stimulus. This means that the conditioned stimulus not only must be contiguous with an unconditioned stimulus, but must also be a good and accurate predictor of the occurrence of the unconditioned stimulus. In chapter I.1 (Saumweber et al., 2011a) of my thesis I took up this idea and analyzed what can *Drosophila* larvae learn. Do they learn only about the presence of a reward, or are they also able to learn anything about the absence of a reward, which is indeed the case. In accordance with the mathematical model of Rescorla and Wagner, which was developed as a powerful tool to predict the strength of associations (Rescorla and Wagner, 1972), the presence as well as the absence of the unconditioned stimulus has an effect on the reinforcement in associative conditioning. Beneath that finding is also true for *Drosophila* larvae, in this study together with Jana Husse and Bertram Gerber we investigated naïve odor preferences before and after conditioning, established a one-odor-version of the associative learning paradigm and looked for a possible dissociation between innate attractiveness and learnability of odors. First, we modified the standard two-odor differential conditioning version of the paradigm (Scherer et al., 2003), which was further analyzed parametrically by Neuser et al., 2005. In that standard paradigm, one group of larvae receives a reward (fructose) in the presence of an odor X, but not in the presence of an odor Y (Train: X+ // Y), whereas another group is trained reciprocally (Train: X // Y+). In a choice test between the trained odors (Test: X -- Y), differences in odor preference between the reciprocally trained groups reflect associative learning performance. We developed a one-odor reciprocal version of this paradigm, where during training and test only one odor is presented: one group of larvae receives the odor and the reward as paired presentation whereas the reciprocally trained group receives the odor and the reward in an unpaired manner. Using this paradigm, we could show that innate attractiveness and the learnability of different odors ca be dissociated, and that paired as well as unpaired presentations of odor and reward establish associative memories leading to conditioned approach and avoidance, respectively. Furthermore, this one-odor paradigm now makes it possible to e.g. perform generalization types of experiment, where a single odor is trained and a non-trained odor is tested, or where one odor, at a particular intensity, is trained and that same odor is tested, at either the trained intensity or at a higher or a lower intensity (for adult flies: Yarali et al., 2009a, for larvae Mishra et al., 2010, Chen et al., 2011). Last but not least, when using a one-odor conditioning paradigm for neurogenetic analyses (Chapter I.5, Saumweber et al. 2011b) one needs to control only the smelling ability of one odor between mutants and wild type.

The second seminal paper in 1966 was published by Garcia and Koelling changing the traditional view of Pavlov. His thought was that conditioning is an entirely general process, no matter which kinds of stimuli are paired the same association is formed. However, Garcia and Koelling could show that also preparedness matters. It is easier to associate a light stimulus paired with food instead of with illness (Garcia and Koelling, 1966). This finding changed the general view of the value of conditioning. The pressure of natural selection made it necessary not to associate all contiguously occurring stimuli, but that preparedness is also necessary to predict future events to adjust behavior. The idea that different kinds of unconditioned stimuli lead to different kinds of associations and further to different kinds of predictions for future events was taken up in chapter I.2 (Niewalda et al., 2008). In cooperation with Thomas Niewalda I focussed on salt processing in the context of choice, feeding and learning. Indeed, salt processing is interesting in several regards. Sodium chloride (NaCl) is required for many physiological processes including neurobiological signalling, but cannot be synthesized or stored. Therefore sodium chloride has a strong innate ‘value’ to most animals, and its intake is tightly controlled. Typically, the appetitive responses to low salt gradually turn into aversion as concentration is increased (for adult flies: Arora et al., 1987; larvae: Miyakawa, 1982; Liu et al., 2003). Given this switch in valence, we compared two kinds of behavioral function in larval *Drosophila*. First, we wanted to know how salt concentration affects reflexive behavior, and second, how salt concentration affects reinforcement function. Looking at choice and feeding behavior of the larvae we found that similar to adults larval choice behavior turns from appetitive to aversive as salt concentration is increased. We next asked for the concentration dependency of sodium chloride as a reinforcer and found, when testing is carried out in the absence of the reinforcer, low and high training concentrations of salt do not support positive learning performance, whereas intermediate concentrations do. In contrast testing in the presence of the reinforcer, learning performance is significantly negative for the highest salt concentration.

While Pavlov interpreted his finding as a stimulus substitution, the CS becomes US Edward Tolman was the first developed the idea that it is not a substitution but a learnt expectation (Tolman, 1932). Dogs hearing the sound learnt to expect food. Additionally in 1950 Erich van Holst together with Horst Mittelstaedt demonstrated the "Reafference Principle". They could show that organism are able to separate self-generated sensory stimuli from externally generated sensory stimuli concerning an interactive processes between the central nervous system and its periphery (v. Holst and Mittelstaedt, 1950). The idea that animals learn to expect things and have knowledge about their own behavior, was taken up

and mainly supported by Jenkins and Moore in 1973. They trained pigeons by pairing light with food in one group and with water in another group (Jenkins and Moore, 1973). The typical behavior of Pigeons expecting food reward is pecking with closed eyes and opened beak, whereas expecting water they pecked with open eyes and closed beak. Similar to this operant conditioning experiment Hendel and Gerber later investigated this phenomenon in *Drosophila* larvae in a pavlovian conditioning experiment (Gerber and Hendel, 2006). They could show that even *Drosophila* larvae only retrieve their memory and show the learnt behavior if they can improve their situation. To predict rewarding food leads to searching behavior in a non rewarding situation, whereas to predict punishing food leads to an escape behavior only in the presence of the negative reinforcer. Their findings were reanalyzed in detail and further investigated together with Michael Schleyer (Schleyer et al., *In Press*). We asked how such outcome expectations organize learned behavior and worked on a behavior-based circuit-model. I will come to this point later in this introduction (see later: *Larval Learning on behavioral and cellular level*).

Before 1966 conditioning was thought to be a basically simple and automated process in which stimuli occurred together were associated. This is indeed not the case. Conditioning is a much more complex process involving not only contiguity, frequency and intensity, but also contingency, preparedness and some others like different kinds of reinforcement, motivation and extinction. This complexity makes it difficult to investigate associative learning in higher animals and makes it necessary to analyze different aspects of associative learning in detail under controlled environmental conditions.

Since the beginning of the 20th century when Thomas Hunt Morgan discovered white eyed flies and could attribute this phenotype to a spontaneous deletion (Hazelrigg et al., 1984) of a part of the then socalled *white* gene (Morgan, 1911), *Drosophila melanogaster* became an attractive model organism for geneticists. Among others, the short generation time (approx. ten days at 25° C: Ashburner and Bergman, 2005), the high number of progeny, and the low cost of keeping facilitated flies as model for genetic research.

At the Kaiser Wilhelm Institut during the Second World War the idea was born to investigate different neurological aspects in different model organism. Oscar Vogt (1870 – 1959) and Nikolaj Vladimirovich Timoféeff-Ressovsky (1900 – 1981) combined in an inventive manner brain research of human neurological and psychiatric diseases with neuroanatomical and genetical studies in *Drosophila* (Schmuhl, 2003). The fruit-fly entered the field of brain research.

Later, Seymour Benzer was the first using forward genetic mutagenesis screens to isolate mutants for behavior (Benzer, 1971) and introduced together with William G. Quinn *Drosophila melanogaster* in the field of learning and memory research. They developed an efficient, reproducible memory assay (Quinn et al., 1974) and made it possible to exploit the integrative approach combining learning psychology and genetic intervention. In the introduced learning experiment, flies were trained to associate an odor with an electric shock. This paradigm was subsequently modified allowing also using sugar as a reward (Tempel et al., 1983; Schwaerzel et al., 2003; Keene et al., 2006; Kim et al., 2003; Kim et al., 2007; Krashes et al., 2007; Schwaerzel et al., 2007; Thum et al., 2007; Krashes et al., 2009). *Drosophila melanogaster* turned into a "work horse" for learning and memory research and an enormous repertoire of other learning paradigm were and are still developed, e.g. the heat box (Wustmann et al., 1996), the flight simulator for visual learning and navigation (Wolf and Heisenberg, 1991), free flight yaw torque (Wolf and Heisenberg, 1991), visual association learning in freely moving flies (Schnaitmann et al., 2010), oviposition choice (Mery and Kawecki, 2002), courtship learning (Siegel and Hall, 1979), spatial learning (Wolf et al., 1998), and conditioning of the proboscis extension reflex (Chabaud et al., 2006).

It was Martin Heisenberg, who started assigning different behaviors to different brain structures in the fly brain (Heisenberg, 1980; Fischbach and Heisenberg, 1981; Heisenberg et al., 1985). This is still one major goal localizing the cellular basis of learning referred as memory formation and to identify the underlying memory traces.

Investigating associative learning in *Drosophila* culminates in two major breakthroughs, which together provide experimental access unrivalled in any higher organism:

In 2000 the full fly genome has been sequenced (Adams et al., 2000) giving access to modern methods of bioinformatics. Knowledge of the whole sequence enables identifying genes and proteins and investigating similarities and homologies of proteins and/ or protein domains between species to predict structures and mechanism at molecular and neuronal level conserved through evolution (see also chapter I.4, Michels et al., 2011).

The other major breakthrough was establishing artificial, bipartite expression systems in the fly, enabling geneticists to express any gene X anywhere and any time in a controlled matter. In general such systems have an activator which directly binds to a target sequence leading to the expression of gene X (Fig. 1).

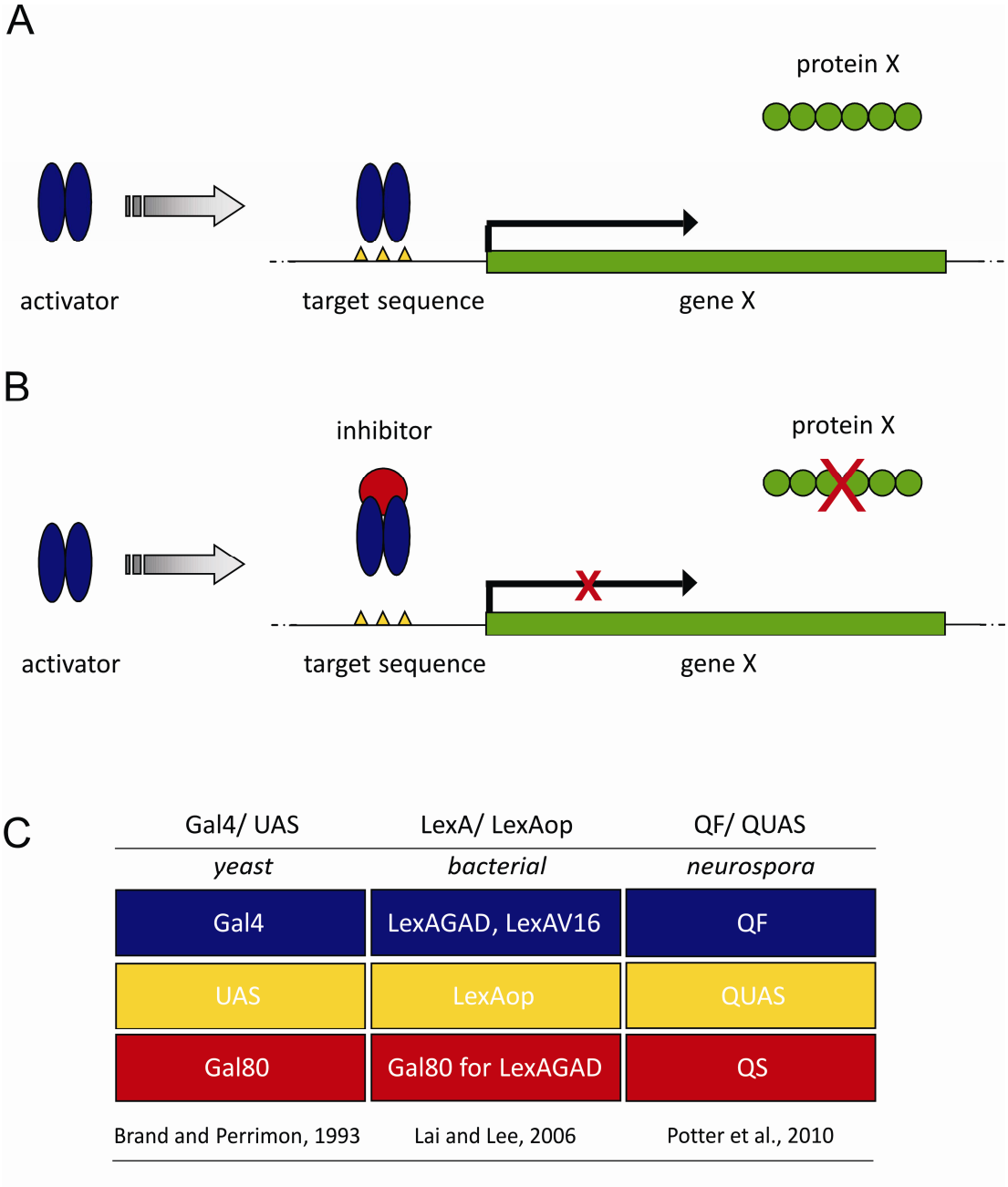


Figure 1: Cartoon of three independent ectopic expression systems in the fly

In general these ectopic expression systems can be used in *Drosophila* to express any gene, anywhere at any time.

A: An activator like a transcription factor can be expressed locally and temporally under control of a specific endogenous enhancer. This activator binds only in these specific cells to its target sequence leading to the ectopic expression of the gene of interest only in this subset of cells.
B: For all three systems there are also inhibitors available to inhibit the activator function.
C: Three systems for ectopic expression are introduced in the fly from three host species, from *yeast*: Gal4/ UAS system, *bacteria*: LexA/ LexOp system and *neurospora*: the QF/ QUAS system, which can be used independent from each other to express different effectors in different tissues in the fly. For more detailed information see text.

Three independent systems to manipulate *Drosophila* genetically are available in the fly: Gal4/ UAS (Brand and Perimon, 1993), LexA/ LexAop (Lai and Lee, 2006) and QF/ QUAS system (Potter et al., 2010). These expression systems work independently from each other and therefore can be used in a combinatorial manner. Using two independent bipartite expression systems give access to compare expression pattern, to identify cells, to reconstitute e.g. the green fluorescent protein (GFP) across synaptic partners using GRASP, to identify connections formed by neurons (Diegelmann et al., 2008).

Here I want to give an overview how such a particular elegant method can be used to express a given gene of interest in the fly using the Gal4/ UAS system.

Excurse: The Gal4/ UAS system:

In 1993 the Gal4/ UAS system was established and introduced in *Drosophila* (Brand and Perrimon, 1993). In the yeast *Saccharomyces cerevisiae* the *Gal4* gene encodes the transcriptional activator Gal4 not found in wild type *Drosophila*. The Gal4 protein consist of an activation domain and DNA-binding domain which directly binds to its defined target sequence, the Upstream Activating Sequence (UAS). Separate a DRIVER strains (*Gal4*-strain) and an EFFECTOR strains (UAS-strain) are generated by cloning a P-element containing the *Gal4*-gene into the genome of one fly strain and a P-element containing the target gene downstream of UAS in the genome of a second fly strain. Depending on where the P-element is inserted under the control of a nearby tissue-specific endogenous enhancer Gal4 is specifically expressed in a subset of cells. Crossing the homozygous strains together yields a progeny containing the DRIVER and the EFFECTOR construct. In these flies Gal4 is expressed where the endogenous enhancer is spatially and temporally active and Gal4 in turn directs transcription of the Gal4 responsive UAS target gene in identical pattern (Phelps and Brand, 1998). With knowledge of endogenous enhancer sequences it is able to clone Gal4 together with the known endogenous enhancer or even parts of it in the fly´s genome to directly express Gal4 in a clearly defined local subset of cells. Today thousands of *Gal4*-strains exist covering different tissues up to single cell level (see e.g. Bloomington stock center at Indiana University; Drosophila Genetic Resource Center, Kyoto Institute of Technology; GETDB [NP Consortium Gal4 Enhancer Trap Insertion Database]; stock collection Janelia Farm, Ashburn, USA). Introducing a temperature-sensitive mutant of Gal80 ($Gal80^{ts}$), which represses GAL4 transcriptional activity at permissive temperatures into the fly enables to restrict the regional Gal4 expression also in a temporally temperature dependent manner (McGuire et al., 2003; Zeidler et al., 2004; see also chapter I.4, Michels et al., 2011).

The UAS determines what kind of EFFECTOR is expressed. In general everything which is gene coded can be expressed in the fly. Initially established

for enhancer trapping to identify regulatory regions of *Drosophila* genes, β-Galactosidase (UAS-*lacZ;* Brand and Perrimon, 1993) and later on the green fluorescent protein (UAS-*GFP;* Yeh et al., 1995) were used as reporter to visualize expression pattern of different Gal4 driver lines (see also chapter I.4, Michels et al., 2011). On the effector side various tools have been developed also for investigating neurobiological questions. To demonstrate the potency of this system some examples are summarized here. The artificial expression of tetanus toxin (UAS-*tnt*) enables to inhibit neuronal synaptic activity by cleavage of synaptobrevin (Sweeney et al., 1995), or a dominant negative form of dynamin called *shibire*^{ts} allows silencing of neurons by blocking normal endocytosis for synaptic vesicle recycling, thereby causing an impairment of synaptic transmission at high temperature (Kitamoto, 2001). This temperature-induced block of synaptic transmission is reversible by shifting back to the permissive temperature (Koenig and Ikeda, 1989; Kitamoto, 2001 and see also chapter I.4, Michels et al. 2011). As an effective counterpart, *dTrpA1* encodes for a Transient Receptor Potential (TRP) channel that is required in a small number of neurons in the brain for temperature preference in *Drosophila* (Hamada et al., 2008), which can be used to activate neuronal activity in a temperature-dependent manner (Krashes et al., 2009). The UAS-*Cameleon2.1* allowed to monitor Ca^{2+}-levels and thereby the activity of neurons (Diegelmann et al., 2002). Since the advent of the Channelrhodopsin, a directly light-activated cation-selective ion channel (Nagel et al., 2003), it is possible to directly activate specific neurons by only switching blue light on (Schroll et al., 2006). These methods can now be used to figure out the molecular function of any gene, protein or cell type of interest not only in adult flies, but also in larvae.

In this thesis two further features on the EFFECTOR side were used. RNA interference, first published for *C. elegans* (Hannon, 2002) can be used to knock-down a gene of interest specifically in any genetically defined subset of cells. In the last years, several stock centres for RNAi lines have been established (Dietzl et al., 2007; e.g. Vienna Drosophila RNAi Center) to investigate the necessity of proteins locally or to mimic effects of null mutants in different kinds of behaviors.
In chapter I.5 for SAP47 (Funk et al. 2004; Saumweber et al. 2011b) as well as in chapter I.4 for Synapsin (Michels et al., 2011) RNAi strains were generated, crossed to a pan-neural *Gal4*-strain (*elav*-Gal4). The yielding progeny was used to mimic the defective associative learning of the respective null mutant larvae of SAP47 (*Sap47*^{156}) and Synapsin (*syn*^{97}).
The second used feature is generating rescue strains investigating the sufficiency of proteins. The Gal4/ UAS system enables to restore the expression of proteins in that null mutant background locally to figure out, if and where this protein is sufficient to bear its function. With knowledge of the coding region of *Sap47* its cDNA can be expressed artificially in genomic null mutants (*Sap47*^{156}) generated by jump-out mutagenesis (Funk et al., 2004). This means that although *Sap47*^{156} mutants cannot express SAP47 genomically, because a deletion in this gene leads to a total absence

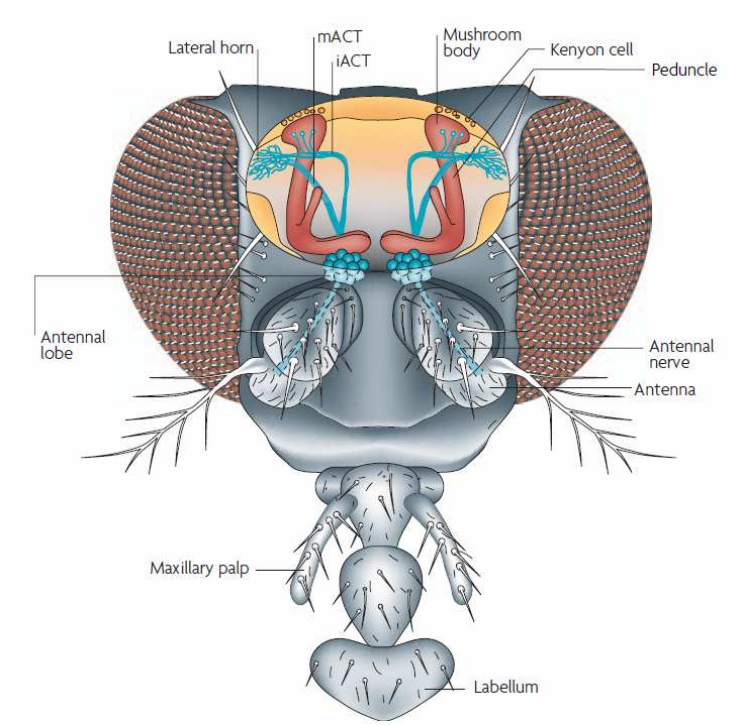
of SAP47; but artificially, Gal4/ UAS-directed expression of *Sap47* in the whole nervous system of these mutants restores its function. In chapter I.4 (Michels et al., 2011) the conserved phospho-protein Synapsin was transgenically expressed in *synapsin* mutants (syn^{97}; Godenschwege et al., 2004; Michels et al., 2005) locally using different mushroom body specific Gal4 strains to figure out the cellular site, where in the larval brain a Synapsin-dependent memory trace is localized. To see whether the defect in associative function upon lack of Synapsin is indeed due to an acute requirement of Synapsin, we further induced expression acutely before the behavioral experiment using *tub*-$Gal80^{ts}$ for temporally control. On the molecular level to investigate the molecular mechanism, we test whether also mutated forms of the Synapsin protein lacking functional phosphorylation sites, are able to function associative learning.

To summarize these methods are powerful tools to regulate gene expression in *Drosophila* unrivalled in any higher organism.

I focused on olfactory conditioning in the fly, because of two reasons. First, olfaction is a vitally important sense for mostly all animals. Sophisticated olfactory systems have evolved to detect and encode identities and intensities of odors and interpret them for orientation, finding food and social interactions. Second, enormous knowledge about the chemosensory pathway, involved structures and cells emerged since *Drosophila* entered the field of learning and memory.

The chemosensory system of Drosophila

As reviewed (Stocker, 1994; Davis, 2004; Ache and Young, 2005; Gerber et al., 2009), the anatomical organization of the chemosensory system of flies, mice and even humans shares many fundamental similarities. The convergence is due to the connectivity of involved cell types, which can be divided in first, second and third order neurons. It implies that this circuitry is an optimal solution to detect and discriminate different odors (Ache and Young, 2005). But remarkably, the insect olfactory systems, in particular in *Drosophila*, comprise much fewer numbers of cells than the vertebrate systems (Fig. 2 and 3B; see also Gerber and Stocker, 2007; Vosshall and Stocker, 2007; Keene and Waddell, 2007; Stocker, 2009).



from Keen and Waddell, 2007

Figure 2: Cartoon *Drosophila melanogaster* head

Dorsal view of an opened fly head showing the main elements of the olfactory pathway. Odors are sensed by olfactory sensory neurons in the antennae and maxillary palps. These neurons project axons along the antennal nerve to the antennal lobe glomeruli. From there the olfactory information is relayed by projection neurons to the higher brain centers, the mushroom body and the lateral horn. Gustatory stimuli are sensed by gustatory receptor neurons in the labellum on the tip of the proboscis, the elongated fly mouthpiece and processed further to the suboesophageal ganglion.
mACT: medial antennocerebral tract; iACT: inner antennocerebral tract.

Flies have two olfactory organs, antennae and maxillary palps (Fig. 2) beset with three morphological types of sensilla, basiconic, trichoid, and coeloconic (Stocker, 1994). Whereas basiconic sensilla are found on both olfactory organs, trichoid and coeloconic sensilla are located exclusively on the antenna. Basiconic olfactory sensory neurons sense general odorants, trichoid neurons respond poorly to odorants, but respond mainly to pheromones (Clyne et al., 1997; Hallem and Carlson, 2006; van der Goes van Naters and Carlson, 2007). These sensilla house ~ 1300 olfactory sensory neurons. As in most animals also in the fly each olfactory sensory neuron usually expresses a single type of olfactory receptor. All olfactory sensory neurons expressing the same olfactory receptor converge upon single glomeruli in the primary olfactory center, the antennal lobe homologous to the olfactory bulb in mammals (Davis, 2004). From there the information is processed further by uniglomerular outcome neurons, projection neurons in the fly and mitral cells in mammals, to higher brain centers, the mushroom body and the lateral horn in the fly (reviewed in Stocker, 1994; Keen and Wadddel, 2007; Gerber et al., 2009) and the olfactory cortex in mammals. This contains

the anterior olfactory nucleus, olfactory tubercle, enthorinal cortex, piriform cortex and cortical amygdale (reviewed in Mori and Sakano, 2011).

In contrast to the vertebrate system, where all kinds of olfactory receptors are G protein-coupled receptors (Spehr and Munger, 2009) in *Drosophila* three kinds of chemosensory receptors, being involved in detecting olfactory cues, are described so far. Firstly described were the also G protein coupled receptors encoded by the *Or*-gene family. It comprises 62 members selectively expressed in subsets of olfactory neurons in the antennae and maxillary palps (Vosshall et al., 1999; Touhara and Vosshall, 2009). These receptors have an inverted membrane topology, constituting a key difference between the olfactory systems of insects and other animals (Benton et al., 2006; Lundin et al., 2007; Deng et al., 2011). One of them, *Orco (synonymous to previously Or83b),* is co-expressed with the other 61 conventional olfactory receptors in single olfactory neurons (Krieger et al., 2003; Larsson et al., 2004; Jones et al., 2005). *Orco* was identified as an essential constant subunit of the heteromultimeric olfactory receptors that form a receptor complex with the variable ligand-binding other 61 *Or*-gene coded receptors (Benton et al., 2006; Larsson et al., 2004; Neuhaus et al., 2005). Subsequently a family of divergent seven-transmembrane-domain receptor genes, distantly related to the olfactory receptors, was isolated (Clyne et al., 2000) and named gustatory receptor family, because many of the gustatory receptor genes were expressed in taste organs such as the labial palps (Scott et al., 2001; Dunipace et al., 2001). But additionally a few members of the gustatory receptor family are expressed in olfactory organs, where some have been found to mediate response to CO_2 (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004). Recently a third family of ~ 60 receptors called ionotropic receptors has been identified, of which several are expressed in olfactory sensory neurons of coeloconic sensilla (Benton et al., 2009). In contrast to other receptor repertoires containing seven transmembrane domains, these ionotropic receptors are related to ionotropic glutamate receptors and are predicted to contain three transmembrane domains a pore loop (Benton et al., 2009). It was further demonstrated that these receptors act in combinations of up to three subunits, comprising individual odor-specific receptors and one or two broadly expressed coreceptors, similar to the olfactory receptor coded by the *Or*-gene family (Abuin et al., 2011). Misexpression of ionotropic receptor neurons is sufficient to confer novel odor responsiveness, supporting the hypothesis that they function directly in odor detection (Benton et al., 2009). Furthermore, at least one population of these ionotropic receptor-expressing neurons converges on to a single glomerulus in the antennal lobe, similar to the wiring logic established for olfactory receptor-expressing neurons (Benton et al., 2009).

The plurality of receptors allows the fly detecting a vast number of odor identities as well as intensities. The discrimination ability further depends on one hand on combinatorial coding and on the other hand potentially on circuit-level interactions at multiple steps of olfactory processing. As in mammals, olfactory sensory neurons expressing the same receptor are collected in one of ~ 50 glomeruli in the antennal lobe (Vosshall et al., 2000; Couto et al., 2005; Fishilevich and Vosshall, 2005). Within the antennal lobe both, inhibitory as well as excitatory multiglomerular local interneurons modify the activity pattern (Ng et al., 2002, Wilson et al., 2004; Wilson and Laurent, 2005; Shang et al., 2007; Asahina et al., 2009; Huang et al., 2010). There is likely also peptidergic input in the antennal lobe (Busch et al., 2009; Selcho et al., 2009; Carlsson et al., 2010; Pauls et al., 2010a). Individual odors activate distinct subsets of olfactory receptors. This results in a distinct glomerular activation pattern for each odor. From there uniglomerular projection neurons then carry olfactory information further towards the central brain, namely to the mushroom bodies and the lateral horn. These two higher centres are thought to control distinct olfactory functions. The mushroom bodies represent key regions for olfactory learning, whereas the lateral horn appears to be involved in innate olfactory behavior (reviewed in Gerber et al., 2009). Mushroom bodies are bilaterally symmetric structures and consist of approximately 2500 intrinsic neurons per brain hemisphere, so called Kenyon cells. They can be classified into three major types based on their axonal projections: γ neurons form a single medial lobe and α/ β neurons, whose axons branch to form a vertical (α) and a medial (β) lobe. Additionally there are α′/ β′ neurons, which also form a vertical (α′) and a medial (β′) lobe (Crittenden et al., 1998). Several studies demonstrated that the mushroom bodies can not only be subdivided immunohistochemically on the basis of the expression pattern of different reporter genes (Yang et al., 1995; Crittenden et al., 1998; Strausfeld et al., 2003; Blum et al., 2009) but also functionally (Zars et al., 2000; Pascual and Preat, 2001; Riemensberger et al., 2005; Akalal et al., 2006; Yu et al., 2005; Krashes et al., 2007; Wang et al., 2004; Blum et al., 2009). Output from the mushroom bodies then projects to different target regions including premotor areas. In adults there are first hints for mushroom body output neurons (Ito et al. 1998; Tanaka et al., 2008; Séjourné et al., 2011; Tanimoto H, MPI für Neurobiologie, München; Gerber B, Universität Leipzig and Thum A, Université de Fribourg; personal communication), but the target areas in detail have to be verified. Such architecture, the convergence from many olfactory sensory neurons to only a few antennal lobe glomeruli on one hand and from few projection neurons to many Kenyon cells on the other hand seems to be convenient to accomplish a good signal-to-noise ratio and the ability to discriminate between many olfactory cues (Gerber et al., 2009).

The gustatory system of the fly is complex and not as well described as the olfactory system. Flies have a functional homologue of our tongue, the proboscis. A total of approx. 660 receptors are encoded by the *Gr*-gene family (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Some of them are found in hairs on legs, wings, the labellum, and even the ovipositor (Stocker, 1994). Gustatory receptor neurons of *Drosophila* occur in three different types of sensilla, called taste hairs, taste pegs and hairless sensilla (Stocker, 1994; Rodrigues and Siddigi, 1978). Anatomical studies have shown that gustatory receptor neurons from different peripheral tissues project to different areas of the suboesophageal ganglion and tritocerebrum, but lack a glomerular organization like that in the antennal lobe (Edgecomb and Murdock 1992; Kent and Hildebrand, 1987). Interestingly, on the behavioral level flies can sense relatively few modalities, sweet, salt and bitter. They show similar behavior towards them as mammals from attraction to repulsion. In the suboesophageal ganglion gustatory receptor neurons may directly contact and stimulate modulatory neurons conveying the reinforcer properties of the gustatory stimuli. These modulatory neurons then interconnect the suboesophageal ganglion to higher brain centers (reviewed in Keen and Waddel, 2007).

The reward and punishment signalling in adult Drosophila *and other insects*

Dopamine and octopamine, two biogenic amines in insects are the key player mediating punishment in aversive or reward in appetitive learning, respectively (Giurfa, 2006). In honeybees and crickets pharmacological blocking of dopamine receptors impairs aversive olfactory memory formation, whereas octopamine receptors seems to be required for appetitive memory formation (Farooqui et al., 2003; Unoki et al., 2005; Vergoz et al., 2007; Mizunami et al., 2009). Injection of octopamine into the mushroom body calyces or the antennal lobe produces a lasting, pairing-specific enhancement of proboscis extension reflex, whereas injection of octopamine into the lateral protocerebral lobe, does not (Hammer and Menzel, 1998). Electrical stimulation of a single octopaminergic neuron, the VUM_{mx1} neuron, identified by Hammer, 1993, was also shown to be sufficient to substitute the reinforcing function of sucrose in an appetitive olfactory learning paradigm (Hammer, 1993).

In the fly, nothing is known yet, about the sensing of the electric shock, but Schwaerzel et al., (2003) could show that blocking synaptic output of dopaminergic neurons during training impairs aversive, but not appetitive olfactory learning. These neurons are strongly activated by electric shocks shown by functional imaging experiments on dopaminergic neurons innervating the mushroom body. The induced activation by odors is prolonged after odor/ shock pairing (Riemensperger et al., 2005). In turn, $T\beta H^{M18}$-mutants,

which lack *tyramine-β-hydroxylase*, the last necessary enzyme for octopamine synthesis, are impaired in appetitive olfactory function, whereas aversive associative function is not significantly reduced (Schwaerzel et al., 2003). Expression of $T\beta H^{M18}$ cDNA in a set of putatively octopaminergic/ tyraminergic neurons similar to the VUM cluster of the honeybee (Sinakevitch and Strausfeld, 2006; Busch et al., 2009) is sufficient to rescue this mutant learning phenotype (Thum et al., 2007).

Taken together in adult flies, it seems likely that dopamine signalling is sufficient for mediating an aversive and octopamine an appetitive unconditioned stimulus. The identification of defined subset of cells remains to be proven.

Using *Drosophila* as model organism for learning and memory research enables further to figure out cellular sites and the molecular mechanism, which are topics of ongoing research to get hopefully a comparably detailed idea of these processes in adult flies (Heisenberg, 2003; Gerber et al., 2004a, 2009).

Molecular mechanisms of associative function

Learning is the capability to change behavior based on individual experience. This is supposed to come about through changes in neurons, and memory guided behavior relies on these changes (Lechner and Byrne, 1998; Martin et al., 2000; Cooke and Bliss, 2006; Gerber et al., 2004a; Heisenberg and Gerber, 2008). In this brief overview I will focus on olfactory associative learning and even on short-term memory on the molecular level. Notably, *Drosophila* is able to associate also other stimuli, like e.g. different kinds of visual pattern with positive or negative reinforcement signals, and also shows longer-term memories (Margulies et al., 2005; Tully et al., 1994; Blum et al., 2009; Knapek et al.; 2010). The molecular mechanism (see also Fig. 6 in Chapter I.4, Michels et al., 2011) is thought to be a modulation of synaptic transmission at Kenyon cell synapses to mushroom body output neurons. Studies of associative function first in *Aplysia* and later also in *Drosophila* revealed that activation of the cyclic adenosine monophosphate (cAMP) signaling pathway plays a critical role in learning and memory processes (Abrams and Kandel, 1988; Wu et al., 1995; Gervasi et al., 2010; Michels et al., 2011). In *Drosophila* the adenylate cyclase, a doubly-regulated enzyme synthesizing cAMP (Levin et al., 1992), is encoded by the *rutabaga* gene, acting as a molecular coincidence detector of the to-be-associated stimuli (Dudai et al., 1988; Abrams et al., 1998; Heisenberg, 2003). Only the simultaneous arrival of the conditioned stimulus (e.g. an odor signal, via calcium/ calmodulin) and the reinforcement signal (activation of G-protein coupled octopamine or dopamine receptors) at the presynapse

activates the adenylyl cyclase (Abrams et al., 1998). Adenylate cyclase activation then leads to an increase of cAMP level, which then activates the protein kinase A (PKA). Further, PKA phosophorylates its target proteins, including *Synapsin* (Knapek et al., 2010; see also chapter I.4, Michels et al., 2011). Phosphorylation of Synapsin leads to a release of synaptic vesicles from the reserve pool and further to a recruitment to the readily releasable pool. Upon a subsequent presentation of the learned odor, more transmitter can be released (Hilfiker et al., 1999). This strengthened output is proposed to mediate conditioned behavior towards the odor at the test situation.

Localizing a memory trace

The localization of memory traces has occupied neuroscientists throughout this century (Lashley, 1929). Functionally, several experiments showed that the mushroom bodies house an olfactory memory trace for electroshock associated short term memory in adult *Drosophila* (Zars et al., 2000; Blum et al., 2009; Krashes et al, 2009). In Gerber and Heisenberg discussed four criteria for localizing a memory trace (Gerber et al., 2004a; Heisenberg and Gerber, 2008)

1. Neuronal plasticity occurs in these cells and is sufficient for memory
2. The neuronal plasticity in these cells is necessary for memory
3. Memory cannot be expressed if these cells cannot provide output during test
4. Memory cannot be established if these cells do not receive input during training

1) Neuronal plasticity is the process of neurons to change their biochemical, physiological and morphological properties dependent on conditioning procedures. Although physiological techniques improved impressively, at the moment no direct measurement of neuronal plasticity is available neither for central neurons of adult flies nor larvae *in vivo*. The best approach seems to be manipulating molecular components underlying neural plasticity such as AC-cAMP-PKA signalling cascade locally (see also chapter I.4, Michels et al., 2011). Indeed, for the mushroom bodies it was shown that they have the potential for AC-cAMP-PKA dependent plasticity (Davis, 1996; Abrams et al., 1998; Gervasi et al., 2010; Akalal et al., 2011). Rescue experiments concerning this cascade showed that its function in the mushroom bodies is sufficient for olfactory associative learning (McGuire et al., 2003; Zars et al., 2000; Mao et al., 2004; Keene et al., 2004; for review see Heisenberg, 2003; Keene and Waddel, 2007 and Newquist, 2010).

2) Disrupting the regulation of the AC-cAMP-PKA cascade in the mushroom bodies by transgenically expression of a dominant negative $G\alpha_s$ protein subunit ($G\alpha_s^*$) short term

memory is abolished, whereas expression of wild type $G\alpha_s$ does not affect learning (Connolly et al., 1996). This means that regulation of cAMP levels is necessary and hence that this plasticity is necessary within the mushroom body Kenyon cells for memory trace formation (Heisenberg and Gerber, 2008).

Whereas criteria 1 and 2 refer to the AC-cAMP-PKA cascade within these mushroom body cells, the next two criteria address its function in a neuronal network by blocking synaptic output using the temperature sensitive *shibire*^{ts} tool.

3, 4) It was shown that, blocking mushroom body output during test (McGuire et al., 2001; Dubnau et al., 2001; Schwaerzel et al., 2003), and blocking input to the mushroom body during training (Schwaerzel et al., 2003) prevents flies from expressing any memory. Recently it was further shown that only a few mushroom body efferent neurons, the MB-V2 neurons, which connect the mushroom body to the lateral horn and middle superior medial protocerebrum are responsible for aversive olfactory memory retrieval (Séjourné et al., 2011).

Due to these four criteria, it has been proposed that the memory trace for the association between odor and shock is localized within Kenyon Cells. If during an activation of a pattern of Kenyon cells representing an odor a modulatory reinforcement signal like octopamine or dopamine occurs simultaneously, then output from these activated Kenyon cells onto mushroom body output neurons is suggested to be strengthened (Heisenberg, 2003; Séjourné et al., 2011). This strengthened output is thought to mediate conditioned behavior towards the odor.

Advantages of Drosophila larva:

A suitable model organism to investigate learning and memory

I focused on larval *Drosophila* throughout my thesis. It combines the advantages of the genetic toolkit available for *Drosophila,* working as well in the larva, and the even much more simple system because of lower cell numbers in comparison to the adult fly. Flies lay their eggs on ripe fruit, where all further development takes place until pupation. After egg laying, embryogenesis and larval hatching, *Drosophila* undergo three larval stages until pupariation after about 6-7 days. After pupal metamorphosis, which takes another 4-5 days, the adult fly emerges to renew the life cycle upon sexual maturity. Given that the larvae are the feeding stage, they are specialized for tracking down suitable food patches within their host piece of fruit, and their effective exploitation in the context of their equally hungry conspecifics as well as of parasitiod, fruit feeding and insectivor feeding pressure. Still, given the relatively few dimensions of behavioral demand as compared to adult flies, its brain has ten to a hundred times fewer cells than that of adult flies (see Fig. 3 e.g. in the olfactory

system; Stocker, 2001; Python and Stocker, 2002). Determination of the number of neuroblasts and the number of cell divisions suggest that there are ~10,000 – 15,000 neurons in the larval brain (Scott et al., 2009).

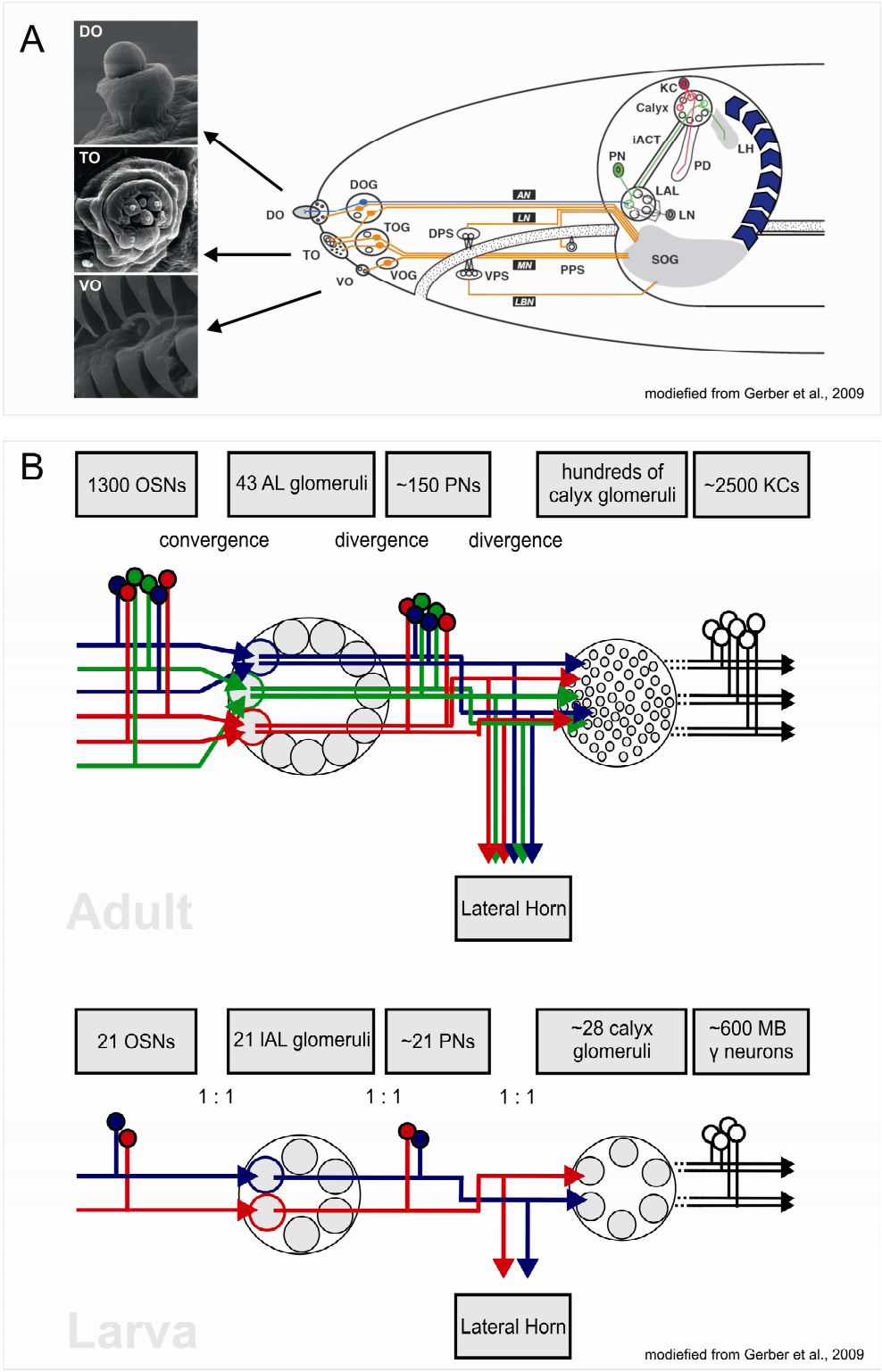


Figure 3: Overview of the larval chemosensory pathway

A: Shown is the olfactory pathway and its projection into the brain. Odor molecules are detected by olfactory receptors (*Ors*) located in the dorsal organ (DO), which send their axons to the

larval antennal lobe (lAL). Olfactory information is further processed via projection neurons (PNs) to higher brain centres, the lateral horn (LH) and the mushroom bodies (MBs). Gustatory afferents are collected in various regions of the suboesophageal ganglion (SOG).
Scanning electron microscopy (SEM): Shown are the domeshaped DO, the terminal organ (TO) and the cirri surround the open mouth and cover the ventral organ (VO) in the larva.
B: Comparison of the approximate number of olfactory sensory neurons (OSNs), AL glomeruli, PNs, MB calycal glomeruli and Kenyon cells (KCs). Note that local interneurons in the antennal lobe are present in both adult and larva, but are omitted in this figure.

Despite this reduced cell number, the layout and connectivity of these cells, especially of the olfactory system appears strikingly similar between larvae and adults as well as to mammals (Fig. 3, [Gerber et al., 2009]). Combined with the genetic tractability, this makes the larva to an obviously suitable model organism to investigate chemosensation and learning.

The chemosensory system of Drosophila larva

The cephalic chemosensory system of larvae comprise three external organs – the dorsal, terminal and ventral organ - and three inner ones – the dorsal, ventral and posterior pharyngeal sense organs (see also Fig. 3; DPS, VPS and PPS; [Singh and Singh, 1984; Python and Stocker, 2002; Gendre et al., 2004; Colomb et al., 2007a]). All these organs are mostly gustatory in nature and the well described sensory neurons in these organs include 180 gustatory and only 21 olfactory sensory neurons. Thermosensory (Liu et al., 2003), hygrosensory and mechanosensory neurons may be also present (Gerber et al., 2009). The ionotropic receptors recently described by Benton et al., (2009) may mediate also chemosensory information but the expression patterns and functions of the larval-expressed *Ir* genes remain opaque.

The gustatory system

Despite its numerical prominence, the larval taste system is relatively poorly described. The axons of external and internal taste organs are terminate in various regions of the suboesophageal ganglion, the primary gustatory centre as in adults (Gerber et al., 2009). Four major target subregions have been identified via single-cell labelling in various Gal4 driver lines (Colomb et al., 2007a; Scott et al., 2001). These regions seem to be correlated with the peripheral locations of the gustatory sensory neurons (Isono and Morita, 2010). As the genes are the same in adult and larva the larval gustatory receptors are also members of the G-protein coupled receptor family. Interesting candidates of potential taste afferents might be a set of 20 neurons in the suboesophageal ganglion expressing the *hugin* gene (Bader et al., 2007; Melcher and Pankratz, 2005). These neurons establish dendritic arborizations that partially overlap with the terminals of taste receptor neurons and send projections to the

protocerebrum, the ventral nerve cord, the ring gland and the pharyngeal apparatus (Bader et al., 2007; Colomb et al., 2007a).

Interestingly, on the behavioral level larvae are similarly responsive to gustatory cues as adult flies. They show clear preferences for various sugars (Schipanski et al., 2008), although this behavior is maybe mediated by different gustatory receptors than in adults (Colomb et al., 2007a). They show avoidance of various substances that to humans taste bitter (Hendel et al., 2005; Meunier et al., 2003; Schleyer at al., *In Press*), and show an dose-dependent switch from attraction at low concentrations of salt to avoidance of high concentrations of salt (Miyakawa, 1982; Liu et al., 2003; see chapter I.2, Niewalda et al., 2008, and chapter I.3, Schleyer et al., *In Press*). Interestingly some if not all of these behaviors are altered as the larvae mature into pupal stages. Concomitantly with an over-all less movement this involves not only a lack of behavior during pupariation, but rather a switch from e.g. light avoidance to attraction (Gong et al., 2010; see also Xu et al., 2008), from negative to positive geotaxis, and from a general attraction to odors to a general avoidance.

The olfactory system of larval Drosophila

Much more is known about the olfactory system of *Drosophila* larvae. They have one pair of olfactory organ, which is called the dorsal organ. Specifically its prominent dome is innervated by only 21 olfactory sensory neurons, in contrast to ~1300 olfactory sensory neurons in adult flies (Heimbeck et al., 1999; Oppliger et al., 2000; Kreher et al., 2005 and 2008). Single-unit recordings from many insect species including moths, honey bees, mosquitoes, and flies have revealed that different olfactory sensory neurons respond to different odors and that they also differ in response properties such as signalling mode (whether the response is excitatory or inhibitory) and response dynamics (Clyne et al., 1997; deByrne et al., 1999; Meijerink et al., 1999; van den Broek et al., 1999; Shields and Hildebrand, 2000 and 2001; deByrne et al., 2001; Meijerink et al., 1999; Laurent et al., 2002; Nikonov and Leal, 2002; Ochieng et al., 2003; Stensmyr et al., 2003a and b; Hallem et al., 2006). As in adult flies Kreher and colleagues identified the conventional, ligand-specific expressed *Or* genes in the *Drosophila* larvae encoding for the seven transmembrane domain proteins (Vosshall et al., 1999) like their mammalian counterparts (Buck and Axel, 1991). All of the 21 olfactory sensory neurons in the larvae as well as the majority of adult olfactory sensory neurons express additionally the *Orco* gene (synonymous to previously *Or83b* in Benton et al., 2006; Larsson et al., 2004; Vosshall et al., 1999). Or83b is an obligatory

coreceptor that associates with the conventional olfactory receptors forming a heterodimeric Or/ Or83b complex required for receptor function (Larsson et al., 2004; Benton et al., 2006; Pellegrino and Nakagawa, 2009). Each type of olfactory receptor is only expressed in one single olfactory sensory neuron and determines the response spectrum to different odors (Kreher at al., 2005 and 2008). As one can see in Fig 3, the larval central olfactory pathway largely shares the layout and types of neurons of its adult counterpart, but is much simpler in terms of cell numbers (Python and Stocker, 2002). Each of the 21 olfactory sensory neurons sends its axon to a single glomerulus in the antennal lobe (Fishilevich et al., 2005; Kreher et al., 2005; Ramaekers et al., 2005). Local interneurons interconnect the 21 larval antennal glomeruli to shape olfactory coding (Ramaekers et al., 2005). To date it remains to be verified that there are also cholinergic excitatory local interneurons in the larval antennal lobe (Thum A, Université de Fribourg; personal communication). Each glomerulus appears to be innervated typically by only one projection neuron (Ramaekers et al., 2005), suggesting the number may be not much higher than ~21. Similar to the situation in adult flies there are two target areas of projection neurons, first the lateral horn most likely involved generating innate odor responses, and second the mushroom body. The mushroom body calyx comprises only about 30 – 40 relatively large calyx glomeruli (Marin et al., 2005; Masuda-Nakagawa et al., 2005; Ramaekers et al., 2005). Each of them is innervated by only one or exceptionally two projection neurons. Most of the ~ 600 larval Kenyon cells per brain hemisphere (numbers vary in different studies from about 600 to ~1200; see also Technau and Heisenberg, 1982), get input from usually six randomly selected glomeruli (Masuda-Nakagawa et al., 2005; Murthy et al., 2008). This means that there is a local divergence – convergence connectivity, because projection neurons diverge onto multiple Kenyon cells, and most Kenyon cells receive input from multiple projection neurons (Masuda-Nakagawa et al., 2005; Murthy et al., 2008). Given that the numbers of olfactory sensory neurons, antennal lobe glomeruli, projection neurons and calyx glomeruli are almost the same, the larval olfactory pathway lacks convergent and divergent connectivity like in adults up to the mushroom bodies (Ramaekers et al., 2005). In this context, it is interesting that Louis and colleagues found chemotaxis behavior with only one single functional olfactory neuron on either side of the head (Louis et al., 2008). Therefore they developed a method to create stable odorant gradients in which odor concentrations were experimentally measured. Interestingly one single functional neuron (*Or42a* functional in the empty neuron system *Or83b* -/-) seems to provide sufficient information permitting larval chemotaxis behavior to a single and multiple odor source. Local heading and turning bias analysis suggested that larvae also are able to

detect the direction of local odor gradients and that the overall accuracy of navigation is enhanced by the increase in the signal-to-noise ratio conferred by bilateral sensory input (Louis et al., 2008). Although it has been hypothesized that odor quality and intensity are encoded by the combinatorial activation of different types of odorant receptors (Hallem and Carlson, 2004; Kreher et al., 2005; Kreher et al., 2008), the conclusions of Louis et al., 2008 suggest that combinatorial coding may be advantageous in expanding the sensitivity range of the overall olfactory system beyond the capacity of a single type of odorant receptor.

In general on behavioral level regarding olfaction, larvae are typically attracted by odors, but may, for some odors and at high concentrations, also show aversion (Rodrigues, 1980; Cobb and Domain, 2000; Boyle and Cobb, 2005; Kreher et al., 2008).

Learning and Memory in the Drosophila *larvae*

Despite the numerical simplicity of the larval chemosensory system, larvae have the capacity of associative learning. Similar to the adult learning paradigm reciprocal paradigms for larval learning were introduced. One group of larvae is trained X+ // Y and tested X -- Y and another group of larvae is trained reciprocally X // Y+ and also tested X -- Y.

In pioneering studies, larvae were trained to associate odors with electric shock as a aversive reinforcer (Aceves-Pina and Quinn, 1979; Heisenberg et al., 1985; Tully et al., 1994; Pauls et al., 2010a). Given that the boundary conditions to obtain robust learning scores in this paradigm remained unclear (Forbes, 1993), other kinds of olfactory learning paradigm using a different reinforcer were introduced:

1. An associative learning paradigm investigating whether larvae would learn to associate distinct odors with food types, predation risk, and temperature instead of electric shock. Dukas could show that larvae learned to avoid odors associated with predation and to prefer odors associated with high-quality food, but failed to learn about odors associated with optimal temperature (Dukas, 1999). He suggested that larvae possess a general ability to evaluate a suite of environmental parameters and associate preferred conditions with relevant stimuli.
2. A reciprocal, differential conditioning paradigm for individually assayed larvae, in which larvae associated odorants with fructose as reward (Scherer et al., 2003; Hendel et al., 2005).

3. A reciprocal, differential conditioning paradigm for individually assayed larvae, in which 'light' or 'dark' visual conditions are associated with fructose as reward (Gerber et al., 2004b).
4. An *en mass* version of odor-reward learning (Neuser et al., 2005), including a simplified one-odor variant of that paradigm to involve only one kind of gustatory reinforcer and one odor (Saumweber et al., 2011a); this is the paradigm used throughout this study.
5. Initially it was thought that odor-tastant memories in larvae can be formed for rewards only, because high-concentration salt and quinine, although aversive, did not seem to have any effect as negative reinforcers (Hendel et al., 2005). It turned out, however, that both high-concentration salt and quinine actually are effective as reinforcers, but that the respective memories are not automatically expressed in behavior (Gerber and Hendel, 2006; see chapter I.2, Niewalda et al., 2008). Rather, larvae behaviorally express aversive memory only in the presence of the negative reinforcer. Thus, conditioned escape is a part of an escape behavior which is expressed only when escape is warranted. Following the same logic, it was then asked whether, in turn, larvae trained in an appetitive manner would not show learned behavior in the presence of the reward, as was indeed obseved. Thus, conditioned approach behavior is part of a search routine which is disabled if the sought-for reward is actually present. This leads to a two-step account of conditioned behavior: First, irrespective of the test situation, the odor activates its memory trace. In a second, previously unrecognized evaluative step a comparison is made between the value of this memory trace and the value of the test situation. Only if the value of the memory trace is higher than that of the test situation, tracking down the odor can be expected to improve the situation. It is this expectation of outcome, rather than the activated memory trace *per se*, which drives conditioned behavior (Gerber et al., 2009).

Larval Learning on behavioral and cellular level

With knowledge about the chemosensory pathways and taken up the idea from Tolman together with Michael Schleyer I reanalyzed some of the data from Gerber and Hendel (2006) and performed a series of experiments investigating how olfactory memory traces, once established, actually organize behavior, and how innate and learned olfactory behavior are integrated (see chapter I.3, Schleyer et al., *In Press*). The major question in this regard is, if

conditioned olfactory behavior will be expressed according to the value of the odor (e.g. Fiala, 2007) or if conditioning will lead to goal-oriented behavior in the sense of directing escape from a repulsive situation or directing search for a more suitable situation (Gerber and Hendel, 2006; Dickinson, 2001; Elsner and Hommel, 2001; Hoffmann, 2003). To tackle this kind of questions we first analyzed innate olfactory and innate taste behavior systems for possible interactions to further specifically focus on how tastants can modulate the behavioral expression of odor-taste memory traces. It turned out that gustatory behavior is 'insulated' against olfactory processing. Tastants therefore appear of inherent, odor-independent value to the larvae being the direct basis for innate gustatory behavior. However, different kinds of associative training with odors and tastants modifies olfactory behavior (Scherer et al., 2003; Neuser et al., 2005; Gerber and Stocker, 2007), supporting that learned olfactory behavior is not an automatic, but rather a regulated process. The behavioral expression of a memory trace is neither determined by the strength of that memory trace alone, nor by the gustatory value of the test situation alone, but by the interaction of both. Thus, the interaction between what the animals expect, based on their olfactory memory and what they observe, based directly on gustatory input during test can provide them with an estimate of their behaviors´ expected gain. Notably, that study then included a behaviorally plausible minimal neuroanatomically circuit model integrating the currently available behavioral and neurobiological data.

Recently on cellular level in *Drosophila* larva Pauls et al., (2010a) looked for functional differences between embryonal and larval born cells in larval odor-reward learning. During mushroom body development proliferation of four mushroom body neuroblasts gives rise to ~250 – 300 Kenyon cells of embryonic origin and to a further ~2000 Kenyon cells of larval origin (Technau and Heisenberg, 1982; Ito and Hotta, 1992). They form the three main compartments of the larval mushroom bodies, the calyx, pedunculus, and lobes (Armstrong et al., 1998; Lee et al., 1999; Kurusu et al., 2002; Strausfeld et al., 2003). Newly born Kenyon cells send their axons into the core region of the pedunculus and push earlier born fibers to the surface, creating a characteristic layering of the pedunculus (Kurusu et al., 2002). Pauls et al., (2010a) tested first instar wild type larvae comprising exclusively embryonic born Kenyon cells and found that they are able to form appetitive olfactory associations. Correspondingly, second and third instar larvae whose mushroom bodies were chemically deprived performed not significantly different from untreated controls. These findings suggest that larval born Kenyon cells are sufficient for this learning task. In turn, they asked for the necessity of embryonic- *versus* larval-born Kenyon cells by using different mushroom body-Gal4 to drive

the temperature-sensitive dominant-negative *shibire*^{ts1} (Brand and Perrimon, 1993; Kitamoto, 2001; Duffy, 2002). It turned out that appetitive olfactory learning is impaired by blocking synaptic output of embryonic-born Kenyon cells. These experiments argue that the formation of appetitive olfactory association relay on embryonic-born Kenyon cells (Pauls et al., 2011a).

Regarding reinforcement processing, Schroll and colleagues used also the Gal4/ UAS system to genetically express *channelrhodopsin-2* (Nagel et al., 2003), a light-activated cation channel, as tool to stimulate neurons optophysiologically, in *Drosophila* larva (Schroll et al., 2006). Light-induced activation of dopaminergic neurons (*TH*-Gal4) was paired with an odor stimulus, resulting in aversive memory formation, whereas activation of octopaminergic/ tyraminergic neurons (*TDC2*-Gal4) induced appetitive memory. Thus, the net effect of dopaminergic system, as covered by *TH*-Gal4, is punishing, whereas the net effect of the octopaminergic/ tyraminergic neurons, as covered by *TDC2*-Gal4, is rewarding. These data are supported by Honjo and Furukubo-Tokunaga, 2009; for detailed discussion see also Gerber and Stocker, 2007, and chapter I.1, Saumweber et al., 2011a. In terms of requirement, an additional twist was added to this story by Selcho et al., (2009) with regard to the dopamine system. As in adult flies also in larva dopaminergic neurons innervate wide areas including protocerebra, mushroom bodies and suboesophageal ganglion. Dopamine receptors are highly enriched expressed in the mushroom bodies (Riemensperger et al., 2005, Selcho et al., 2009). Further Selcho et al., 2009 could show that aversive and also appetitive olfactory learning is strongly impaired either by blocking synaptic output of *TH*-Gal4 positive cells using *shibire*^{ts} and in dopamine receptor mutant larvae. This suggests that different types of dopaminergic neurons might be involved in different types of signalling, necessary for aversive as well as appetitive olfactory memory formation, or for the retrieval of these memory traces. Indeed, Schroll et al. (2006) showed that larvae, in which *TH*-GAL4 neurons are experimentally optogenetically activated by light together with an odor stimulus, tend to display an appetitive memory when tested in the absence of salt (Schroll et al., 2006; loc. cit. Figure S1). Clearly, tackling these kinds of questions, calls for a detailed understanding of the anatomy of the *TH*-GAL4-positive neurons on the single-cell level (Selcho et al., 2009). Taken together similar to the adult case it seems likely also in larva that dopamine signalling play a role in an aversive and octopamine an appetitive memory formation, although it remains to be proven which dopaminergic neurons are those that are mediating aversive and appetitive signalling what is major topic of present research.

Larval Learning on molecular level

Most of what is known to date about the molecular mechanisms of olfactory learning in the larva is based on odor-reward paradigm (Scherer et al., 2003; Neuser et al., 2005). On the genetic level, fairly detailed studies exist concerning the genes *synapsin*, *neurexin*, *foraging*, *presenilin* (reviewed in Gerber et al., 2009), and further *Sap47* (Chapter I.5, Saumweber et al., 2011b).

1. The *synapsin* gene (CG 3985, syn; Michels et al., 2005):
   In the fly Synapsin is encoded by a single gene homologous to three *synapsin* genes in vertebrates (Klagges et al., 1996). This presynaptic phosphoprotein is expressed throughout the whole neuropile of the larva (Michels et al., 2005 and 2011; Godenschwege et al., 2004; Hilfiker et al., 1999). It seems to be involved in the regulation of recruitment of vesicles from the reserve to the readily releasable pool of vesicles in a phosphorylation dependent manner (see later in this section). The syn^{97CS} deletion mutant (Godenschwege et al., 2004) lacking Synapsin protein show reduced learning performance of about 50% as compared to wild type (Michels et al., 2005). Chemosensation as well as motor skills not affected in syn^{97CS} mutant larvae (Michels et al., 2005). Similar results were subsequently also found in the adult fly (Knapek et al., 2010). For further details see Chapter I.4 (Michels et al., 2011).

2. The *neurexin* gene (CG 7050, *dnrx*; Zeng et al., 2007 and Li et al., 2007):
   In vertebrates, Neurexins are presynaptic transmembrane proteins (Dean and Dresbach, 2006) and interact with their postsynaptic binding partners of the Neurolignin protein family. They play an important role organizing the molecular machinery at active zones. In *Drosophila neurexin* is brain-wide expressed in larva and adult flies (Zeng et al., 2007 and Li et al., 2007). Whereas learning is intact in $white^{1118}$ mutants (see also Chapters I.4, Michels et al., 2011, chapter I.5, Saumweber et al., 2011b and chapter II, Yarali et al., 2009b), a lack of the Neurexin protein in the deletion mutant $nrx\text{-}1^{\Delta 83}$ abolishes learning completely (Zeng et al., 2007). Also in that case, the learning impairment is neither due to an impairment in smelling and/ or tasting ability nor to defective motor skills. Importantly, the learning phenotype could at least partially be rescued by spatially extended transgenic expression of *neurexin*

(using *elav*-Gal4; see also Chapters I.4, Michels et al., 2011 and chapter I.5, Saumweber et al., 2011b).

3. The *foraging* gene (CG 10033, *for;* Kaun et al., 2007a):
   The *for* gene encodes a cGMP-dependent protein kinase G and affects larval feeding behaviour (Osborne et al., 1997). Two alleles, (sitters: for^{s} and rovers: for^{R}) are described showing a behavioral polymorphism. In the absence of food locomotion is not different between genotypes, but interestingly in the presence of food larvae carrying the sitter allele forage largely within their food patch, whereas larvae with the rover allele move between patches. Dependent on the distribution of the food source, either of these strategies may be more beneficial. Although sitters and rovers differ in their protein kinase G activity, Kaun and colleagues reported that rover and sitter larvae show the same ability for visual, but not for olfactory learning, suggesting that rover larvae show higher initial performance, but lower later retention (Kaun et al., 2007b). This reduced initial learning performance in sitters can be rescued to rover levels by overexpression of the protein kinase G in the mushroom bodies (see also Pauls et al., 2010a; chapter I.4 Michels et al., 2011). PKG plays a role in food related behaviors, including energy acquisition, nutrient absorption, nutrient allocation, nutrient storage and energy use (Kaun and Sokolowski, 2009). All these aspects are related with motivational state and therefore may also affect associative function (Krashes et al., 2009). However, naïve responsiveness to odors as well as to the reward do not differ between genotypes (Kaun et al., 2007b).

4. The *presenilin* gene (CG 18803, *psn*; Boulianne et al., 1997; Knight et al., 2007): Investigating visual and olfactory learning of larvae lacking Presenelin showed that both are completely abolished (Knight et al., 2007). The $white^{1118}$ strain, which serves as genetic control, performs fine in both tasks (see also Chapters I.4, Michels et al., 2011; I.5, Saumweber et al., 2011b and II, Yarali et al., 2009b). Ones more naïve responses towards the reinforcers and towards the odors do not differ between both genotypes. In the case of olfactory learning, performance can at least partially be rescued by pan-neural expression of *presenilin* (*elav*-Gal4 driver strain; see also Chapters I.4, Michels et al., 2011; I.5, Saumweber et al., 2011b).

In the context of what had been known about the molecular mechanisms of larval odor-taste learning, the contribution of this thesis are as follows:

In cooperation with Birgit Michels I focused on Synapsin and analyzed the cellular site and the molecular mode of Synapsin action in odor-reward learning. As mentioned above this phosphoprotein is associated with synaptic vesicles, contributing to the regulation of synaptic efficacy (Hilfiker et al., [1999]; Sudhof, [2004]). 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 (Akbergenova and Bykhovskaia, 2007; Gitler et al., 2008; Hilfiker et al., 1999; Li et al., 1995). It seems that phosphorylation of Synapsin leads to a recruitment of vesicles from the reserve pool and to the ready releasable pool at active zones for release. As Michels et al., 2005 showed that larvae lacking Synapsin (in the deletion mutant syn^{97}) are impaired in odor-sugar learning, similar results are found in adult odor-shock learning, which is also reduced (Godenschwege et al., 2004; Knapek et al. 2010). Also mutant adults and have the same ability than wild type to recognize gustatory and olfactory stimuli as well as motor performance, sensitivity to experimental stress, sensory adaptation, habituation, satiation (Michels et al., 2005), and basic synaptic transmission is intact. Based on these results, we ask in which cells of the larval brain short-term odor-food reward associative memory traces are established, and what their molecular nature is. We analyzed on the cellular level where in the larval brain a Synapsin-dependent memory trace is localized, and on the molecular level whether mutated forms of the Synapsin lacking functional PKA-consensus motifs, are able to support associative function. Based on our findings similar to the situation in adult odor-shock learning, and other animal species, the type I adenylyl cyclase (AC) is proposed to act as a molecular coincidence detector for odor and reinforcement (see Abrams et al., 1998; Dudai, 1985). If both signals occur together or timely correlated the AC-cAMP-PKA cascade is triggered. We suggest that Synapsin is one of the effector proteins of PKA, such that Synapsin phosphorylation allows recruitment of synaptic vesicles from the reserve pool to the readily releasable pool. Subsequent presentation of the learnt odor leads to more transmitter being released (Hilfiker et al., 1999) and consequentially give stronger output. These processes are proposed to take place within the mushroom body Kenyon cells (adult *Drosophila*: Gervasi et al., 2010; Tomchik and Davis, 2009), and are suggested to be the basis of short-term memory after odor-shock training (Gerber et al., 2004a).

The main project of my Thesis was investigating the Synapse Associated Protein of 47 kDa (SAP47) and its role in behavioral and synaptic plasticity. SAP47 (Reichmuth et al.,

1995; Funk et al., 2004; Hofbauer et al., 2009) was identified by a monoclonal antibody screen from a hybridoma library raised against *Drosophila* brains (Hofbauer et al., 2009). The single gene, comprising 11 exons and 10 introns, codes for a protein with a highly conserved BSD domain between nematode, fly, fish and human (Reichmuth et al., 1995; Funk et al., 2004). This domain is found in BTF2-like transcription factors, SAP47 homologue proteins, and DOS2-like proteins. It is characterized by three predicted α helices and a conserved phenylalanine-tryptophan amino acid pair (Doerks et al., 2002). SAP47 is located in synaptic terminals (Reichmuth et al., 1995; Funk et al., 2004), and therefore appeared to be a good candidate to be involved in synaptic and/ or behavioral function. What we found was that SAP47 is associated with synaptic vesicles using immuno-gold-labelling in electron microscopy, although it has not a transmembrane domain. Using confocal immuno-histochemistry I report that SAP47 is expressed troughout the whole nervous system. After extensive outcrossing, we used the viable and fertile deletion mutant $Sap47^{156}$ generated by Funk et al., (2004) and confirmed the protein-null status of this mutant. Performing two-electrode voltage clamp recordings at the neuromuscular junction, we found that basic synaptic transmission in these mutants is intact, but short-term synaptic plasticity is distorted. A series of behavioral experiments gave evidence that mutants lacking SAP47 after odor-reward conditioning show reduced learning performance compared to wild type. In contrast, sensory and motor faculties as required in this learning task are normal in these mutants (see discussion above). The defect of the $Sap47^{156}$ mutant in associative function was phenocopied by pan-neurally (*elav*-Gal4) driving an UAS-RNAi-SAP47 transgene, hence reducing SAP47 expression throughout the larval brain and using a second deletion allele $Sap47^{201}$showing also the same learning impairment. After generating two rescue strains, I finally found a partial rescue by restoring the isoform of 47 kDa in the $Sap47^{156}$ mutant background, using UAS-*Sap47-RF* transgene driven by the pan-neural *elav*-Gal4 strain. A rescue up to wild-type level was found expressing UAS-*Sap47-RA* the full length isoform of SAP47 with the same driver (*elav*-Gal4).

In the last part of my thesis, I contributed a comparison of larval odor-reward learning between wild type and the $white^{1118}$ mutants to the study of Ayse Yarali (Yarali et al., 2009b). She investigated punishment and relief learning in adult *Drosophila* and looked for a role of White in both kinds of learning. She found, that $white^{1118}$ mutants show increased punishment learning and decreased relief learning, as compared to wild type flies. This is important from a practical point of view, as transgenic flies are usually in the $white^{1118}$ mutant background, such that a *mini-white* gene in the transgenic construct can help to keep track of the construct.

It turned out that the *white*^{1118} mutation has no effect on larval odor-reward learning (see also Chapter II, Yarali et al., 2009b), such that, at least in this paradigm, the behavior of transgenic flies can be interpreted without considering *white* function.

Understanding the behavioral, cellular and molecular basis of memory is a major goal of modern neuroscience, which can be investigated on different levels. This I tried during my PhD. I established a one-odor version of the larval learning paradigm, could show, that innate attractiveness and learnability can be dissociated. Further I could show that larvae can also learn about the absence of the reward on behavioral level. Together with Thomas Niewalda we could confirm that preparedness also in *Drosophila* larva matters. Larvae show a shift from appetitive learning of low salt concentrations to aversive learning of high salt concentrations and that they even show aversive conditioned behavior only in the presence of the negative reinforcer. On cellular level together with Michael Schleyer I investigated the relevant neural circuitry of how outcome changes when a larva learns. And finally, on molecular level I described that Synapsin as well as SAP47 play a crucial role in associative learning performance, whereas in *white*^{1118} mutants associative memory performance at least in the larva is unaffected.

Besides carrying out all these investigations, it turned out that *Drosophila* larva is a fantastic model system for such a rigorous, multi-level analysis of learning and memory.

I Olfactory associative learning in *Drosophila* larva

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Innate Attractiveness and Associative Learnability of Odors Can Be Dissociated in Larval *Drosophila*

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1. Innate attractiveness and associative learnability of odours can be dissociated in larval Drosophila.

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Running title

Attractiveness and learnability

Key words:

Discrimination, *Drosophila*, innate behavior, intensity, learning, olfaction, taste

Abstract

We investigate olfactory associative learning in larval *Drosophila*. A reciprocal training design is used, such that one group of animals receives a reward in the presence of odour X but not in the presence of odour Y (Train: X+ // Y) whereas another group is trained reciprocally (Train: X // Y+). After training, differences in odour preference between these reciprocally trained groups in a choice test (Test: X -- Y) reflect associative learning. The current study, after showing which odour pairs can be used for such learning experiments, (i) introduces a one-odour version of such reciprocal paradigm that allows estimating the learnability of single odours. Regarding this reciprocal one-odour paradigm, we show that (ii) paired presentations of an odour with a reward increase odour preference above baseline, whereas unpaired presentations of odour and reward decrease odour preference below baseline; this suggests that odours can become predictive either of reward or of reward absence. Further, we show that (iii) innate attractiveness and associative learnability can be dissociated. These data deepen our understanding of odour-reward learning in larval *Drosophila* on the behavioural level, and thus foster its neurogenetic analysis.

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Timo Saumweber, Jana Husse and Bertram Gerber

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I Olfactory associative learning in *Drosophila* larva

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Salt Processing in Larval *Drosophila*: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way

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2. Salt processing in larval Drosophila: Choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way.

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Running title
Salt processing in larval *Drosophila*

Key words:
Drosophila larva, feeding, learning, taste, olfaction, sodium chloride

Abstract

Sodium and chloride need to be ingested, and cannot be stored. Therefore, choice of habitat and diet as related to NaCl needs to be tightly regulated. We thus expect that the behavioural effects of salt are organized according to its concentration. Here, we comparatively 'fingerprint' the reflex-releasing (in choice and feeding experiments) versus the reinforcing effects of sodium chloride ('salt') in terms of their concentration dependencies, using larval Drosophila. Qualitatively, we find that the behavioural effects of salt in all three assays are similar: choice, feeding and reinforcing effect all change from appetitive to aversive as concentration is increased. Quantitatively, however, the appetitive effects for choice and feeding share their optimum at around 0.02 M, whereas the dose-response curve for the reinforcing effect is shifted by more than one order of magnitude 'eastward', i.e. towards higher concentrations. Interestingly, a similar shift between these two kinds of behavioural effect is also found for sugars (Schipanski et al., 2008). Thus for salt and for sugar, the sensory-to-motor system is more sensitive regarding immediate, reflexive behaviour than regarding reinforcement. We speculate that this may partially be due to a dissociation of the sensory pathways signaling toward either reflexive behaviour or internal reinforcement.

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Salt processing in larval Drosophila: Choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way.
Thomas Niewalda, Nidhi Singhal, André Fiala, Timo Saumweber, Stephanie Wegener, Bertram Gerber

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and in the printed version of this thesis on pages 65-80.

I Olfactory associative learning in *Drosophila* larva

3. A behavior-based circuit-model of how outcome expectations organize learned behavior in larval Drosophila.

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Key words
Drosophila, learning and memory, retrieval, decision-making, robotics

Abstract

***Drosophila* larvae combine a numerically simple brain, a correspondingly moderate behavioral complexity and the availability of a rich toolbox for transgenic manipulation. This makes them attractive as a study case when trying to achieve a circuit-level understanding of behavior organization. From a series of behavioral experiments, we here suggest a circuitry of chemosensory processing, odor-tastant memory trace formation and the 'decision' process to behaviorally express these memory traces- or not. The model incorporates statements about the neuronal organization of innate versus conditioned chemosensory behavior, and the kinds of interaction between olfactory and gustatory pathways during the establishment as well as behavioral expression of odor-tastant memory traces. It in particular suggests that innate olfactory behavior is responsive in nature, whereas conditioned olfactory behavior is captured better when seen as an action in pursuit of its outcome. It incorporates the available neuroanatomical and behavioral data and thus should be useful as scaffold for the ongoing investigations of the chemo-behavioral system in larval *Drosophila*.**

Introduction

Drosophila larvae, being the major feeding stages of the flies´ life cycle, have a numerically simple brain, maybe ten million times fewer neurons as compared to man, and possess correspondingly moderate behavioral complexity. These features, together with the general potential of *Drosophila* for transgenic manipulation (Sokolowski, 2001; Elliott and Brand, 2008), make them an attractive study case when trying to achieve a circuit-level understanding of behavior, in particular with regard to chemosensory processing and odor-tastant learning (Gerber and Stocker, 2007; Gerber et al., 2009).

Drosophila larvae innately ('innate' throughout this paper is used in the sense of: experimentally naïve) show positive preference for sugars (Schipanski et al., 2008) as well as to relatively low concentrations of salt (Miyakawa, 1982; Niewalda et al., 2008), but negative preference for high salt concentrations (Liu et al., 2003; Niewalda et al., 2008) and for substances that to humans taste bitter (Hendel et al., 2005; Meunier et al., 2003). Regarding olfaction, larvae are typically attracted to odors but may, for some odors and at high concentrations, also show aversion (Rodrigues, 1980; Cobb, 2000; Boyle and Cobb, 2005; Kreher et al., 2008). Given the numerical simplicity of the chemosensory system in the larva (Heimbeck et al., 1999; Ramaekers et al., 2005; Colomb et al., 2007a), a reasonably detailed understanding of innate gustatory and olfactory behavior can be reckoned with (Gerber and Stocker, 2007; Kreher et al., 2008; Gerber et al., 2009) (see Discussion).

To complicate matters, however, olfactory larval behavior can be flexible: Larvae can be differentially conditioned to associate one odor with a sweetened reward substrate, and another odor with a not sweetened substrate. After such training, larvae prefer the previously rewarded over the previously non-rewarded odor in a binary choice assay (Scherer et al., 2003; Neuser et al., 2005). Also, by punishing one odor with a bitter or high-concentration salt taste, larvae can be conditioned aversively to odors (Gerber and Hendel, 2006; Niewalda et al., 2008).

Presently, the cellular site(s) of these kinds of learning, as well as their molecular mechanisms, are the topic of ongoing research, and one can be hopeful that a comparably detailed picture of these processes can be obtained in the larva as it has been obtained for adult *Drosophila* (Heisenberg, 2003; Gerber et al., 2004a, 2009; see Discussion) and bees (Menzel, 2001; Giurfa, 2007). However, there remains a gap in our understanding of how olfactory memory traces, once established, actually organize behavior, and how innate and learned olfactory behavior are integrated. Notably, the psychological nature of olfactory behavior as response or action is under continued debate: Within cartesian tradition,

conditioned behavior often is explained by a change in value of the odor (e.g. Fiala, 2007); that is, as result of appetitive training, the odor itself is something 'good' for the animals and therefore they approach it. In other words, learned olfactory behavior, just as innate olfactory behavior, is regarded as a *response to the odor*.

Alternatively, Gerber and Hendel, (2006) (see also Dickinson, 2001; Elsner and Hommel, 2001; Hoffmann, 2003) suggested that it is more fruitful to view appetitive conditioned behavior as an action, taken *in search of food*: Specifically, at the moment of testing the difference between what the animals 'expect' (based on olfactory memory) minus what they 'observe' (based directly on gustatory input) can provide the animals with an estimate of their behaviors´ expected gain in terms of finding food. If this expected gain is positive, i.e. if memory promises a situation *better than* the current one, the larva moves towards the previously reinforced odor. Thus, Gerber and Hendel (2006) suggested that it is this expected gain of food, rather than the value of the memory trace *per se*, or of the value of the testing situation *per se*, which is the immediate cause of learned behavior. In an analogous manner Gerber and Hendel (2006) interpreted conditioned aversion as escape behavior. In this case, the expected gain takes the form of a relief from punishment.

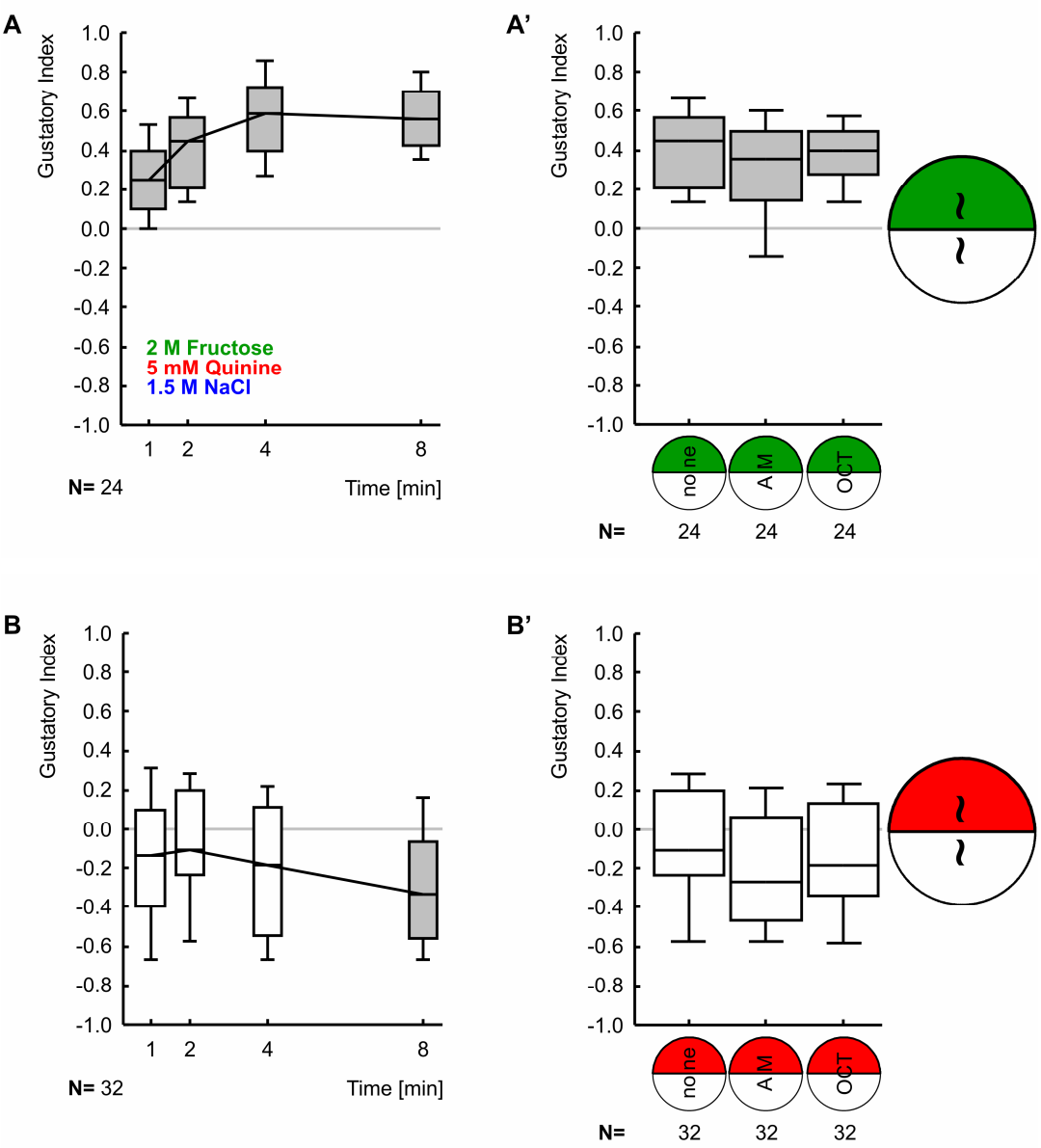
Here, we first ask whether innate gustatory behavior is affected by the presence of odors and whether in turn innate olfactory behavior is affected by the presence of tastants. Regarding associative odor-taste learning, we then ask whether and how learned olfactory behavior is affected by the presence of tastants. We report that learned, but not innate olfactory behavior is affected by the presence of tastants, and propose a minimal, neuroanatomically plausible circuitry that can accommodate the presented behavioral as well as the available neurobiological data. We suggest that the modulating effects that the tastants can exert on learned olfactory behavior at the moment of testing ensures the organization of this learned, but not of innate, olfactory behavior according to its expected outcome.

Results

Experiment 1: Is innate gustatory behavior affected by the presence of odor?

We offer experimentally naïve larvae a choice between two halves of a Petri dish, one filled with pure agarose, the other filled with agarose plus tastant (either 2 M fructose, 5 mM quinine, or 1.5 M salt). Contemplating the time courses of gustatory behavior, which is positive preference with regard to 2 M-fructose (Fig. 1A) and negative preference for 5 mM quinine (Fig. 1B) as well as for 1.5 M-salt (Fig. 1C), we choose the 2-min time point (when

gustatory behavior has not yet reached its asymptote) to test whether the presence of odor (either *n*-amylacetate diluted 1:50 in paraffin or undiluted 1-octanol) would alter gustatory behavior. This is not the case, neither with regard to fructose (Fig. 1A'), nor quinine (Fig. 1B'), nor salt (Fig. 1C') (Fig. 1: Kruskal-Wallis tests; [A'] H= 1.4, df= 2, *P*= 0.51; [B'] H= 2.9, df= 2, *P*= 0.24; [C'] H= 3.6, df= 2, *P*= 0.16). The same holds true when gustatory behavior is scored at later time points (8 min) (not shown), when tastant-effects are over-all stronger.



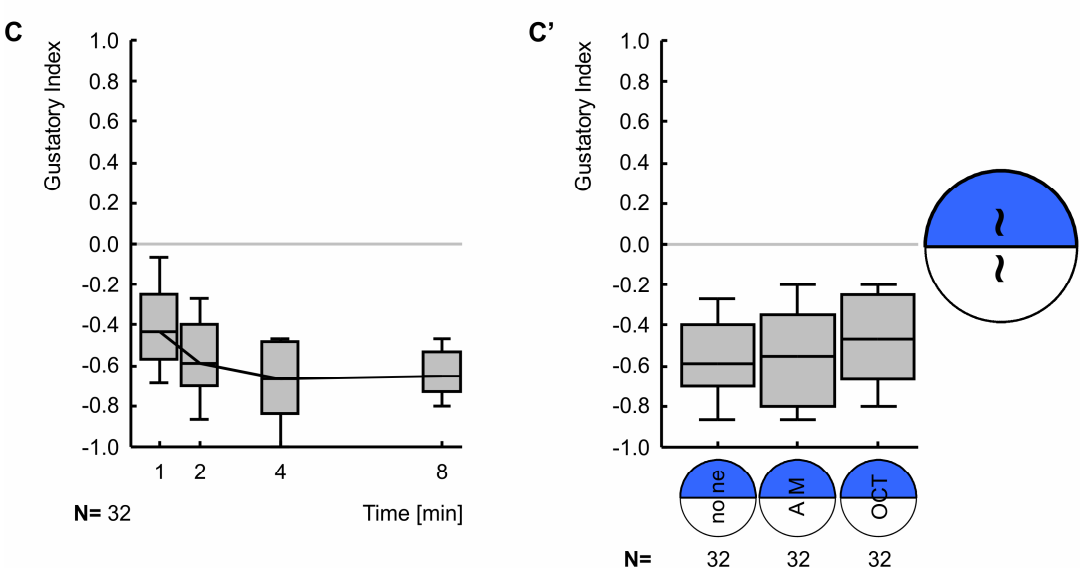


Figure 1: *Is innate gustatory behavior affected by ambient odor?*

Displayed are the tastant preferences towards (A, A') 2 M fructose, (B, B') 5 mM quinine and (C, C') 1.5 M salt. Larvae show positive preference towards fructose and negative preference towards quinine and salt. (A, B, C) show preferences over time, (A', B', C') show preference values after 2 minutes, measured in the presence of either no odor, *n*-amylacetate or 1-octanol. Please note that the 'none' scores in (A', B', C') re-present the '2 min' data from (A, B, C), respectively. The box plots show the median as the bold line, 25 and 75 % quantiles as the box boundaries, and 10 and 90 % quantiles as whiskers. Significant differences from zero ([A, B, C]: $P< 0.05/ 4$, [A', B', C']: $P< 0.05/ 3$, one-sample sign tests) are indicated by shading of the boxes.

Next, following the approach of Shiraiwa (2008), we ask whether behavior towards a fructose concentration which is just-about threshold in the absence of odor (i.e. between 0.005 and 0.015 M; Fig. 2A- C) can be pushed above-threshold by the presence of an odor; this is not the case (Fig. 2: Kruskal-Wallis tests; [A'] H= 0.77, df= 2, $P= 0.68$; [B'] H= 2.9, df= 2, $P= 0.23$; [C']: H= 3.0, df= 2, $P= 0.22$). According to the same rational, we note that odors do not alter near-threshold behavior towards quinine (Fig. 1B').

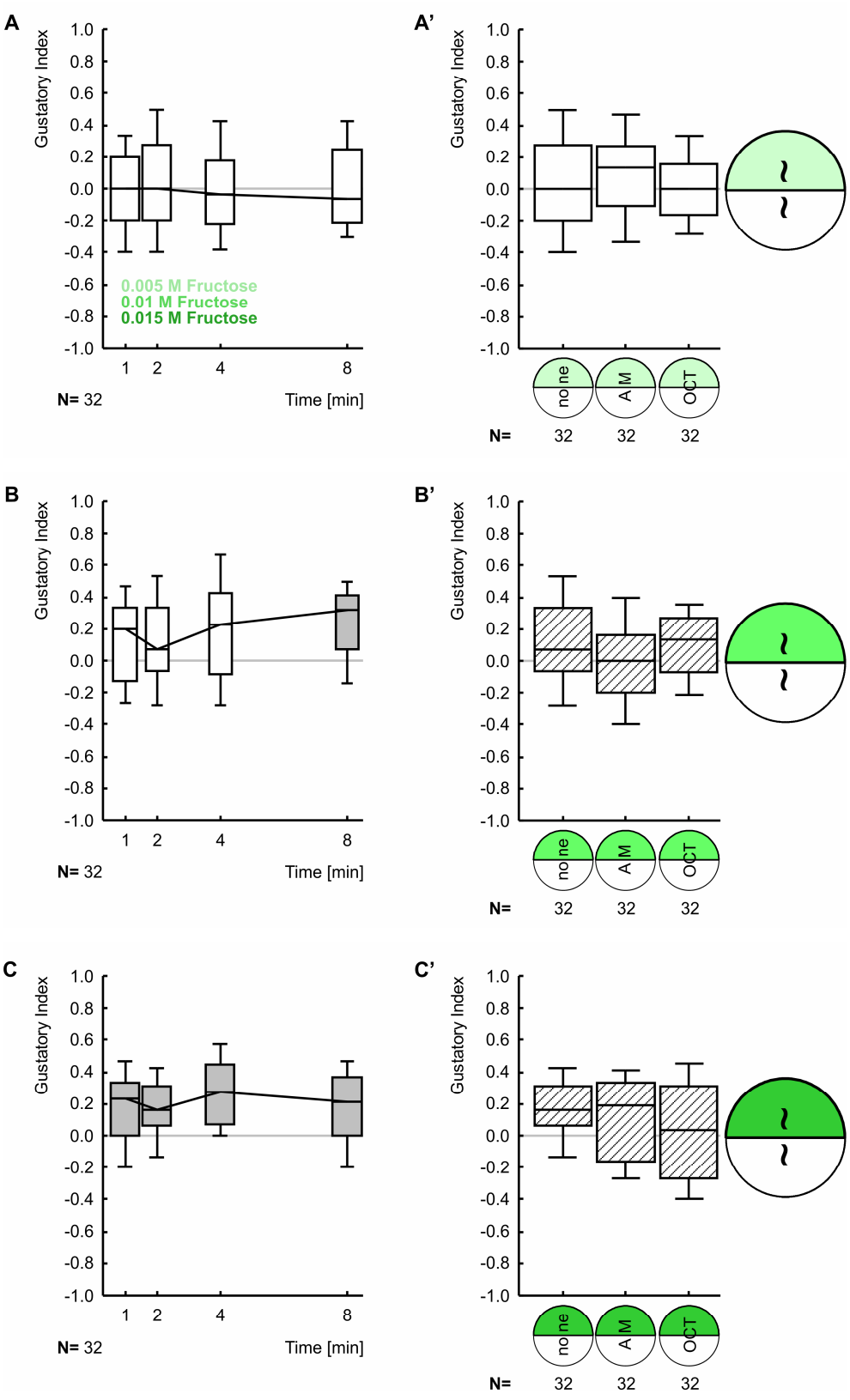


Figure 2: *Do odors affect near-threshold fructose preference?*

Gustatory preference towards (A, A') 0.005 M fructose, (B, B') 0.01 M fructose and (C, C') 0.015 M fructose. (A, B, C) show preferences over time, (A', B', C') show preference values after 2 minutes, measured in the presence of either no odor, *n*-amylacetate or 1-octanol. Please note that the 'none' scores in (A', B', C') re-present the '2 min' data from (A, B, C),

respectively. For a description of the box plots see legend of Figure 1. Significant differences from zero (P< 0.05/ 4, one-sample sign tests) are indicated by shading of the boxes. Hatched shading of the boxes indicates significant differences from zero for the pooled data (B' and C', P< 0.5/ 3 in one-sample sign-tests in both cases). Thus, as intended, at the chosen concentration range fructose preference is just around threshold.

Regarding salt, we correspondingly seek to take advantage of the fact that behavior towards salt changes from negative preference at high salt concentration towards positive preference as concentration is decreased (Niewalda et al., 2008). The point of draw between these two behavioral tendencies is 0.25 M (Niewalda et al., 2008), which we confirm here (Fig. 3A: One-sample sign-tests; P> 0.05/ 4 in all cases). We reasoned that at this salt concentration our assay should be most sensitive when testing for any modulation by odors: At this concentration, the positive and negative behavioral tendencies of salt just cancel out, so it should be particularly easy to 'tip the balance' towards one or the other kind of behavior. Such modulating effect of odor, however, is not observed (Fig. 3B: Kruskal-Wallis test; H= 0.1, df= 2, P= 0.96).

Thus, innate gustatory behavior is 'insulated' against olfactory processing. Tastants therefore appear of inherent, odor-independent value to the larvae; this value is the direct basis for innate gustatory behavior.

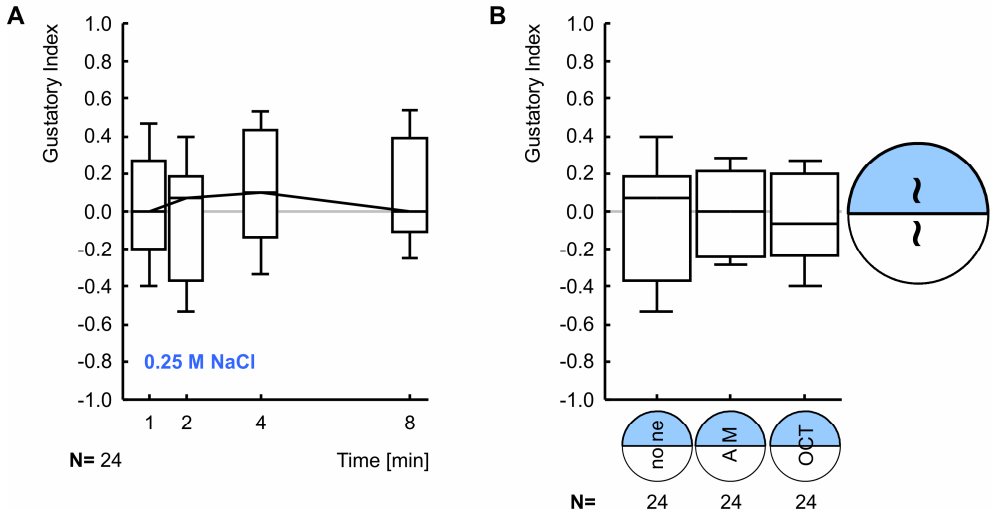


Figure 3: *Do odors tip the balance between attraction and avoidance for salt preference?*

(A) Preferences towards 0.25 M salt in the absence of odor, measured over time.
(B) Preference values after 2 minutes in the presence of either no odor, *n*-amylacetate, or 1-octanol. Please note that the 'none' scores re-present the '2 min' data from (A). For a description of the box plots see legend of Figure 1. Values of none of the groups are significant different from zero ([A] P< 0.05/ 4; [B] P< 0.05/ 3, one-sample sign tests) arguing that as intended the attractive and aversive tendencies of salt at this concentration cancel out.

Experiment 2: Is innate olfactory behavior altered in the presence of tastants?

We next ask whether in turn olfactory behavior is modulated by taste processing. Larvae are tested for their choice between an odor-filled container on one side and an empty container on the other side of a Petri dish. This test we perform on either a pure substrate, or on substrates with added fructose (2 M), quinine (5 mM), or salt (either 0.25 M or 1.5 M). We find that neither for *n*-amylacetate (Fig. 4A) nor for 1-octanol (Fig. 4B) olfactory behavior is modified by the substrate condition (Fig. 4: Kruskal-Wallis tests; [A] H= 6.9, df= 4, *P*= 0.14; [B] H= 4.5, df= 4, *P*= 0.34), even when odors are diluted to yield only moderate levels of attraction which arguably are easier to be modulated (Fig. 4: Kruskal-Wallis tests; [C] H= 0.52, df= 4, *P*= 0.97; [D] H= 8.6, df= 4, *P*= 0.1). We notice a small and non-significant tendency of higher attraction towards *n*-amylacetate (diluted 1:50) in the presence of both fructose and quinine, compared to the values obtained on the pure, tasteless substrate (Fig. 4A); the same trend had also been found in previous experiments (data not shown). However, we see this trend neither using a different concentration of *n*-amylacetate (Fig. 4B) nor for using 1-octanol (Fig. 4C, D).

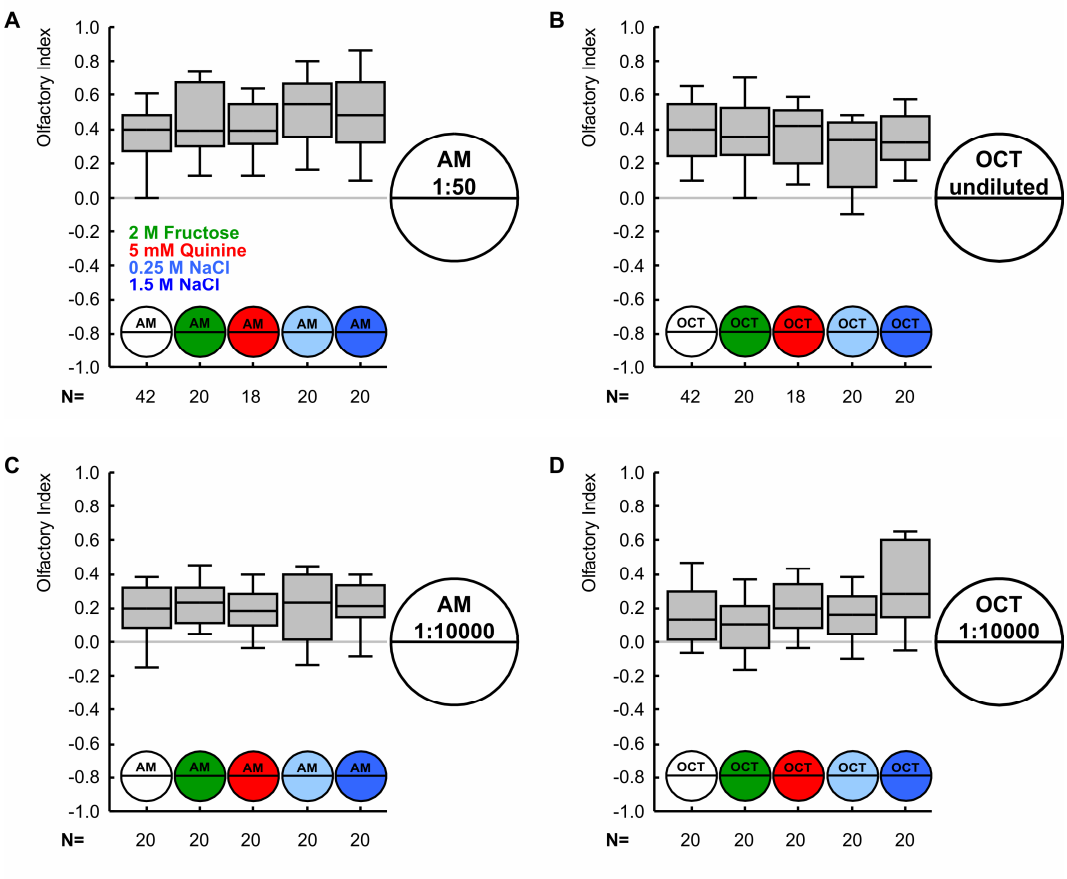


Figure 4: *Is innate olfactory behavior altered in the presence of tastants?*

The Olfactory Index is displayed, measured on the indicated tastant-substrates, regarding (A)

n-amylacetate diluted 1:50 in paraffin oil, (B) undiluted 1-octanol, (C) *n*-amylacetate diluted 1:10000 in paraffin oil and (D) 1-octanol diluted 1:10000 in paraffin oil. For a description of the box plots see legend of Figure 1. Pooled data of each graph are significantly different from zero ($P< 0.05/ 4$, one-sample sign tests).

Thus, to the extent tested, innate olfactory behavior seems to be 'insulated' from taste processing. This suggests that odors are of inherent value to experimentally naïve larvae and that this value, independent of taste processing, is the basis for innate odor attraction. In Figure 11A, we graphically represent this mutual independence between smell and taste behavioral systems.

Experiment 3: Expected gain drives learned olfactory behavior

The above experiments suggest a mutual independence of innate olfactory and gustatory processing; however, associative training with odors and tastants can modify olfactory behavior (Scherer et al., 2003; Neuser et al., 2005; Gerber and Stocker, 2007). Clearly, the formation of an odor-taste memory trace requires an interaction between olfactory processing and a taste-triggered reinforcement signal (Schwaerzel et al., 2003; Schroll et al., 2006) (Fig. 11B; for a discussion see Gerber et al., 2004a; 2009). The following experiments by Gerber and Hendel (2006) had been designed to see whether in addition there is a second kind of odor-taste interaction, during the translation of such memory traces into conditioned behavior (Fig. 5; for the corresponding Olfactory Index values see Fig. S2). They trained fruit fly larvae to associate an odor either with sugar, quinine, or salt (salt being used at either high, medium, or low concentration; this classification is based on the relative preference between quinine and salt [Fig. S1: Kruskal-Wallis test: $H= 178.9$, $df= 8$, $P< 0.05$]). A second odor was always presented without any reinforcer. They then tested for the choice between the two odors in either the absence or presence of that reinforcer which had been used for training. If the training-reinforcer was absent at test (Fig. 5A), larvae behaviorally expressed appetitive memory after sugar as well as after low-salt training; after aversive training with either quinine, high-salt or medium-salt, however, animals did not express any memory (Fig. 5A: Kruskal-Wallis test; $H= 26.4$, $df= 4$, $P< 0.05$). If in turn the training-reinforcer was present during test (Fig. 5B), the inverted pattern of results was found: Larvae showed no conditioned behavior in the presence of the appetitive reinforcers, whereas they did show conditioned aversive behavior in the presence of the aversive reinforcers (Fig. 5B: Kruskal-Wallis test; $H= 20.9$, $df= 4$, $P< 0.05$). Thus, Gerber and Hendel (2006) interpreted behavior towards previously food-associated odors as search for food, being abolished *in the presence of food*. In turn, fleeing a previously quinine-associated odor is pointless as long as there *is no quinine*.

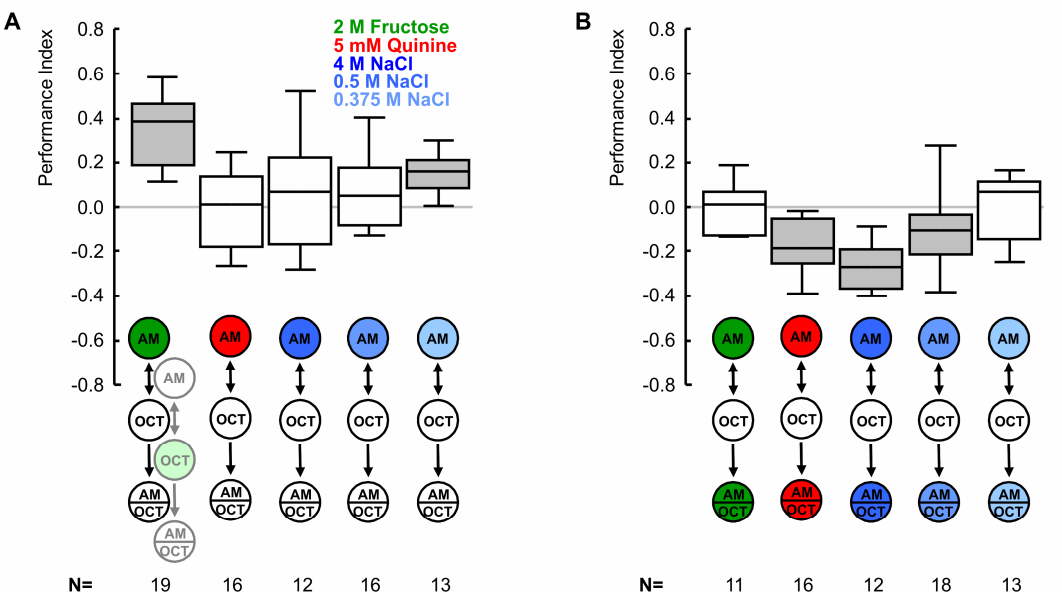


Figure 5: *Expected gain drives learned olfactory behavior (i)*

Behavioral expression of associative memory, as measured by the Performance Index, in (A) the absence or (B) in the presence of the training-reinforcer. Appetitive memories are expressed only in absence, aversive memories only in presence of the training-reinforcer. The sketches below the boxes show the training procedures and test conditions; colored circles represent Petri dishes containing tastant, white circles represent tasteless, pure Petri dishes. For example, in the left-most panel the larvae receive AM with reward and OCT without reward; then, they are tested for their choice between AM and OCT. The reciprocally trained group (dimmed display) receives AM without reward, whereas OCT is rewarded. Fom the difference in preference between the reciprocally trained groups the Performance Index is calculated. Positive Performance Indices indicate appetitive memory, negative values aversive memory. Note that the reciprocally trained groups were run in all cases, but with the exception of the left-most panel are omitted from the sketch for clarity. Also note that in half of the cases the sequence of training trials is as indicated (in the left-most panel e.g. AM+/ OCT and AM/ OCT+), but in the other half is reverse (e.g. OCT/ AM+ and OCT+/ AM). For a description of the box plots, see legend of Figure 1. Significant differences from zero ($P< 0.05/ 5$, one-sample sign tests) are indicated by shading of the boxes.
Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval *Drosophila*. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. 1).

In a next experiment, Gerber and Hendel (2006) extended these findings (Fig. 6; for the corresponding Olfactory Index values see Fig. S3). Three groups of larvae were trained such that for all groups one odor was presented with quinine, and the other odor with salt. What differed between groups was the concentration of salt, which was chosen as either high, medium, or low. Then, all groups were tested in the presence of quinine (Fig. 6A). Only the groups trained with quinine/ medium salt and quinine/ low salt showed significant conditioned aversion of the quinine-associated odor, whereas the group trained quinine/ high salt did not (Fig. 6A: Kruskal-Wallis test; H= 8.0, df= 2, $P< 0.05$). Therefore, Gerber and Hendel (2006) suggested that memories are behaviorally expressed only if doing so can improve the situation. That is, in the case of training with quinine/ high-salt, quinine was the

less bad of the two options (Fig. S1). Therefore, in the presence of quinine, no memory was behaviorally expressed (Fig. 6A). As the salt concentration was reduced, quinine became the worse of the two options (Fig. S1), and hence larvae started to behaviorally express their memory in the presence of quinine (Fig. 6A). If this reasoning is correct, the pattern of results should be inverted if animals were tested in the presence of the respective salt concentrations. This indeed was found (Fig. 6B: Kruskal-Wallis test; H= 11.2, df= 2, $P< 0.05$) (the fact that, although naïve larvae are indifferent between quinine and the medium salt concentration [Fig. S1], larvae express an avoidance of the salt-associated odor after quinine/ medium salt training [Fig. 6A] may suggest that the learning assay is more sensitive to pick up differences in value between quinine and medium salt processing).

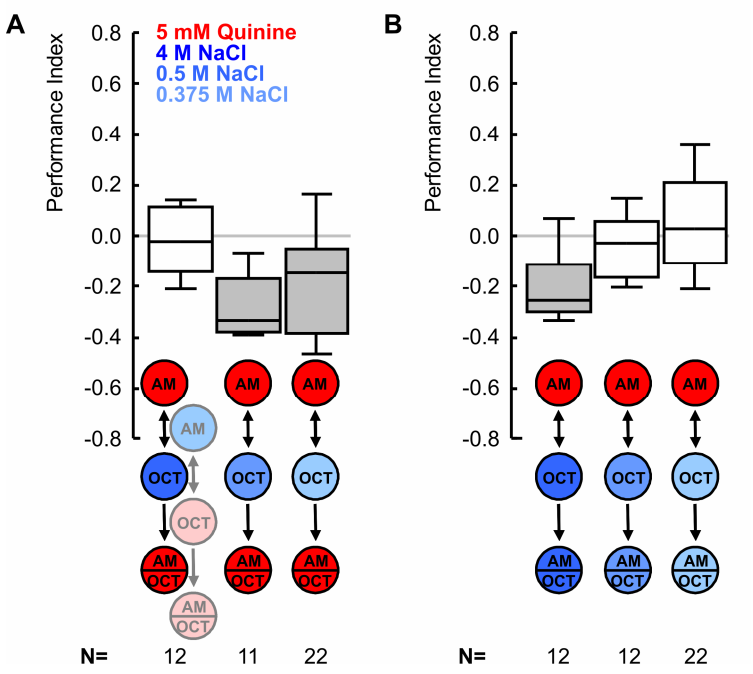


Figure 6: *Expected gain drives learned olfactory behavior (ii)*

All larvae receive one odor paired with quinine, and the other odor with salt. In different groups, the concentration of salt was either high, medium, or low. Testing is performed either in the presence of quinine (A) or in the presence of that salt concentration which had been used for training (B). Memory expression is suppressed if none of the odors predicts a gustatory environment better than the actual test situation. Other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P< 0.05/ 3$, one-sample sign tests) are indicated by shading of the boxes.
Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval Drosophila. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. 2).

We here seek to extend these findings to appetitive memory (Fig. 7; for the corresponding Olfactory Index values see Fig. S4). Using the one-odor version of the learning paradigm (see Materials and Methods), four groups of larvae are trained with a medium concentration of fructose (0.2 M) as appetitive reinforcer. The following test is performed either on a pure, tasteless substrate or on a low (0.02 M), medium (0.2 M) or high (2 M) concentration of fructose (Fig. 7: Kruskal-Wallis test; H= 39.1, df= 4, $P< 0.05$). Larvae show conditioned behavior only when the sugar concentration at the moment of test is lower than the sugar concentration during training (One-sample sign-tests; $P< 0.05/ 5$), whereas animals tested on a substrate with a sugar concentration equal to or higher than during training do not (One-sample sign-tests; $P> 0.05/ 5$). Thus, given that the four left-most groups in Figure 7 all are trained the same and consequentially will all have established the same memory trace, it is not the memory trace *per se* that determines the behavior of the animals.

If, in turn, animals are trained with a high concentration of fructose, but are tested in the presence of the medium sugar concentration (Fig. 7; right-most panel), these animals show a higher level of conditioned behavior compared to animals tested on the same medium sweet substrate, but trained with a medium sugar concentration (Fig. 7; Mann-Whitney U-test; U= 28, $P< 0.05/ 3$). Thus, also the testing situation *per se* is not a sufficient determinant of appetitive conditioned behavior (this is in contrast to the simple modulation of conditioned behavior by satiety as has recently been investigated by Krashes et al. 2009). Rather, both the memory trace and the testing situation need to be considered to accommodate learned behavior; specifically, we suggest that the animals compare the value of the activated memory trace with the value of the testing situation and show appetitive conditioned behavior depending on the outcome of this comparison.

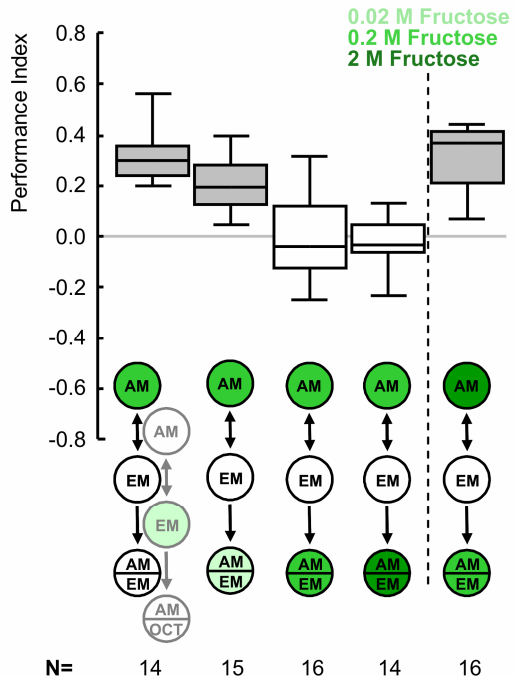


Figure 7: *Expected gain drives learned olfactory behavior (iii)*

Animals are trained using *n*-amylacetate (AM) and empty cups (EM). In all four left-most panels a medium fructose concentration (0.2 M) is used as reinforcer during training; the subsequent test is performed either in absence of fructose, or in presence of a lower-than-trained fructose concentration (0.02 M), the medium training fructose concentration (0.2 M), or a higher-than-trained fructose concentration (2 M). In the right-most panel, a high fructose concentration (2 M) is used during training, but the test is performed in the presence of the medium (0.2 M) fructose concentration. Memory is behaviorally expressed only if the fructose concentration during training is higher than the fructose concentration at the moment of test. Other details as in Figure 5; for a description of the box plots, see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel. Significant differences from zero ($P < 0.05/5$, one-sample sign tests) are indicated by shading of the boxes.

Interim Summary

Thus, in contrast to innate olfactory behavior (Fig. 4), learned olfactory behavior is massively influenced by taste processing (Fig.s 6, 7). That is, learned olfactory behavior is not an automatic (Fig. 11B), but rather is a regulated process (Fig. 11C): A comparison between what the animals 'expect' (based on olfactory memory) and what they 'observe' (based directly on gustatory input) can provide them with an estimate of their behaviors´ expected gain:

(i) Expected Gain= Expected Value - Observed Value

Learned olfactory behavior requires this expected gain to be positive. In other words, the behavioral expression of a memory trace involves a two-step process: First, the odor activates its memory trace. Second, in an evaluative step, a comparison is made between the value of that memory trace and the gustatory value of the testing situation. If the value of the memory trace for an odor is higher than that of the gustatory situation, i.e. if there is something to gain, the larva will track down the learnt odor. If the gustatory situation, however, already is as valuable as what the memory trace is promising, conditioned behavior remains suppressed.

Notably, Honjo and Furukubo-Tokunaga (2009), in contrast to the results of Gerber and Hendel (2006) (and also to our findings below), reported that quinine-induced aversive memory can be behaviorally expressed also in the apparent absence of quinine. The authors, however, bathe larvae for 30 min in quinine-solution, which despite extensive washing may induce a lingering bitter after-taste during the test (see also Discussion).

Experiment 4: Independence of appetitive and aversive memory

We next extend the above account by an 18-group experimental design in which larvae are trained differentially using one of three kinds of training regimen:

- One odor is presented with a fructose reward and the second odor without any reinforcement.
- One odor is paired with quinine punishment and the other odor without any reinforcement.
- A push-pull experimental design is used, such that one odor is rewarded and the other punished.

Animals that underwent one of these three kinds of training regimen are then tested for their choice between the trained stimuli in one of three different testing situations: On a tasteless, pure substrate, on a fructose substrate, or on a quinine substrate. Lastly, all experiments are performed using either the two-odor version or the one-odor version of the learning paradigm (see Materials and Methods for details) (Fig. S5 shows the corresponding Olfactory Index scores of all groups of larvae within this experiment).

In keeping with the above account (i), scores after quinine-only training (Fig. 8A, A': Kruskal-Wallis tests; [A] H= 23.9, df= 2, $P< 0.05$; [A'] H= 16.4, df= 2, $P< 0.05$) do not reveal conditioned avoidance of the quinine-associated odor on a pure or on a sweet substrate, thus behavior on both substrates do not differ (Fig. 8A, A': Mann-Whitney U-tests; [A] U= 97, $P= 0.52$; [A'] U= 103, $P= 0.50$). This is because the 'observed' pure and the 'observed' fructose are both better than the 'expected' quinine, such that in both cases the quinine-

memory trace is not expressed in behavior. In turn, we observe conditioned avoidance in presence of quinine, different from animals´ behavior on the pure substrate (Fig. 8A, A': Mann-Whitney U-tests; [A] U= 22, $P< 0.05/ 2$; [A'] U= 34, $P< 0.05/ 2$).

Scores after training with reward-only (Fig. 8B, B': Kruskal-Wallis tests; [B] H= 21.7, df= 2, $P< 0.05$; [B'] H= 19.5, df= 2, $P< 0.05$) are higher for the pure test situation than in the presence of fructose (Fig. 8B, B': Mann-Whitney U-tests; [B] U= 14, $P< 0.05/ 2$; [B'] U= 34, $P< 0.05/ 2$), because the 'observed' pure substrate is less valuable than the 'expected' fructose, leading to the behavioral expression of the fructose-reinforced memory trace on the pure substrate. Interestingly, scores are equal in the presence and absence of quinine (Fig. 8B, B': Mann-Whitney U-tests; [B] U= 94, $P= 0.44$; [B'] U= 124, $P= 0.52$), which is somewhat surprising: The above account (i) predicts that the value of quinine, if present at the moment of testing, is offset against the value of a fructose-reinforced memory trace. Thus, conditioned behavior should be expressed particularly strongly when larvae are tested in the presence of quinine after training with fructose, because the difference in value between the 'observed' quinine and the 'expected' fructose is particularly large. This, however, is not observed; we therefore argue that the behavioral expression of a fructose-reinforced memory trace is independent of quinine processing.

Correspondingly, one may ask whether the behavioral expression of a fructose-reinforced memory trace is possible although the behavioral expression of a quinine-reinforced memory trace is suppressed. If this were possible, scores on a pure testing substrate (Fig. 8C, C': Kruskal-Wallis tests; [C] H= 25.3, df= 2, $P< 0.05$; [C'] H= 20.6, df= 2, $P< 0.05$), which allows for the behavioral expression of a fructose-reinforced memory trace but not of a quinine-reinforced memory trace, should be equal after fructose-only and fructose-quinine training. This is indeed what we find (Fig. 8C, C': Mann-Whitney U-tests; [C] U= 112, $P= 0.98$; [C'] U= 114, $P= 0.32$), suggesting that the behavioral expression of the quinine-reinforced memory trace can remain suppressed even if the fructose-reinforced memory trace is in effect.

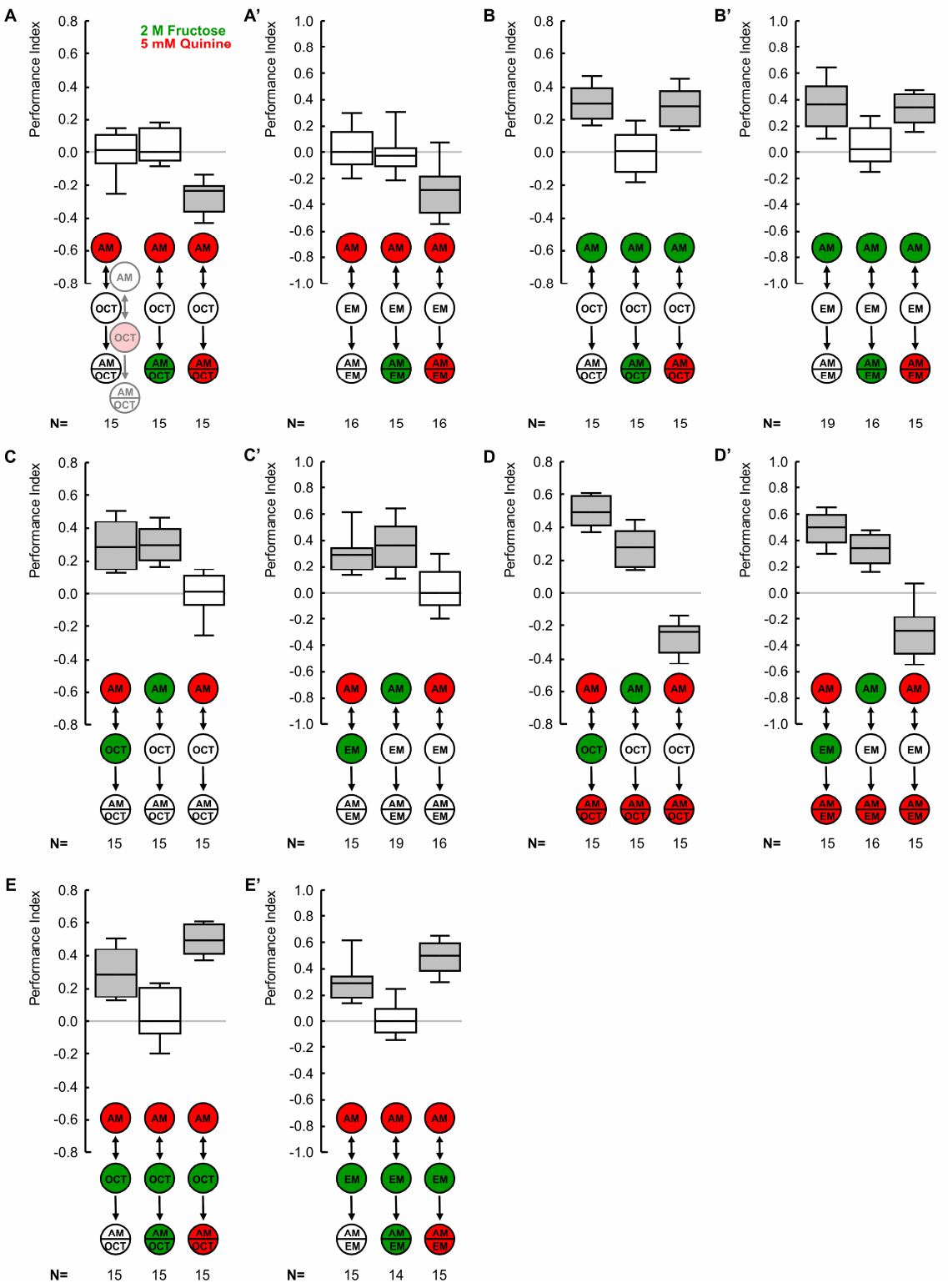


Figure 8: *Independence of appetitive and aversive memory systems (i)?*

(A, B, C, D, E) show results of a two-odor paradigm using both *n*-amylacetae (AM) and 1-octanol (OCT), whereas (A', B', C', D', E') show the corresponding results of an one-odor paradigm, using only *n*-amylacetate and empty cups (EM).
(A, A') After aversive-only training, larvae behaviorally express memory only in the presence of quinine. The presence of fructose has no effect.
(B, B') In contrast, after appetitive-only training, memory is behaviorally expressed only in absence of fructose, whereas the presence of quinine has no effect.

(C, C') If animals are tested in the absence of any reinforcer, expression of aversive memories is abolished, but expression of appetitive memories remains intact.
(D, D') Animals tested on quinine show memory after all kinds of training regimen. Importantly, scores after training in a push-pull regimen using both punishment and reward are higher than those after appetitive-only training, suggesting that both an appetitive and an aversive memory is behaviorally expressed.
(E, E') After push-pull training, scores for animals tested on quinine are higher than for those tested on pure, confirming that only under these conditions both appetitive and aversive memories are behaviorally expressed.
For convenience, some data of this 18-group experiment are included in more than one graph. Other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P < 0.05/3$, one-sample sign tests) are indicated by shading of the boxes.

Given these arguments for independence, we propose separate systems for steering conditioned search and conditioned escape (Fig. 11C, [8, 9]). Within either system, it is determined separately whether conditioned behavior is expressed- or not. That is, conditioned search is expressed if the appetitive memory promises a gain in the sense of yet-more-reward than actually is present:

(ii) Conditioned search if: Appetitive Memory > Observed Reward

Conditioned escape, however, remains suppressed as long as the actual situation is less bad than what aversive memory suggests, i.e. unless escape offers a gain in the sense of relief:

(iii) No conditioned escape if: Aversive Memory > Observed Punishment

This prompted us to ask what will happen if both conditioned search and conditioned escape tendencies are activated. On a quinine substrate, which as we have shown above allows the behavioral expression of both quinine- and fructose-reinforced memory traces, we compare the scores of groups with differing histories of training (Fig. 8D, D': Kruskal-Wallis tests; [D] $H = 34.4$, $df = 2$, $P < 0.05$; [D'] $H = 34.1$, $df = 2$, $P < 0.05$). Scores in this experiment turn out to be higher after fructose-quinine training than after fructose-only training (Fig. 8D, D': Mann-Whitney U-tests; [D] $U = 32$, $P < 0.05$; [D'] $U = 44$, $P < 0.05$). Along the same lines, we find that after fructose-quinine training (Fig. 8E, E': Kruskal-Wallis tests; [E] $H = 25.4$, $df = 2$, $P < 0.05$; [5E'] $H = 26.7$, $df = 2$, $P < 0.05$) scores are higher in the quinine than in the pure testing condition (Fig. 8E, E': Mann-Whitney U-tests; [E] $U = 48$, $P < 0.05$; [E'] $U = 38$, $P < 0.05$). We suggest that this is because in the presence of quinine both memory traces can be expressed behaviorally: Conditioned escape from quinine is expressed because quinine *is present*, and

conditioned search for fructose is expressed because fructose *is absent*. These two effects can both steer behavior independently of each other, and eventually summate in terms of the distribution of the larvae between the previously rewarded and the previously punished odor.

To further confirm our findings we partially repeat the last experiment using a high concentration sodium chloride (1.5 M; for further details see legends of Fig. 9) instead of quinine (Fig. 9; for the corresponding Olfactory Index values see Fig. S6). In replication of the results of Gerber and Hendel (2006), after punishment-only training with high salt larvae show conditioned behavior when tested in the presence of high salt but not on a tasteless Petri dish (Fig. 9A: Mann-Whitney U-test; U= 3, $P< 0.05$). Notably, after push-pull training with high salt and sugar (Fig. 9B), values are significantly higher when tested on high salt compared to the tasteless test condition (Fig. 9B: Mann-Whitney U-test; U= 51, $P< 0.05$). Thus, under appropriate testing conditions fructose-induced appetitive and salt-induced aversive memory traces can summate in behavior: On a too salty Petri dish animals both search for sugar and try to escape the high salt concentration.

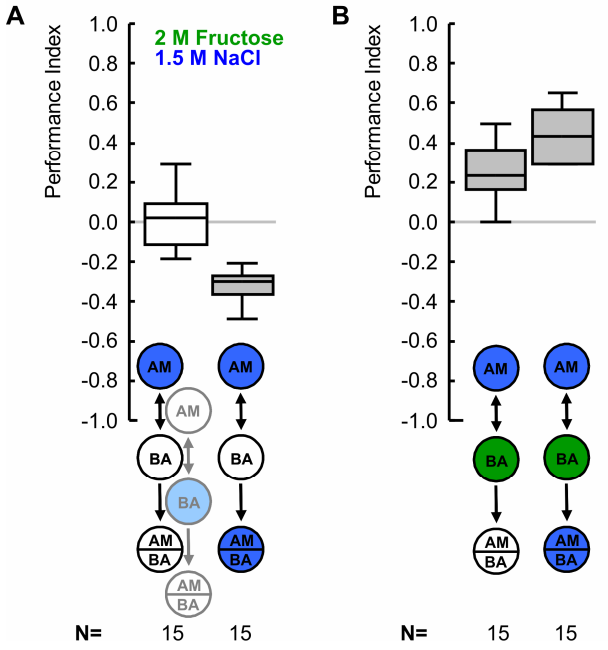


Figure 9: *Independence of appetitive and aversive memory systems (ii)?*

The experiment partially repeats and extends the one shown in Figure 8, using a slightly different protocol: As odors, *n*-amylacetate (AM), diluted 1:250 in paraffin, and undiluted benzaldehyde (BA) are used. Unrewarded, tasteless Petri dishes contain 2.5 % agarose, reward Petri dishes have 2 M fructose added and punishment Petri dishes have 1.5 M sodium chloride added.
(A) Larvae receive aversive training with salt as punishment and are tested either in absence or presence of salt. Larvae show conditioned behavior when tested in the presence of salt but not on a tasteless Petri dish.
(B) After push-pull training with salt punishment and sugar reward, performance indices are higher when tested on salt compared to the tasteless test condition, corresponding to the results of Figure 8.

All other details as in Figure 5; for a description of the box plots see legend of Figure 1. Note that the sketches below the boxes show only one possible training regimen; the reciprocally trained group is indicated by a dimmed display in only the left-most panel of (A). Significant differences from zero ($P < 0.05/2$, one-sample sign tests) are indicated by shading of the boxes.

Discussion

We first briefly sketch what is known neurobiologically about the establishment of smell-taste associative memory traces to provide a point of reference for our behavior-based model in the second part of this Discussion.

Associating smell and taste

Larvae can learn to associate an odor with taste reinforcement. This implies convergence between olfactory and taste processing. However, no such convergence has been reported to date: The olfactory system passes on its information from the dorsal organ via only 21 olfactory sensory neurons (Heimbeck et al., 1999; Kreher et al., 2005) to the larval antennal lobe (Fig. 10A), each of them targeting just one of 21 spherical 'glomerulus' compartments (Ramaekers et al., 2005). The information from a given glomerulus is conveyed further by typically just one projection neuron (Rameakers et al., 2005; but see Marin et al., 2005), connecting to both the lateral horn, a presumed premotor centre, and the mushroom bodies (Python and Stocker, 2002). In the mushroom bodies´ olfactory input region, the calyx, which is organized into reported 28- 34 glomeruli (Marin et al., 2005; Masuda-Nakagawa et al., 2005 and 2009; Ramaekers et al., 2005), projection neurons transmit their signal to several of a total of approximately 600 mature mushroom body neuons (also called Kenyon cells) (Lee et al., 1999; but see Technau and Heisenberg, 1982). A given projection neuron innervates only one calyx glomerulus, and a given Kenyon cell collects input from between one to three (Ramaekers et al. 2005) or up to six (Masuda-Nakagawa et al., 2005) calyx glomeruli. The Kenyon cells then connect to relatively few (a reasonable guess may be between one to dozens; Pauls et al., 2010a) output neurons that have projections into the lateral horn and other potential premotor centres (Pauls et al., 2010a; for the situation in adults: Ito et al., 1998; Tanaka et al., 2008; Sejourne et al., 2011). These output neurons likely receive input

from many if not all mushroom body cells, thus 'summing up' the total level of activation in their input section of the mushroom body.

The gustatory system, in turn, conveys taste information from three external taste organs (terminal organ, ventral organ, and the bulge of the dorsal organ) and three internal taste organs (ventral, dorsal, and posterior pharyngeal organ) (Singh and Singh, 1984; Python and Stocker, 2002; Gendre et al., 2004), comprising a total of approx. 90 gustatory sensory neurons per body side (Colomb et al., 2007a), to the subesophageal ganglion and then the ventral nerve cord (Fig. 10B; Melcher and Pankratz, 2005; Colomb et al., 2007a). Thus, there is no apparent direct connection between smell and taste pathways- consistent with the lack of interaction between innate gustatory and innate olfactory behavior we report in this study.

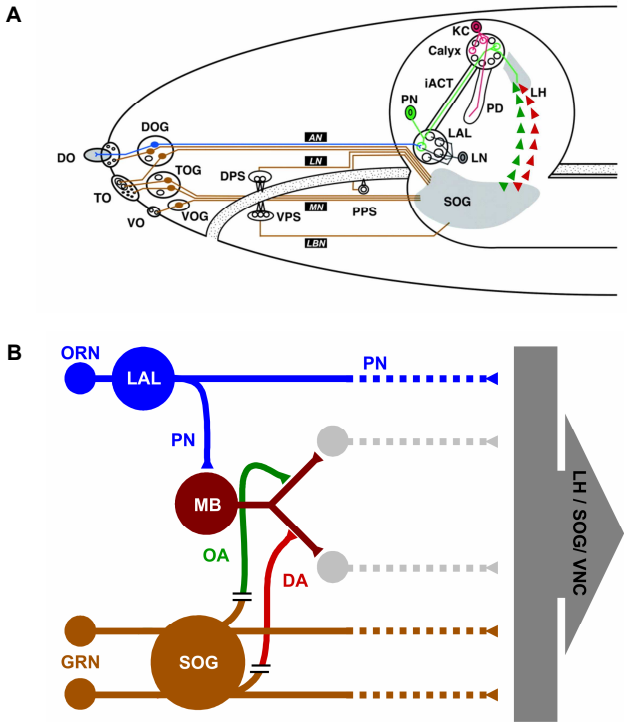


Figure 10: *Neuroanatomy of the larval chemosensory system.*

(A) Schematic diagram of the chemosensory pathways in the larval head. Modified from: Stocker RF (2008). Design of the larval chemosensory system. In: Technau GM, ed. Brain Development in *Drosophila melanogaster*. ©2008 Landes Bioscience and Springer Science+Business Media.
(B) Simplified diagram of the chemosensory pathways in the larval brain.
From the three external chemosensory organs, the dorsal organ (DO) comprises both olfactory (the 'dome'; gray) and gustatory sensilla (little circles). The terminal organ (TO), the ventral organ (VO), and the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, PPS) include mainly taste sensilla. The cell bodies of the sensory neurons are collected in ganglia below each sense organ (DOG, TOG, VOG). Olfactory receptor neurons (ORNs, blue) project into individual glomeruli of the larval antennal lobe (LAL), which are interconnected by local interneurons (LN). Projection neurons (PNs, green) carry signals from the LAL to two higher olfactory centers, the mushroom body (MB) calyx and the lateral horn (LH). One intrinsic MB Kenyon cell (KC) is shown in red. Gustatory receptor neurons (GRN, brown) extend to the subesophageal ganglion (SOG). Octopaminergic neurons (OA, green) are proposed to 'short-circuit' a taste-driven reward signal from the SOG towards the MB, dopaminergic neurons (DA, red) carry punishment signals

towards the MB. The exact neuronal elements to select particular motor programs when facing tastants and odors are unknown, but likely involve the lateral horn (LH) and ventral nerve cord (VNC).

Given this architecture, the ability of the larva to form an odor-taste associative memory trace may appear surprising. Hammer (1993) in the honeybee identified the octopaminergic VUM_{mx1} neuron, which likely receives gustatory input in the subesophageal ganglion and provides output to the antennal lobe, the mushroom body calyx and the lateral horn. This single, identified neuron is sufficient to mediate the rewarding function of sugar in honeybee olfactory learning (Hammer, 1993) (for a description of this neuron in the fly see Busch et al., [2009]; this neuron exists in larval *Drosophila* as well, A.T., unpubl.). As in the bee, also in *Drosophila* there is evidence that at least some octopaminergic neurons 'short-circuit' taste with smell pathways to mediate reinforcement signaling (Fig. 10B): Adult flies lacking octopamine are impaired in odor-sugar learning but not in odor-shock learning. In turn, blocking synaptic output from a subset of dopaminegic neurons impaired odor-shock learning but not odor-sugar learning (Schwaerzel et al., 2003). In larvae, the net effect of driving subsets of octopaminergic or dopaminergic neurons can substitute for reward or punishment, respectively, in olfactory learning (Schroll et al., 2006; this is not at variance with the observation that specific other subsets of these neurons serve different functions, see below). Whether and which of these neurons, in turn, are required for these two forms of learning is less clear (Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009). In any event, important for the current context is that the memory-forming convergence is not between the olfactory and the gustatory pathway itself, but between the olfactory pathway and a modulatory valuation signal ('good' or 'bad', respectively). Such convergence likely happens in the mushroom bodies (Honjo and Furukubo-Tokunaga, 2005 and 2009; Pauls et al., 2010a; Selcho et al., 2009; Michels et al., 2011; concerning adults see Riemensperger et al., 2005; Claridge-Chang et al., 2009; reviews by Heisenberg et al., 2003; Gerber et al., 2004a, 2009; concerning honeybees see Hammer and Menzel, 1998). If an odor is presented, a particular pattern of olfactory sensory neurons is activated, leading to the activation of a particular combination of glomeruli in the antennal lobe (Kreher et al., 2005), as well as of the projection neurons and the corresponding mushroom body neurons (Masuda-Nakagawa et al., 2005 and 2009). At the same time, a tastant, e.g. sugar, activates gustatory sensory neurons that trigger the value signal ('good') *via* e.g. some of the octopamine neurons (in the case of high-concentration salt or quinine: *via* e.g. some of the dopaminergic neurons) and send it to many, if not all Kenyon cells of the mushroom bodies (Honjo and Furukubo-Tokunaga, 2009;

Selcho et al., 2009). Conceivably, only in that subset of Kenyon cells which are activated coincidently by both the odor signal and the value signal, a memory trace is formed (Gervasi et al., 2010; Tomchik and Davis, 2009; see also Gerber et al., 2004a for discussion). The memory trace then is thought to consist of a strengthening of connection between the Kenyon cells and their output neurons: If a learnt odor is presented, Kenyon cell output is strong enough to drive the output neurons for triggering conditioned behavior. Indeed, mushroom body output is a requirement for conditioned behavior (regarding adult *Drosophila*: Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2003). Following Selcho et al. (2009) and Aso et al., (2010), we stress that the genetic tools available at present to manipulate octopaminergic and dopaminergic neurons, respectively, cover anatomically and functionally heterogeneous sets of neurons. Current research is trying to identify from these sets those neurons conferring reinforcement signaling, and to tell them apart from neurons mediating other effects, e.g. regarding olfactory processing *per se*, gustatory processing *per se*, and signaling of satiety states (Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009; also see Aso et al., 2010; Claridge-Chang et al., 2009; Krashes et al., 2009 for adult *Drosophila*).

Regarding the below discussion, two further aspects should be noted: First, for innate olfactory behavior the mushroom body loop is dispensable (deBelle and Heisenberg, 1994), but the projection neurons are required (Heimbeck et al., 2001). This suggests that innate olfactory behavior is supported largely by the direct antennal lobe-lateral horn pathway, whereas conditioned olfactory behavior takes the indirect route *via* the mushroom bodies (see also Saumweber et al., 2011a). Second, there is no evidence to argue that a given odor would not activate the same one subset of Kenyon cells during aversive as well as appetitive learning; this implies that appetitive and aversive memory traces for a given odor may be localized in the same Kenyon cells, but in distinct subcellular compartments (Fig. 11B: [6]) (see discussion in Schwaerzel et al., 2003).

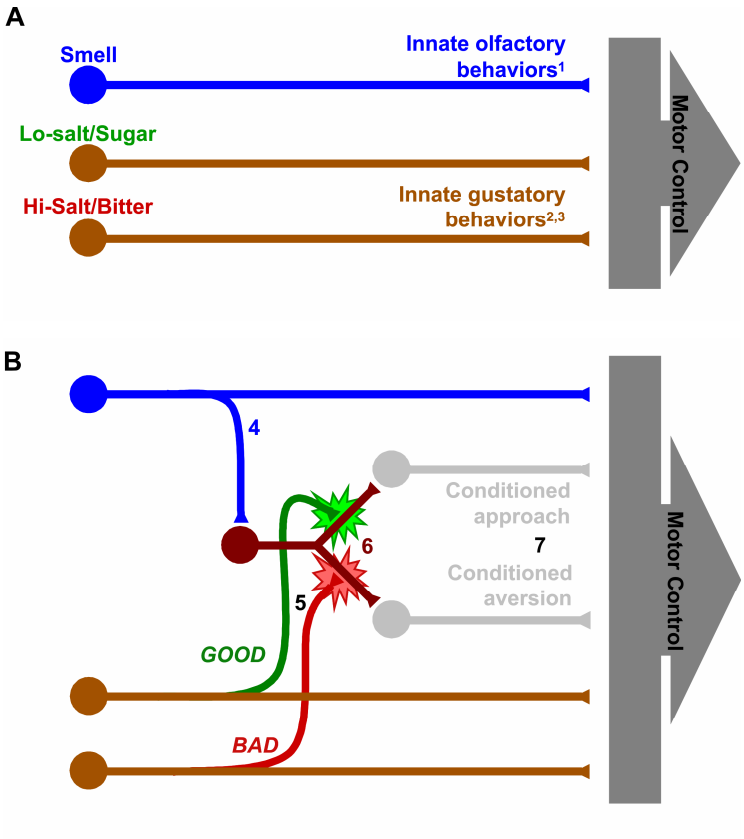
Integrating behavior

Our experimental analyses of chemosensory processing focussed on four kinds of behavior:

- innate taste behavior;
- innate olfactory behavior;
- conditioned olfactory behavior after appetitive learning (conditioned search);
- conditioned olfactory behavior after aversive learning (conditioned escape).

In this study, we asked whether the organization of these kinds of behavior is functionally independent of each other.

Clearly, both olfactory and gustatory stimuli support innate behavior. Larvae can show attraction or aversion to odors (Fig. 11A, [1]) (in order to make it easier to relate the behavioral evidence referred to in the text to the diagrams in the Figures, we add the numerals [1-8] to the figure), and show positive or negative preference for tastants, dependent on identity and concentration of the odors and tastants, respectively (Fig. 11A: [2, 3]). We could, despite effort, not find any evidence of interaction between these two pathways: Neither does ambient taste seem to affect olfactory attraction (Fig. 4), nor does in turn ambient odor have an effect on gustatory preference (Fig. 1). The latter may at first sight appear somewhat surprising, as Shiraiwa (2008) had found in adult *Drosophila* that the proboscis extension reflex, an element of feeding behavior, can be facilitated by odors. However, our results certainly do not rule out that odors may, also in the larva, have a potentiating effect on feeding behavior.



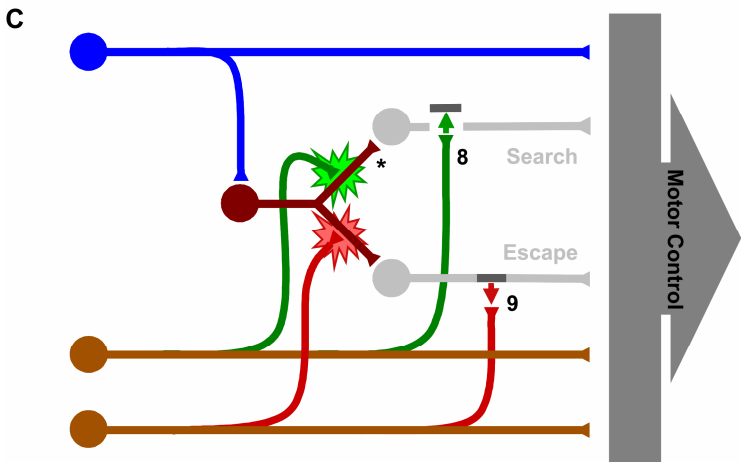


Figure 11 *Behavioral-based circuit of larval chemosensory behavior.*

The figures illustrate, in a sequential way, which kinds of circuitry have to be proposed to accommodate the behavioral experiments reported in this study. To make it easier to relate behavioral evidence referred to in the body text to these diagrams, we add numerals [1-8].
(A) Innate olfactory und innate gustatory behavior are mutually 'insulated' (Fig.s 1, 4). [1] Odors are usually attractive (Fig. 4), except at very high concentrations (e.g. Cobb and Domain, 2000; Colomb et al., 2007b). [2] Larvae show negative preference in the case of high concentrations of salt and of quinine (Fig. 1B, C; Hendel et al., 2005; Niewalda et al., 2008) and positive preference in the case of low concentrated salt and sugar (Fig. 1A; Schipanski et al., 2008). [3] We present joint cellular pathways for sugar/ low concentrated salt processing, and for bitter/ high concentrated salt processing, respectively, based on Hiroi et al., 2004. Separated cellular pathways for sugar/ low concentrated salt *versus* bitter/ high concentrated salt are based on Marella et al., 2006.
(B) Establishment of the memory trace and sketch of conditioned olfactory behavior. Larvae can associate an odor with a reward, leading to conditioned approach towards this odor (Fig. 8B; Scherer et al., 2003; Neuser et al., 2005; Schipanski et al., 2008), or with a punishment, leading to conditioned aversion to this odor (Fig. 8A; Gerber and Hendel, 2006; Niewalda et al., 2008). Trivially, this requires convergence of the to-be-associated signals. [4] The bifurcation of the olfactory pathway is based on Heimbeck et al., 2001; [5] separated cellular processing of internal reward- and punishment-signals is based on Schwaerzel et al. (2003) and Schroll et al. (2006); [6] separated sub-cellular target regions of internal reward- and punishment-signals are based on the suggestion by Schwaerzel et al., 2003; [7] separated cellular processing of retrieval of aversive and appetitive memory traces is based on Sejourne et al., (2011).
(C) Reconsidering the nature of learned behavior as conditioned search and conditioned escape behavior. Olfactory memory traces are behaviorally expressed only if animals expect to improve their situation: [8] the presence of a reward signal at the moment of testing which is at least as 'good' as predicted blocks the expression of conditioned search behavior (Fig. 8B); [9] in turn, only if at the moment of testing a punishment signal is present which is at least as 'bad' as predicted, conditioned escape behavior is expressed (Fig. 8A).
Please note that the suppressing effect of satiety on appetitive conditioned behavior (Krashes et al., 2009) is proposed to come about by preventing processing beyond the point marked by an asterisk in (C) and thus likely is a process distinct from the one we investigate here.

In any event, as assayed in this study, the innate locomotor tendencies supported by odors and tastants seem mutually insulated (a situation similar to what we have found for visual and olfactory processing: Yarali et al., 2006). However, the joint presentation of odor and tastant does support the establishment of an associative memory trace, clearly requiring

some convergence of both kinds of signalling in the larval brain (also, the joint presentation of visual cues and tastants associatively alters visual behavior: Gerber et al., 2004b). As discussed above this type of interaction is mediated by modulatory interneurons to 'short-circuit' taste and smell processing, employing distinct sets of neurons to signal reward and punishment (Fig. 11B: [5]). As for a given odor there likely is but one set of Kenyon cells available to enter into association with reward and punishment, these reward and punishment signals likely signal onto different cellular compartments of these cells (Fig. 11B: [6]), from which appetitive and aversive memory traces likely are retrieved via different sets of mushroom body-extrinsic neurons (Fig. 11B: [7]).

In addition to these interactions of olfactory processing and taste-triggered reinforcement signals during training, we identify a second type of interaction. That is, whether these memory traces are behaviorally expressed or not is determined neither by the strength of the memory trace *per se*, nor by the circumstances of testing *per se*, but rather depends on a comparison between the respective memory trace and the value of the test situation: Conditioned search behavior is expressed unless it is *disabled* by the presence of an at least as-good-as-predicted sugar (Fig. 11C: [8]). In contrast, only the presence of quinine or salt at an intensity at least as-bad-as-predicted *enables* the expression of conditioned escape behavior (Fig. 11C: [9]). We would like to stress that these processes require the memory trace to be 'read-out' to allow for this comparison with the value of the test situation; therefore, obviously, these comparisons have to take place downstream of the site of the memory trace. This is critically different from the proposed effect of satiety: Krashes et al. (2009, *loc cit* Fig. 7) suggest that satiety prevents the very read-out of the memory trace, i.e. is acting effectively upstream of the memory trace (site labeled with * in Fig. 11C). Thus, potentially, there may be two mechanisms at operation, one regulating whether a memory trace is addressed and read-out at all (depending on satiety), and another one regulating the behavioral expression of an activated memory trace, dependent on the comparison between memory trace and the testing situation.

Generality?

As discussed above, we propose that animals express an aversive memory if they are motivated to escape from the test situation. Presenting an aversive reinforcer, as we did in our experiments, may not be the only way to induce such motivation. Indeed, there exist a broad range of studies on aversive conditioned behavior in insects that do not report the necessity of

an apparent aversive reinforcer at the moment of test. Arguably, however, in these cases there may exist other sources of escape motivation:

Honjo and Furukubo-Tokunaga (2009) used a non-reciprocal quinine-learning paradigm and found aversive memory scores in the absence of quinine. However, as in that study animals were bathed in a liquid quinine solution during training, there may be a lingering bitter after-taste at the moment of test. That is, although no quinine is presented on the test Petri dish, animals may still regard the substrate as unpleasantly bitter and seek to escape from it. Such a lingering taste may also be an explanation for the reported differences in stability of appetitive and aversive memory (half-maximal effects after 90 and 10 minutes, respectively: Honjo and Furukubo-Tokunaga, 2005 and 2009): with the lingering taste decreasing, scores of conditioned aversion quickly decay.

Regarding larval electroshock-learning, Pauls et al., (2010b) report that associative aversion scores are more negative if the last training trial included electric shocks, compared to animals that received electric shock in the previous-last training trial. This again may hint at a residual effect of electric shock that may motivate the animals´ escape during test, which may be stronger for those groups that had received shock just prior to testing (in the related study by Khurana et al. [2009] data were not analyzed regarding sequence effects).

Similar arguments may apply in the case of olfactory electroshock learning in adult flies (Tully and Quinn, 1985), especially as the intensity of electric shocks in this paradigm is often close to causing physical damage. Interestingly, Tempel et al. (1983) report that aversive memory scores induced by electroshocks are much less stable than appetitive memory scores induced by sugar (half-maximal effects are found after 2 and 18 hours, respectively). Again, this may hint at a carry-over effect of the aversive reinforcer that vanishes after training, such that the driving force behind associative aversion may get lost. Also, before the arms of the T-maze are opened in this type of assay, about one hundred fairly stressed flies are crowded in a volume approximately as small as a cherry (appr. 1.5 cm^3), a situation that should be unpleasant to the flies. Along the same lines, the training and testing situation in sting-extension reflex conditioning of honeybees fixates the animals horizontally on their backs (Giurfa et al., 2007, *loc cit* Fig. 1), which may not be a leisurely body posture for them.

On the other hand, after conditioning crickets with saline solution (Matsumoto and Mizunami, 2002, *loc cit* Fig. 3) animals avoid the punished odor in a not apparently unpleasant situation. This observation seems to not fit to the rule of escape motivation suggested above. We note, however, that in this paradigm odor and punishment are presented

not only in very close temporal but also in very close spatial proximity, potentially prompting the odor to stand-in for the punishment, rather than becoming a signal-for punishment. A similar argument may apply to odor-taste learning in *Spodoptera littoralis* larvae (Salloum et al., 2011).

Thus, we hesitate to judge whether the behavioral organization of learned behavior as found in this study is an exceptional case or whether it reveals a principle that had remained opaque in previous assays that may have "implicitly" provided a bad-enough testing situation. Indeed, in a recently developed paradigm of association between odor and mechanosensory disturbance as punishment in larval *Drosophila*, learned behavior likewise is only revealed in the presence of that punishment (C. Eschbach, B. Gerber, unpubl.). Also, Schnaitmann et al. (2010), analyzing visual learning in adult flies, report that after punishing animals with formic acid during training, aversive memory is behaviorally expressed in the presence but not the absence of formic acid (*loc cit* Fig. 10). Importantly, in this paradigm 50-100 flies can freely move about a large, 9 cm diameter test arena, such that there may not be any reason to escape unless "explicitly" provided by the experimenter- by adding formic acid to the test situation. Similarly, flies trained to associate visual landmarks with a comfortably cool spot in an otherwise uncomfortably hot arena search at the trained location if the testing arena is uniformly hot (Ofstad et al., 2011), but not nearly as well when it is uniformly cool (Reiser M, HHMI JFRC; personal communication).

Outlook

Contemplating the neuronal architecture of the insect olfactory system (Fig. 10; for a recent review: Galizia and Rössler, 2010), one of the striking features is that antennal lobe output has two target areas, the mushroom bodies and the lateral horn. These, we propose from our behavior analysis (Fig. 11), correspond to one flexible, open processing stream, and one more rigid, closed one:

- Along the mushroom body route, olfactory processing is integrated with at least two kinds of gustatory signal, namely a reinforcement signal to induce associative plasticity in the mushroom bodies, and a value signal regarding the current status of the gustatory environment. When encountering a conditioned odor, this ‘triadic’ architecture accommodates a regulatory step, an element of ‘pondering’ if you will, between the activated memory trace and behavior control: It integrates the past experience of the larva (in the form of the memory trace activated by the odor), its present matter of concern (in terms of the present gustatory environment), and its

options for future action (in terms of the premotor neurons for conditioned behavior). This endows the animal with the option to express conditioned olfactory behavior- or not. The flexibility and openness of this architecture, we suggest, is a basic feature of behavior organization, reflecting the fundamental uncertainty in the world as we find it in general, and the uncertainty of what will be the best action under any given set of circumstances in particular.

- In contrast, the direct antennal lobe-lateral horn pathway is relatively rigid and closed: It is effectively 'insulated' against gustatory processing (as well as against visual processing: Yarali et al., 2006). There are few if any degrees of freedom along this processing stream, such that a given olfactory stimulus is, without much reference to what goes on in the 'rest' of the brain, able to organize behavior. Such relatively hard-wired organization, we argue, reflects the outcome of evolutionary trial and error, a phylogenetic curbing of the initially open and flexible organization of behavior to those few odor-behavior relationships that fit under almost all circumstances.

Considering the contrast to the relatively rigid, closed processing stream along the antennal lobe-lateral horn pathway, we suggest that conditioned olfactory behavior organized along the mushroom body loop assumes characteristics of a 'decision' (Zhang et al., 2007), in our case in the sense that conditioned behavior can be expressed- or not. Such a 'decision' appears simple enough to allow experimental access- and complex enough to remain interesting.

For now, the proposed functional circuitry is merely a working hypothesis, a scaffold to investigate the cellular sites of associative plasticity, the tastant-signals modulating, as well as the downstream motor effectuators organizing learned olfactory behavior. It should thus, we hope, bring us closer to a comprehensive understanding of what makes a larva do what a larva ´s got to do- and to the implementation of this understanding into a bio-inspired robot.

Materials and Methods

General

Drosophila melanogaster of the Canton-S wild-type strain are used and kept in mass culture, maintained at 25 °C, 60-70 % relative humidity and a 14/ 10 h light/ dark cycle. Experiments are performed under a fume-hood at 20- 24 °C room temperature and use five-day old feeding-stage larvae collected from the food slurry and gently washed in tap water before the start of the experiments. Petri dishes used (Sarstedt, Nümbrecht, Germany) are of 85 mm diameter (except in the case of the experiments displayed in Figures 1- 3, which use 52-mm

Petri dishes); they are prepared freshly the day before experiments and contain solidified 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany) (only the experiment displayed in Fig. 9 uses 2.5 % agarose). As the respective experiments require, tastants (fructose [FRU; CAS: 57-48-7; purity 99 %; Roth, Karlsruhe, Germany]; quinine hemisulfate [QUI; CAS: 6119-70-6; Sigma-Aldrich, Seelze, Germany], or sodium chloride [NaCl; CAS: 7647-14-5; purity 99.5%; Roth, Karlsruhe, Germany]) are added to the agarose at the respectively indicated concentrations to create sweet, bitter or salty substrates. Odors (*n*-amylacetate [AM; CAS: 628-63-7; Merck, Darmstadt, Germany]; 1-octanol [OCT; CAS: 111-87-5; Sigma-Aldrich, Seelze, Germany]; benzaldehyde [BA; CAS: 100-52-7; Fluka, Buchs, Switzerland]) are presented by custom-made Teflon containers with 5 mm diameter, covered by a lid with seven 0.5-mm holes as soon as 10 µl of odor has been loaded; dilutions are made in paraffin oil (CAS: 8012-95-1; Sigma-Aldrich, Seelze, Germany). Only the experiments displayed in Figures 1- 3 use another way of odor application (see below).

Is innate gustatory behavior affected by ambient odor?

For assaying innate gustatory behavior, 52-mm diameter Petri dishes are divided into two halves by using a vertical barrier cut from overhead transparencies (Hendel et al., 2005). One half of the dish is filled with only solidified 1 % agarose and the other with agarose in addition containing either of three tastants (FRU, QUI, NaCl). Shortly before the substances solidify, the barrier is removed and Petri dishes stored for use on the following day.

Larvae are put in the middle of such a split Petri dish. After 1, 2, 4 and 8 minutes their location is repeatedly determined as either on the tastant side ($\#_{TASTANT}$), on the agarose-only side ($\#_{PURE}$) or in an approximately 1-cm wide 'middle' stripe of the plate ($\#_{MIDDLE}$; for clarity, this middle stripe is not displayed in the sketches of the Figures) as well as the total number of larvae ($\#_{TASTANT} + \#_{PURE} + \#_{MIDDLE} = \#_{TOTAL}$). Given that for these experiments the small-diameter Petri dishes are used, and that we repeatedly score larval behavior at fairly short intervals, only fifteen larvae are used in all gustatory behavior assays. From these data, a Gustatory Index (GI) [-1; 1] is calculated as:

(iv) $$GI = (\#_{TASTANT} - \#_{PURE})/ \#_{TOTAL}$$

Thus, positive values indicate positive preference to the tastant, negative values negative preferences to the tastant.

To test whether innate gustatory behavior is altered by the presence of an odor, the Gustatory Index is determined (a) in the absence of odor, (b) in the presence of *n*-amylacetate (AM; diluted 1:50) or (c) in the presence of 1-octanol (OCT; undiluted).

To prevent an accumulation of the larvae at and immediately around the odor containers, in only this experimental series no odor containers are used. Rather, two 0.5 cm x 0.5 cm filter papers are attached to the inner side of the lid of the Petri dish, each on one side of the plate, using double faced adhesive tape. Filter papers are loaded either both with 5 µl of AM or both with 5 µl of OCT. As control condition, no odor is added to the filter papers.

Is innate olfactory behavior altered in the presence of tastants?

To test whether, in turn, innate olfactory behavior is influenced by the presence of tastants, behavior of experimentally naïve larvae towards odor is assayed on a Petri dish containing either (a) agarose-only or on Petri dishes which in addition contain (b) fructose (2 M), (c) quinine (5 mM), (d) low-salt (0.25 M), or (e) high-salt (1.5 M).

Petri dishes (85 mm diameter) are filled with either 1 % solidified agarose or with agarose plus an added tastant and are used the following day. Two Teflon containers are placed at the circumfence of the Petri dish, on opposing sides; one is loaded with 10 µl of odor (either AM or OCT, at the respectively indicated dilution), while the other container serves as control with no odor added (empty, EM). For all olfactory behavior assays, the large-diameter Petri dishes are used to yield sufficient distances for odor gradients to form. As in addition no temporal resolution of larval behavior is attempted, all olfactory behavior experiments, including all learning experiments, use groups of thirty larvae. Larvae are transferred to the middle of Petri dish; after 3 min, we determine the number of animals at the odor side ($\#_{ODOR}$), the number at the no-odor side ($\#_{EM}$) as well as in a 1-cm wide middle stripe ($\#_{MIDDLE}$; for clarity, this middle stripe is not displayed in the sketches of the Figures), and the total ($\#_{ODOR} + \#_{EM} + \#_{MIDDLE} = \#_{TOTAL}$) number of larvae and calculate an Olfactory Index (OI) [-1; 1] as:

(v) $$OI = (\#_{ODOR} - \#_{EM})/ \#_{TOTAL}$$

Thus, positive values indicate attraction to the odor, negative values aversion.

Conditioned olfactory behavior: Two-odor paradigm

Three kinds of training are used: (a) appetitive training, (b) aversive training, or (c) a 'push-pull' combination using both reward and punishment. For appetitive training, larvae receive either of two training protocols: Either AM is presented with reward and OCT without reward (AM+/ OCT), or they are trained reciprocally (AM/ OCT+). For aversive training, the procedure is analogous (AM-/ OCT or AM/ OCT-). For the push-pull experimental design, one odor is rewarded and another odor is punished (AM+/ OCT- or AM-/ OCT+). In all cases, we measure the choice between AM *versus* OCT in a final test. As reward and punishment, respectively, we use fructose, quinine or salt added to agarose, in the concentrations mentioned in the Results section.

Specifically, two odor containers are loaded with odor (unless mentioned otherwise, either with AM diluted 1:50 in paraffin oil, or with undiluted OCT) (diluting AM ensures that innate responses to AM and OCT are about equally strong [compare Figures 4A and 4B]) and placed onto a Petri dish that either does or does not contain a tastant-reinforcer. These two containers (both loaded with the same odor) are placed at the outer circumfence of a Petri dish, on opposing sides. For the first training trial, larvae are transferred to the Petri dish; after 5 min they are transferred to a fresh dish with the alternative odor-substrate combination for the second training trial. For example, during appetitive training larvae are first exposed to AM in the presence of fructose (AM+), and then to OCT in the absence of fructose (OCT) (AM+/ OCT training). This training cycle is repeated three times. Then, animals are placed in the middle of a Petri dish with AM on one side and OCT on the other. This test plate may or may not contain a tastant-reinforcer, as is mentioned along the Results section.

After 3 min, we determine the number of animals at the AM side ($\#_{AM}$), the number at the OCT side ($\#_{OCT}$), the number of larvae on the middle stripe ($\#_{MIDDLE}$) and the total ($\#_{AM} + \#_{OCT} + \#_{MIDDLE} = \#_{TOTAL}$) number of larvae and calculate an Olfactory Index (OI) [-1; 1] as:

(vi) $$OI = (\#_{AM} - \#_{OCT})/ \#_{TOTAL}$$

Then, a second group of larvae is trained reciprocally, such that OCT is rewarded and AM is not (AM/ OCT+). From these alternately run, reciprocally trained groups we calculate a Performance Index (PI) [-1; 1] as:

(vii-a) $$PI = (OI_{AM+/\ OCT} - OI_{AM/\ OCT+})/ 2$$

Thus, positive PIs indicate appetitive, negative values aversive conditioned behavior.

In half of the cases the sequence of training trials is as indicated in the previous example (i.e. AM+/ OCT and in the reciprocal group AM/ OCT+), and in the other half of the cases the sequences are reversed (i.e. OCT/ AM+ and in the reciprocal OCT+/ AM). Notably, the sequence of training trials does not have an effect on behavior at test (Schleyer, 2009; Saumweber et al., 2011a), and hence the assignment of data for the calculation of the PI is unproblematic (see also appendix of Hendel et al., 2005). For aversive training and push-pull-training, training is performed in an analogous way and the Performance Indices are calculated respectively as:

(vii-b) $PI = (OI_{AM-/\ OCT} - OI_{AM/\ OCT-})/\ 2$ after aversive training

(vii-c) $PI = (OI_{AM+/\ OCT-} - OI_{AM-/\ OCT+})/\ 2$ after push-pull training

Conditioned olfactory behavior: One-odor paradigm

In order to confirm our findings we repeat the kinds of experiment described above in a number of cases, but using only one odor. That is, training and test are run in the very same way as described, but OCT is omitted throughout, such that instead of loading the respective container with OCT, an empty container (EM) is used. Thus, appetitive training follows the logical structure of training as either AM+/ EM or in the reciprocal AM/ EM+. Aversive training is run as either AM-/ EM or in the reciprocal as AM/ EM-, and the push-pull version uses either AM+/ EM- or AM-/ EM+ training. Again, the sequence of trial types is reversed in half of the cases (see above). Then, larvae are tested for their choice between AM and EM on the respectively mentioned type of substrate and data are analyzed as detailed above.

Data analysis

Given that behavioral data typically are not normally distributed (and in particular as data within restricted intervals by definition are not normally distributed), non-parametric statistics (one-sample sign test, Kruskal-Wallis test, Mann-Whitney U-test) are applied throughout, using Statistica 7.1 (StatSoft, Tulsa, USA) for the PC (the one-sample sign-test uses a web-based statistic tool provided on http://www.fon.hum.uva.nl/Service/Statistics.html). When multiple one-sample or pair-wise comparisons are made within an experiment, a Bonferroni correction keeps the experiment-wide error rate below 5 % by dividing the critical *P*-value by the number of tests (e.g. for three tests $P < 0.05/\ 3$); this is a conservative approach to significance-testing. Data are displayed as box plots, where the middle line shows the median, the box boundaries the 25, 75 % quantiles, and the whiskers the 10, 90 % quantiles.

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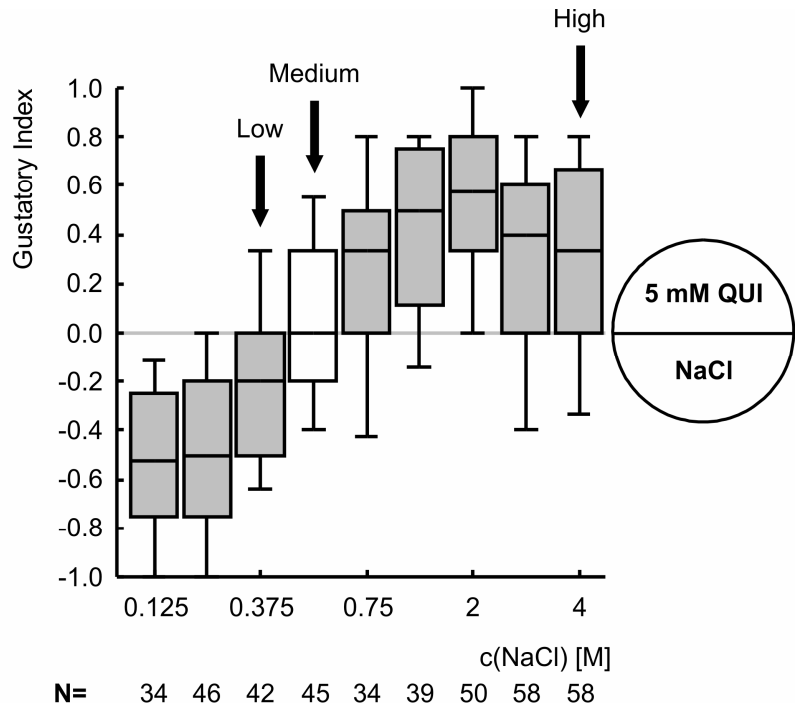
Supplementary Material

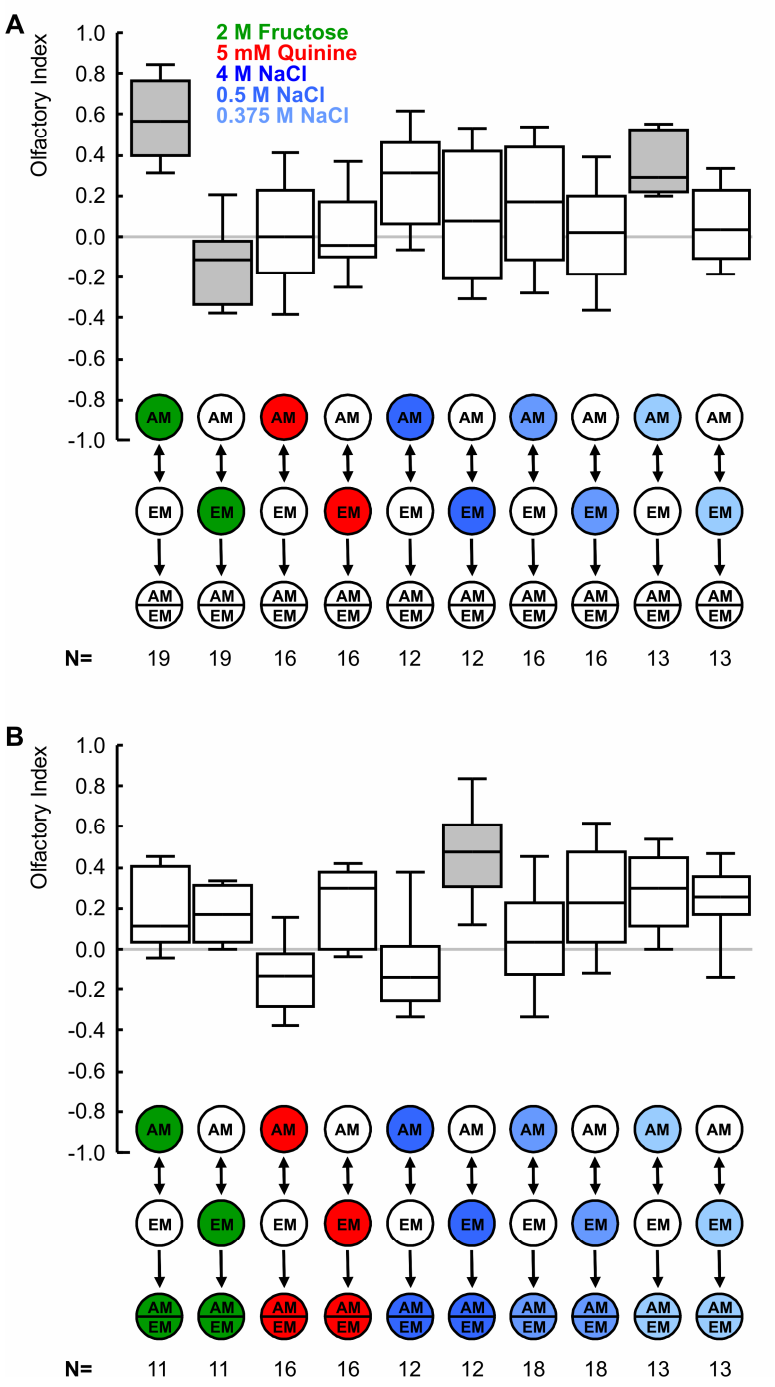


Supplementary Figure 1: *Choice between QUI and salt.*

Innate relative preferences of larvae between 5 mM quinine and various concentrations of salt. One half of a split Petri dish is filled with agarose containing 5 mM quinine solution (QUI) and the other half with agarose containing the indicated concentration of NaCl (SALT). A relative Gustatory Index (GI_R) [-1; 1] is calculated as:

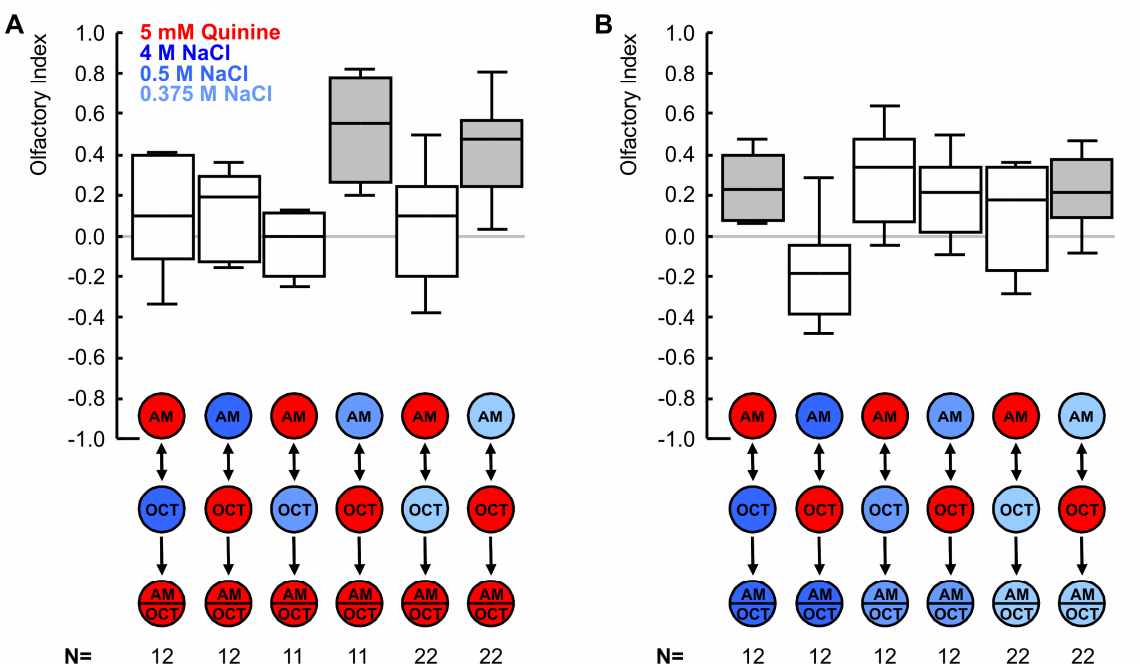
(S-i) $GI_R = (\#_{QUI} - \#_{SALT})/ \#_{TOTAL}$

Thus, positive values indicate that animals prefer quinine, negative values indicate they prefer salt. Salt concentrations are classified as 'low' if the animals prefer the salt side, and as 'high' if they prefer the bitter side. As the larvae are indifferent when facing the choice between quinine and 0.5 M salt, this concentration is classified as 'medium'. The concentrations of salt used in Figures 5 and 6 are indicated by arrows. For a description of the box plots see legend of Figure 1. Significant differences from zero ($P< 0.05/ 9$, one-sample sign tests) are indicated by shading of the boxes.
Data taken from: Gerber B, Hendel T. Outcome expectations drive learned behavior in larval Drosophila. Proceedings of the Royal Society B; ©2006 The Royal Society (loc. cit. Fig. S1).



Supplementary Figure 2

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 5. The sketches below the boxes show the training procedures and test conditions; colored circles represent Petri dishes containing tastant, white circles represent tasteless, pure Petri dishes. Note that differences in OI scores between two corresponding reciprocally trained groups (e.g. the two left-most panels in A) result in Performance Index (PI) scores different from zero (see Fig. 5A, left-most panel). Also note that in half of the cases the sequence of training trials is as indicated (in the left-most panel of (A) e.g. AM+/ OCT), but in the other half is reverse (e.g. OCT/ AM+). For a description of the box plots see legend of Figure 1. Significant differences from zero (P< 0.05/ 10, one-sample sign tests) are indicated by shading of the boxes.



Supplementary Figure 3

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 6. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P < 0.05/ 6$, one-sample sign tests) are indicated by shading of the boxes.

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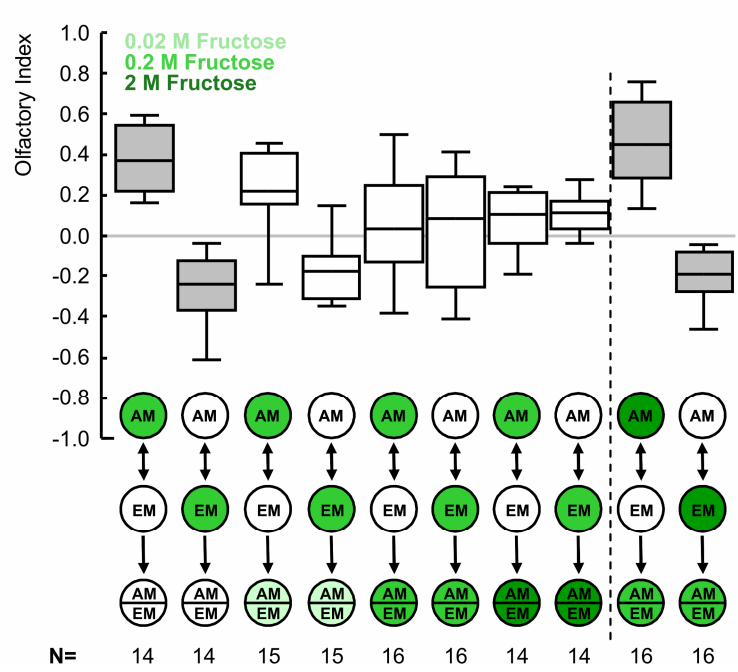

Supplementary Figure 4

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 7. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P < 0.05/ 10$, one-sample sign tests) are indicated by shading of the boxes.

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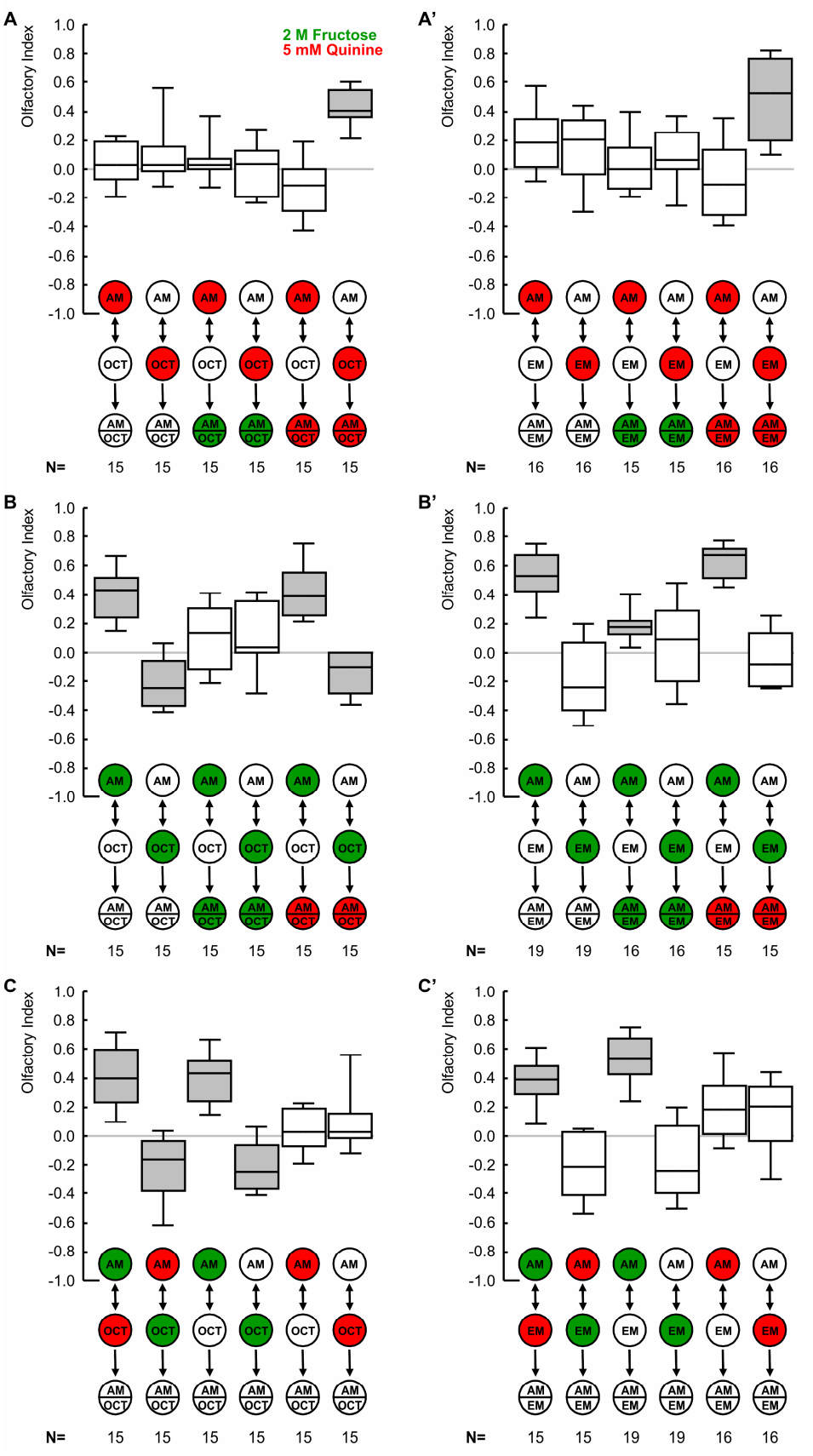
A
A'
B
B'
C
C'
Olfactory Index
2 M Fructose
5 mM Quinine
AM
OCT
EM
N=
15 15 15 15 15 15
16 16 15 15 16 16
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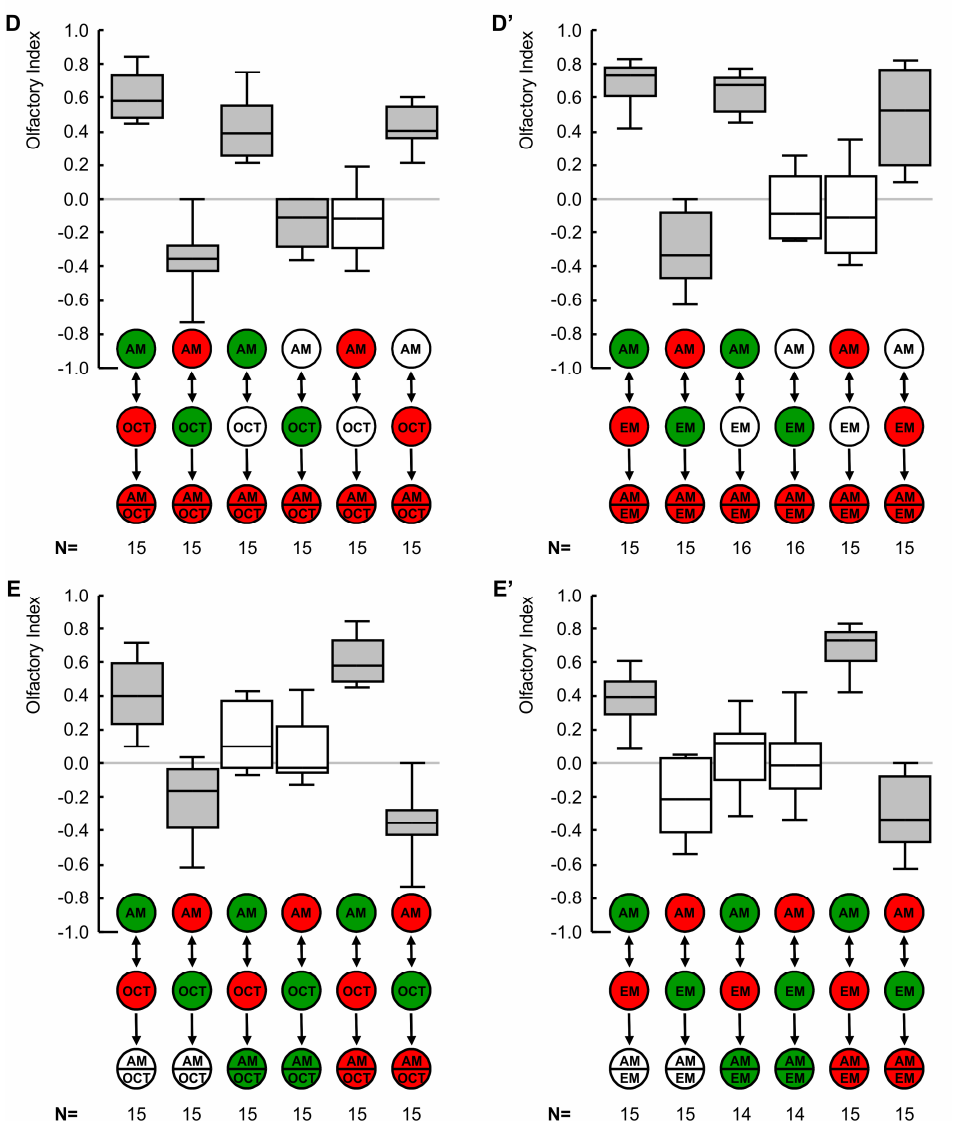
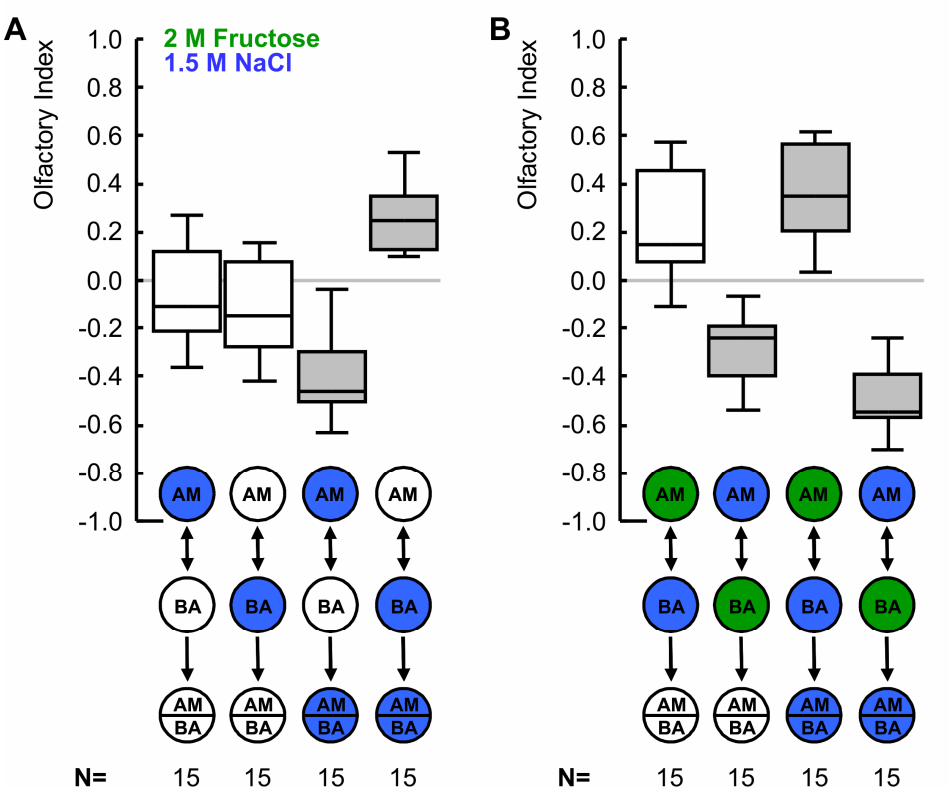
19 19 16 16 15 15
15 15 19 19 16 16



Supplementary Figure 5

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 8. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P < 0.05/6$, one-sample sign tests) are indicated by shading of the boxes.



Supplementary Figure 6

Olfactory Index (OI) values of all groups of larvae from the experiments displayed in Figure 9. All other details as in Figure S2; for a description of the box plots see legend of Figure 1. Significant differences from zero ($P < 0.05/4$, one-sample sign tests) are indicated by shading of the boxes.

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I Olfactory associative learning in *Drosophila* larva

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Research

Cellular site and molecular mode of synapsin action in associative learning

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4. Cellular site and molecular mode of synapsin action in associative learning

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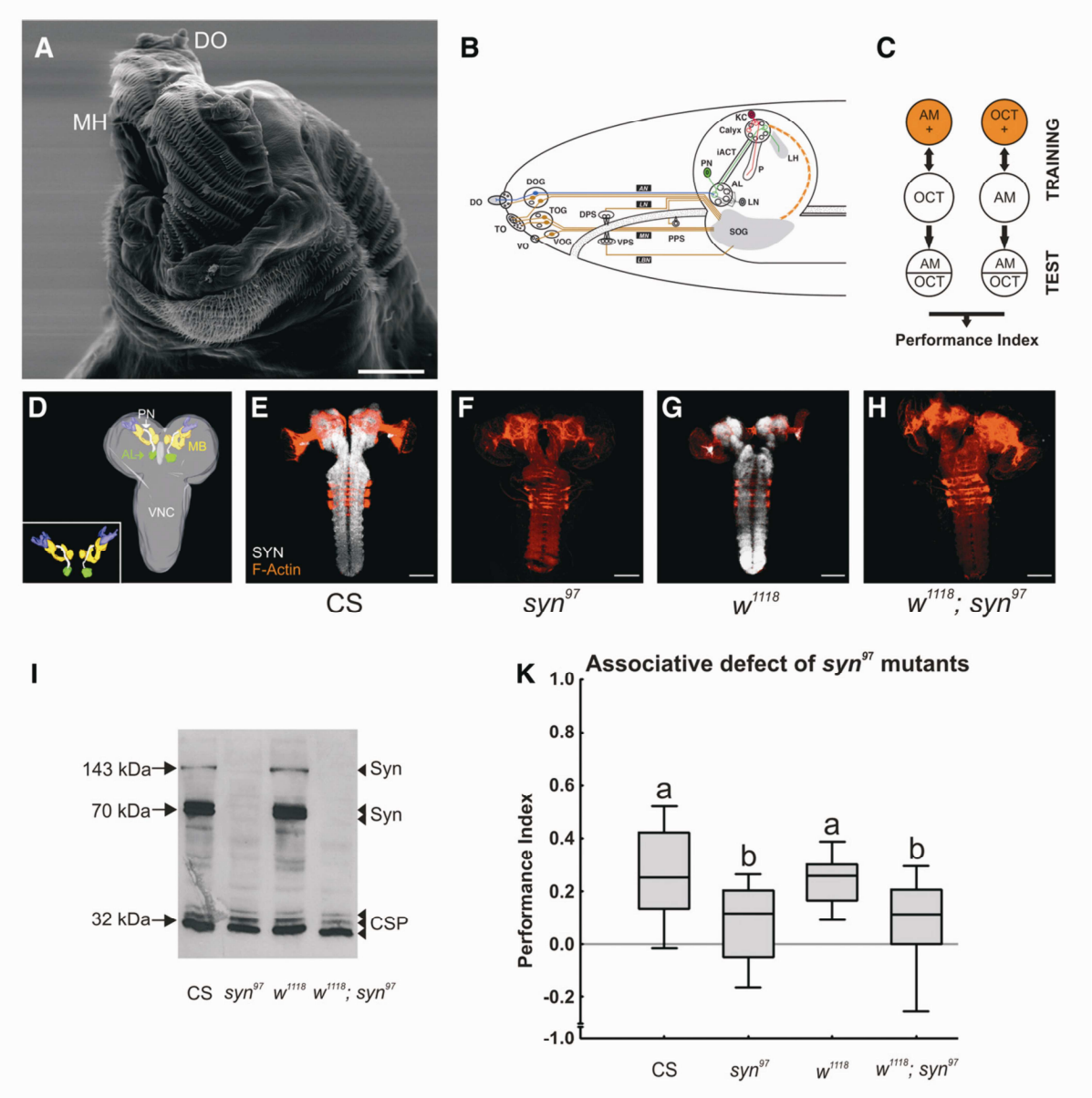
Keywords:
Synapsin, synaptic plasticity, olfactory learning, mushroom body, *Drosophila* larva, PKA

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.



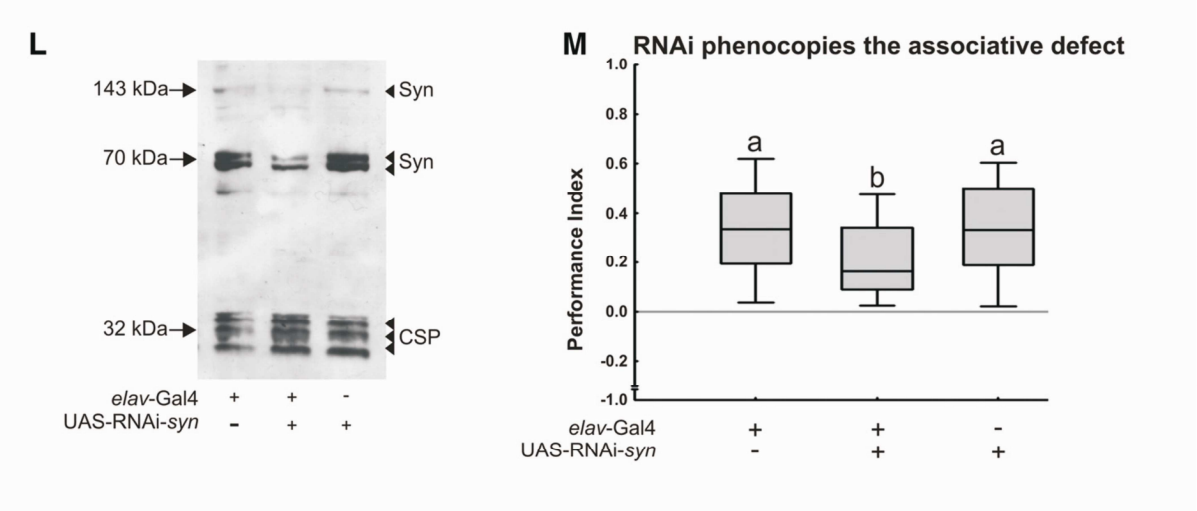


Figure 1:

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.

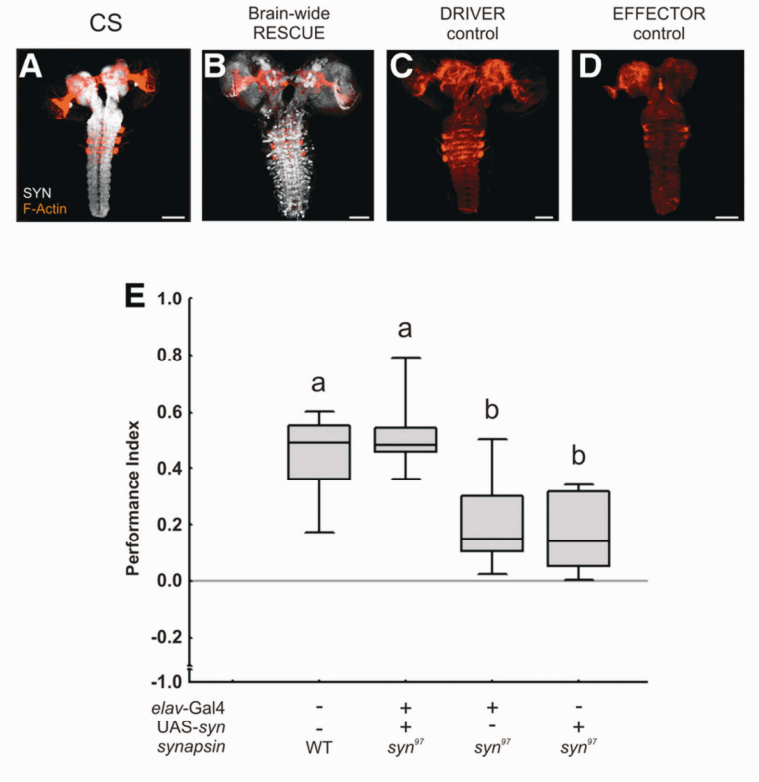
Brain-wide rescue

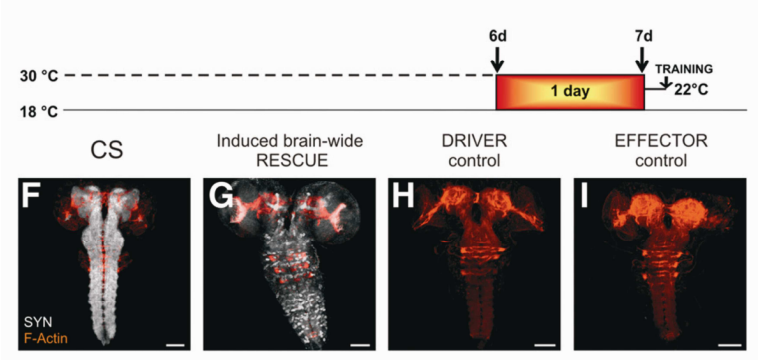
In brain-wide RESCUE larvae, synapsin expression is restored throughout the brain (Fig. 2B; S1B-D; Movie S2). Comparing performance scores between genotypes shows a difference in associative ability (Fig. 2E; KW: P< 0.05; H= 19.03; df= 3; N= 9, 7, 7, 10). Specifically, the brain-wide RESCUE larvae perform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





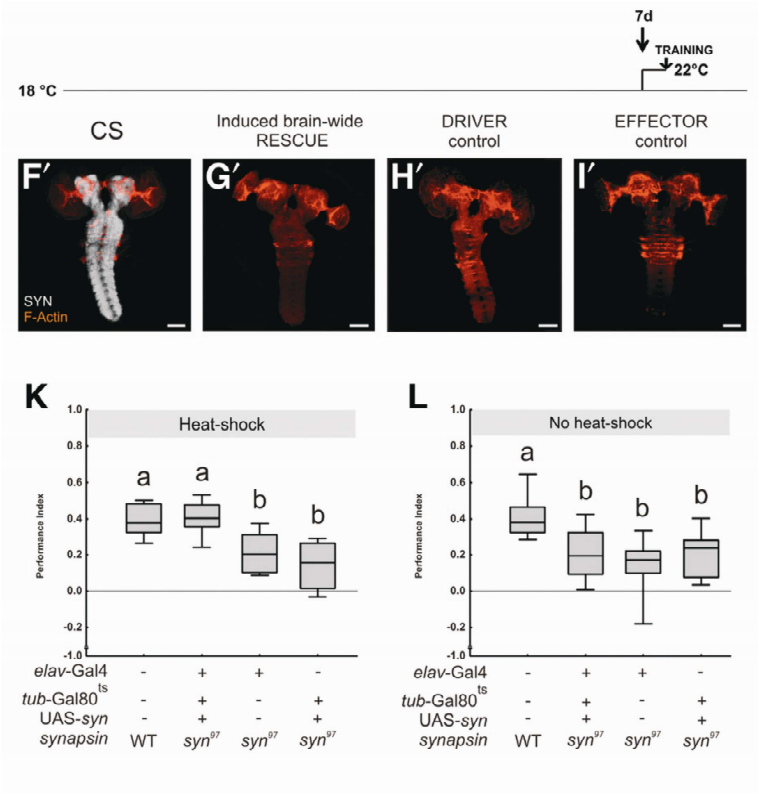


Figure 2:

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

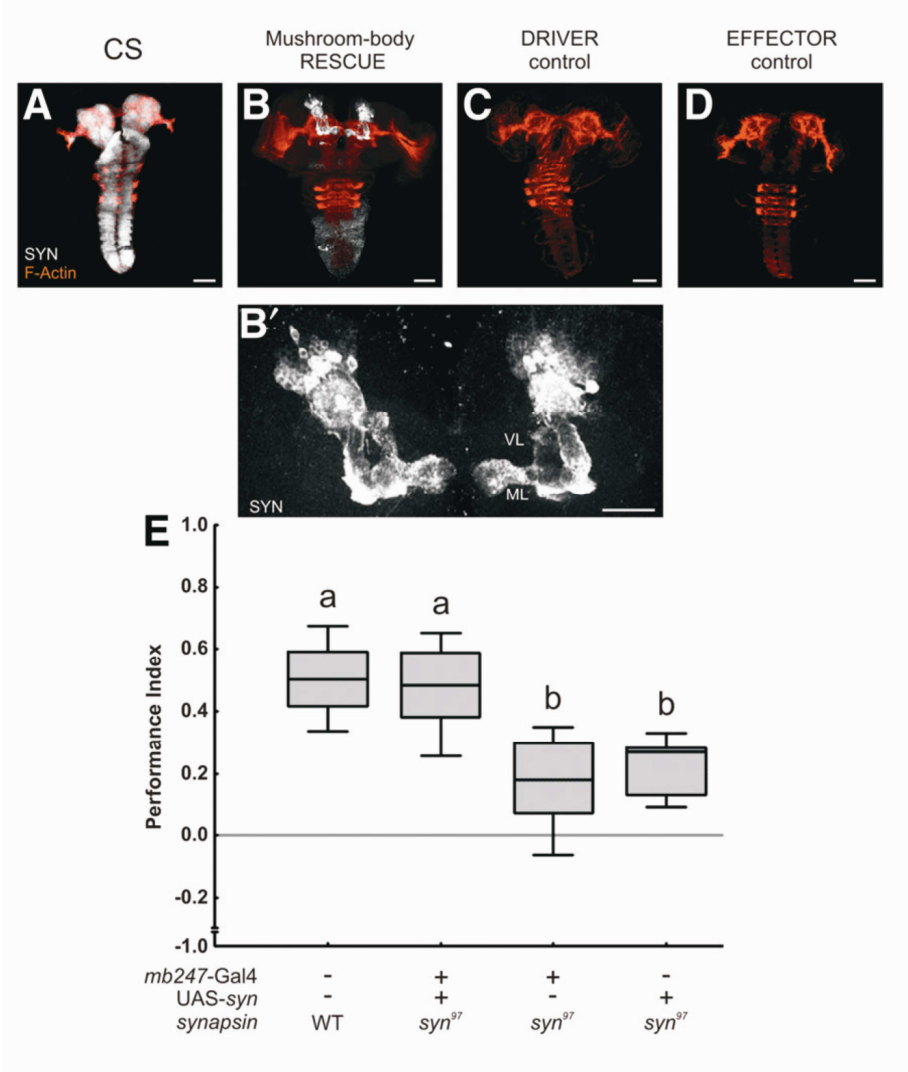
We next ask whether synapsin expression in only the mushroom bodies will restore the defect of the *syn*^{97} mutants in associative function. Associative performance scores differ between wild-type CS, mushroom-body RESCUE strain, DRIVER control, and EFFECTOR control (Fig. 3E; KW: P< 0.05; H= 21.39;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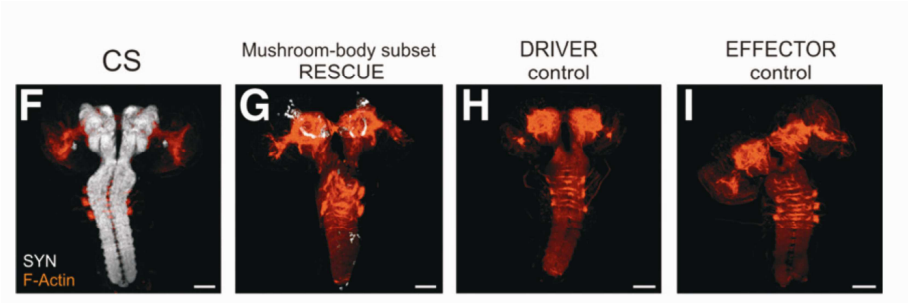
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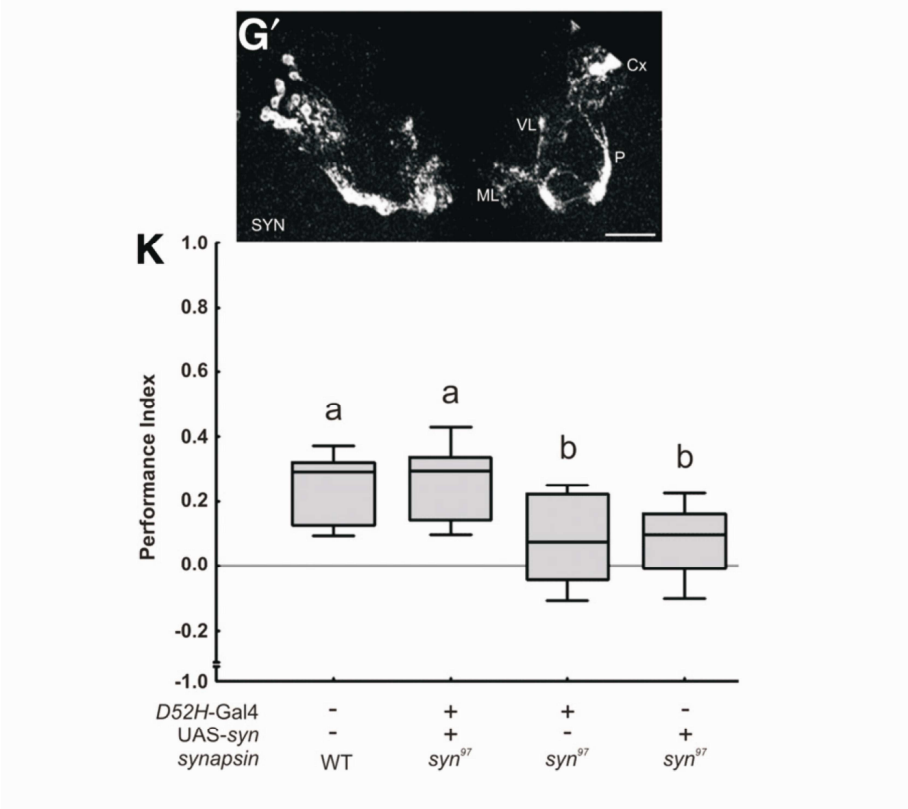


Figure 3:

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

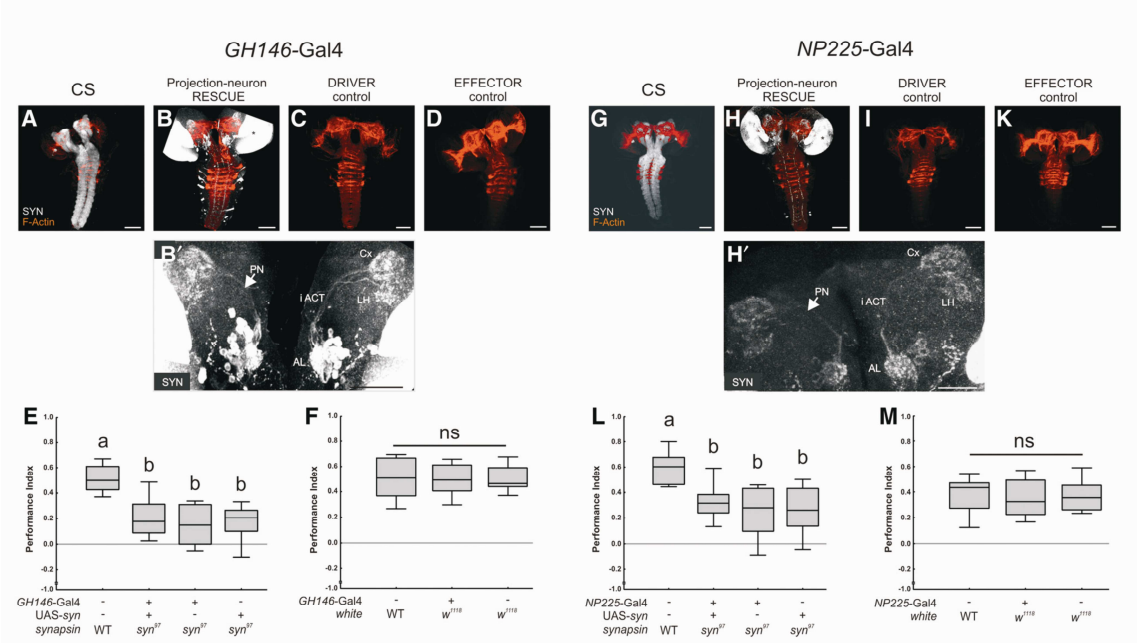
However, as is the case for any lack-of-rescue, the insertion of the driver construct may produce haploinsufficiency in the gene(s) neighbouring it, and this haploinsufficiency may lead to a learning defect masking an actually successful rescue. Therefore, we compare larvae heterozygous for the used 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.



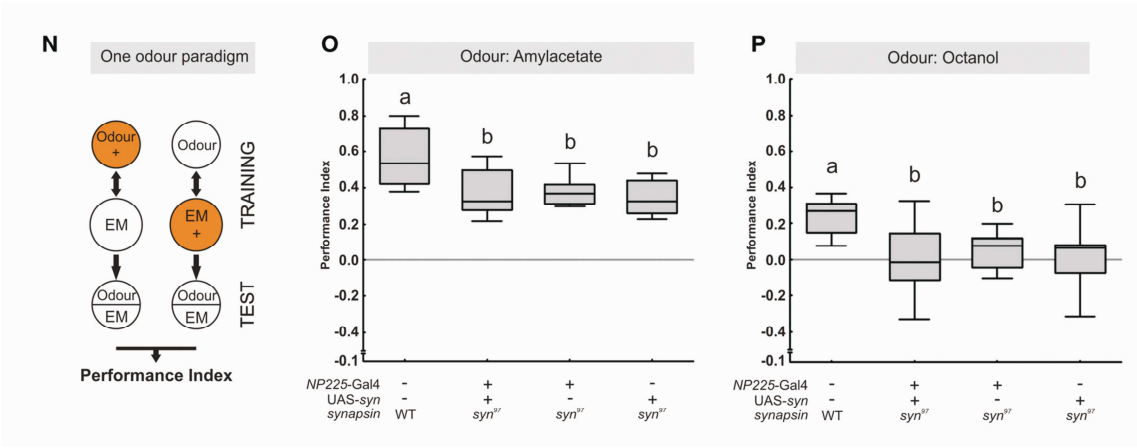


Figure 4:

No rescue in the projection neurons.

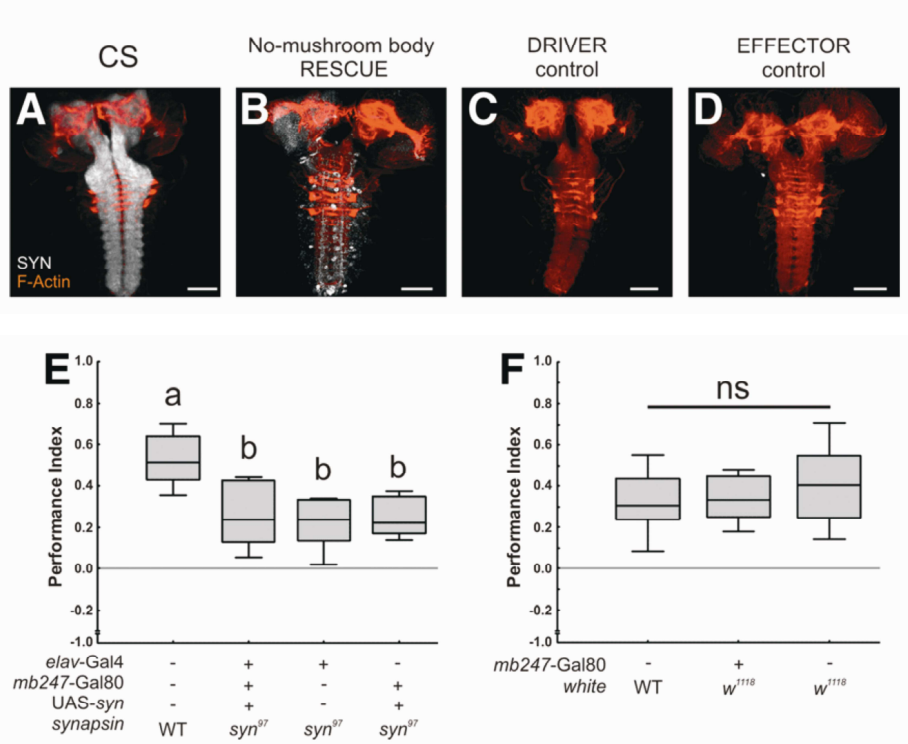
A-D, G-K Anti-synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes. In (B´, H´), magnified views of the projection neurons from the RESCUE strains are presented.
E synapsin expression in projection neurons (driver *GH146*-Gal4) is not sufficient to restore 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.



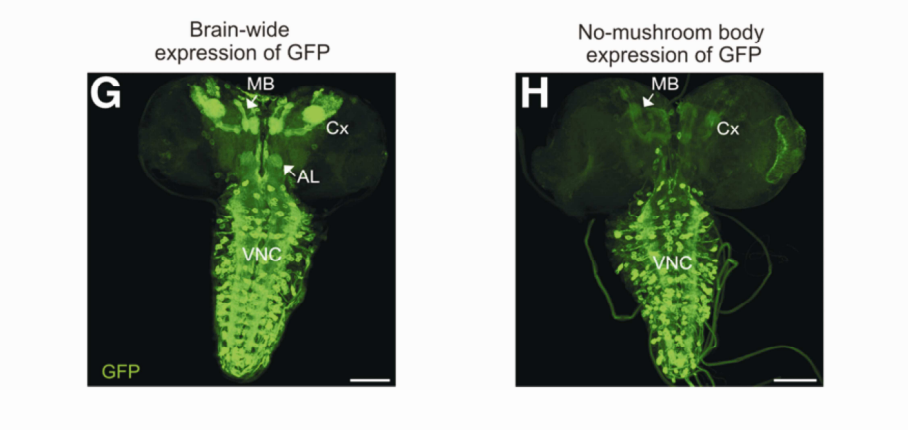


Figure 5:

No rescue by synapsin expression outside of the mushroom bodies.

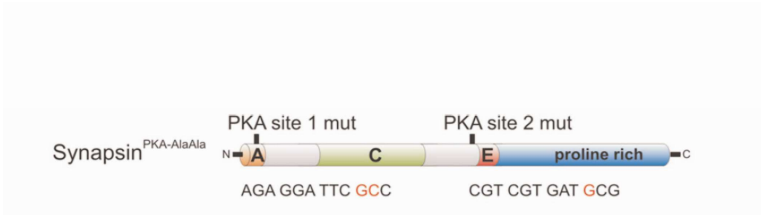
A-D Anti-synapsin (white) and anti-F-Actin (orange) immunoreactivity of brains of the indicated genotypes.
G, H Expression of GFP in *elav*-Gal4 flies (G) and *elav*-Gal4, *mb247*-Gal80 flies (H), each crossed to UAS-*GFP*. Antennal lobe (AL), mushroom body (MB), calyx (Cx) ventral nerve cord (VNC).
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.

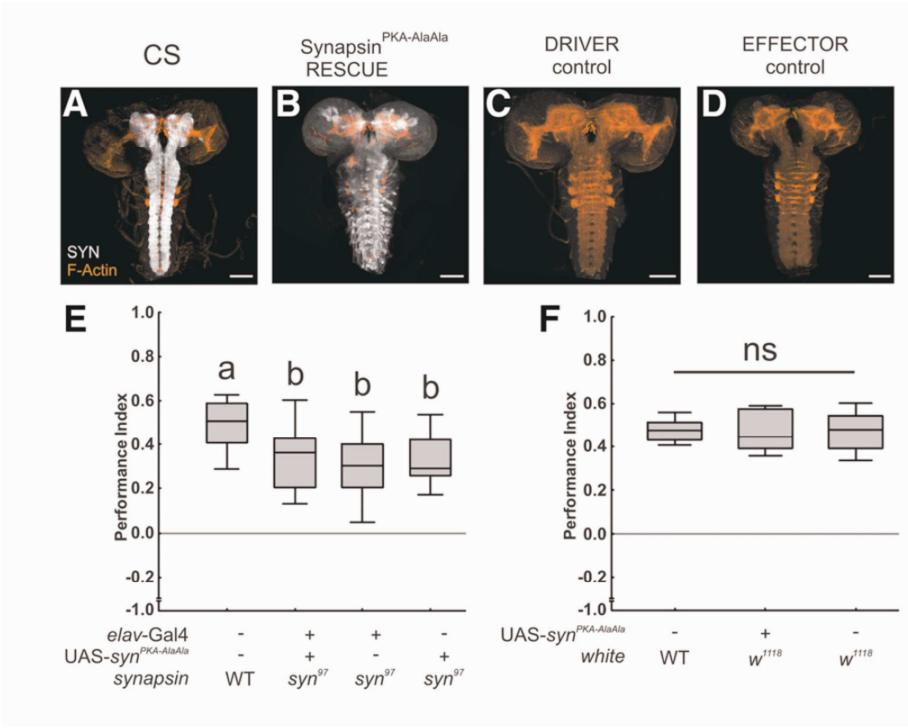
Scale bars: 50µm. All other details as in the legend of Fig. 1 (see also Fig. S3).

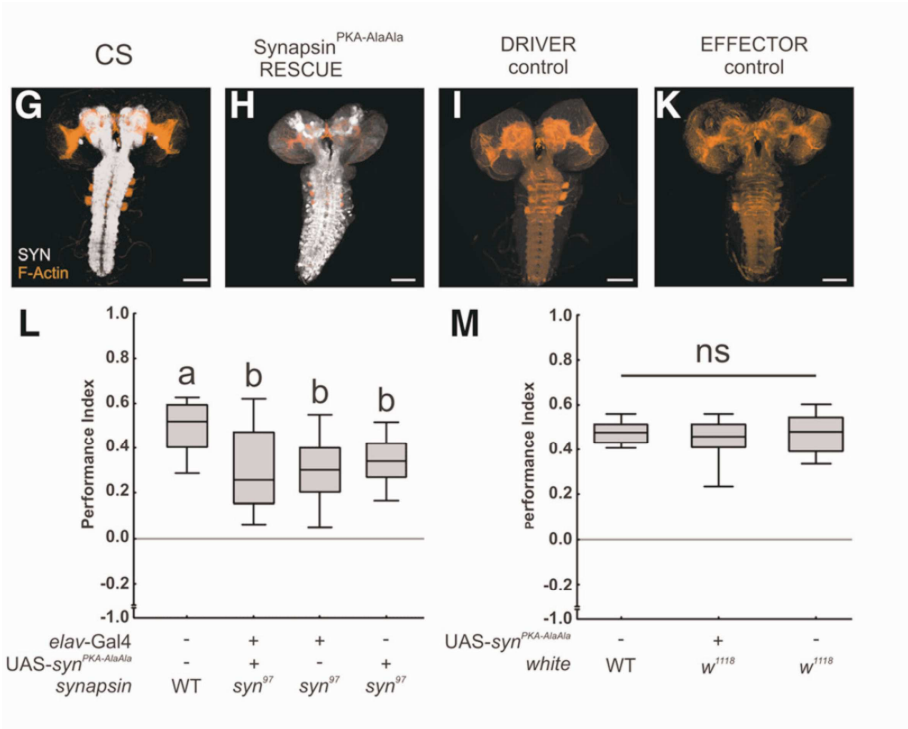
No rescue with PKA-site defective synapsin

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 not perform as well as wild-type CS (Fig. 6E; MW: $P< 0.05/ 3$; $U=70$); rather, associative ability is as poor as in the genetic controls (Fig. 6E; Synapsin$^{PKA\text{-}AlaAla}$-RESCUE versus EFFECTOR control: MW: $P> 0.05/ 3$; $U= 130.5$; Synapsin$^{PKA\text{-}AlaAla}$-RESCUE versus DRIVER control: MW: $P> 0.05/ 3$; $U= 121$). Such lack-of-rescue cannot be attributed to a haploinsufficiency caused by the insertion of the UAS-*syn*$^{PKA\text{-}AlaAla}$ construct (Fig. 6F; KW: $P> 0.05$; $H= 0.04$; $df= 2$; $N= 12$ for all groups) (for

a repetition of these experiments with an independent insertion of the same effector construct see Fig. 6G-M). Thus, intact PKA-sites of synapsin are required to restore associative ability in the *syn*^{97}-mutant.







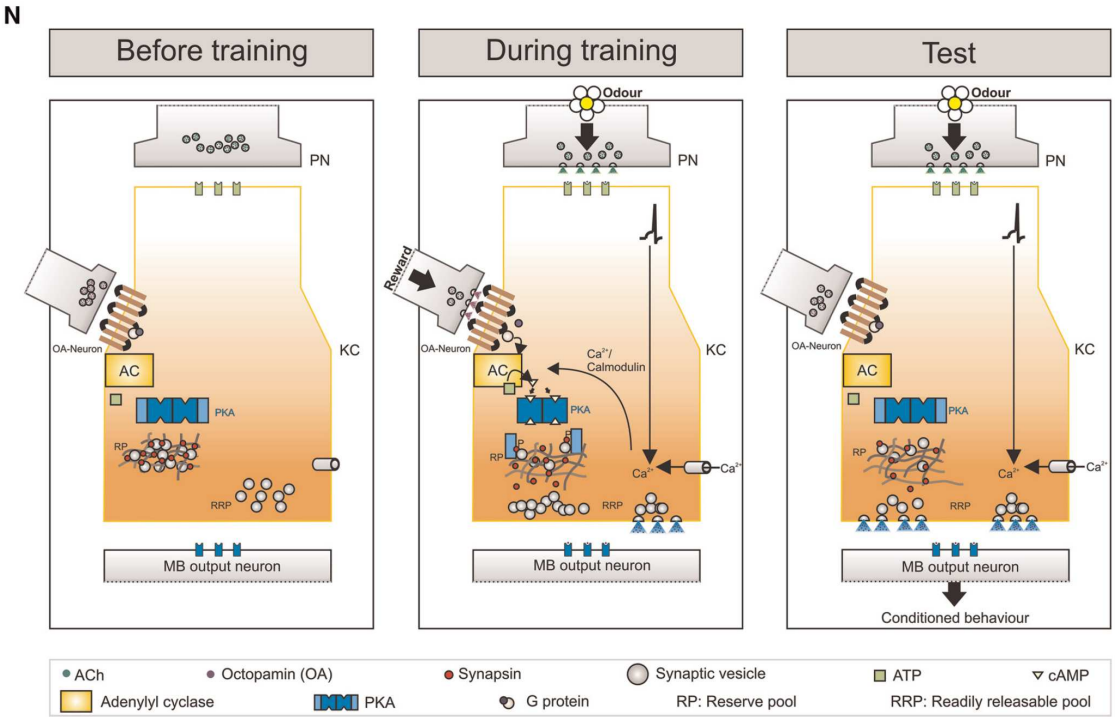


Figure 6:

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:

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

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").

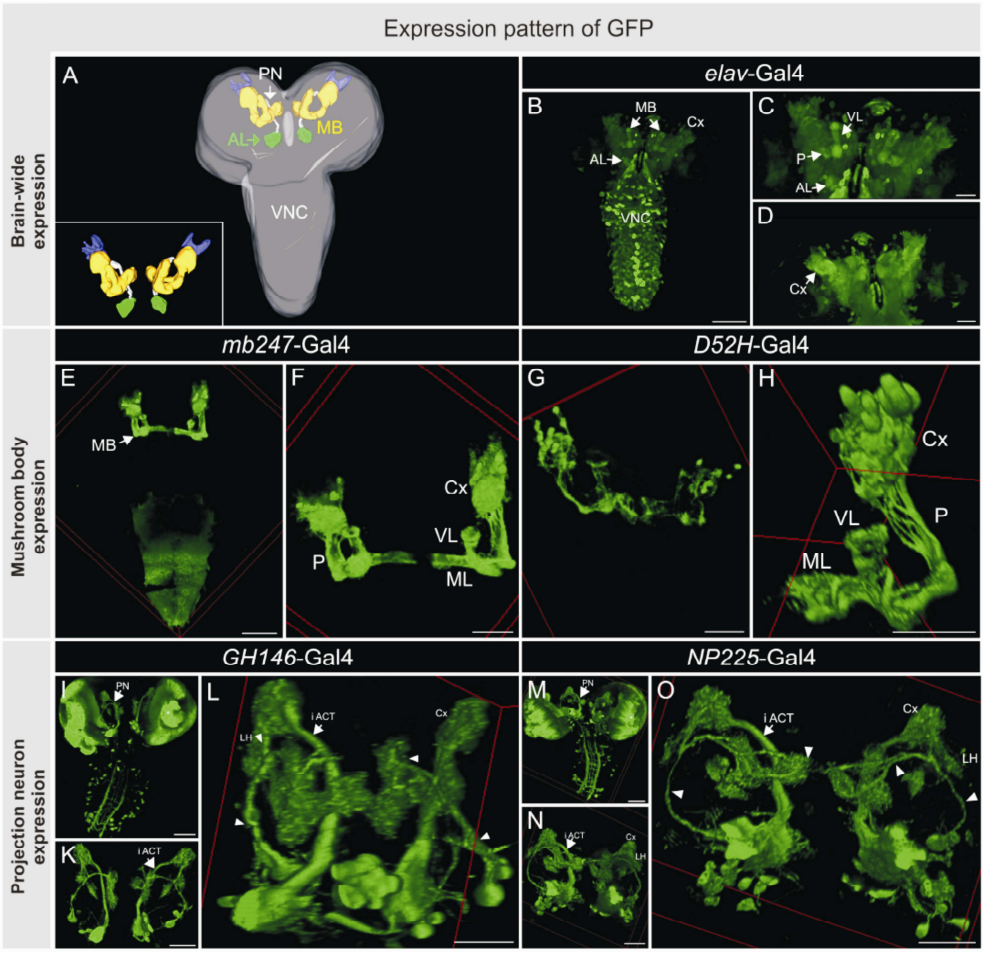
Statistical analyses

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.

Acknowledgements

Supported by Deutsche Forschungsgemeinschaft (IRTG 1156, CRC 554-A10 and CRC TR58-A6, German Excellence Initiative Grant *Graduate School of Life Sciences* University of Würzburg [Y-c.C., B.G., B.M., T.S.]; Emmy Noether Program [H.T.]), the Bernstein Focus Program *Insect Inspired Robotics* (to B.G. and H.T.), and the Max Planck Society (to H.T.). The support and comments of B. Poeck, A. Yarali, M. Heisenberg, E. Buchner. B. Klagges and M. Koblofsky is gratefully acknowledged. B.G. is a Heisenberg Fellow of the DFG.

Supplementary Material



Supplementary Figure 1:

Expression pattern of various Gal4-strains used for behavioral experiments.

Three-dimensional reconstructions of anti-GFP immunoreactivity (green) of whole-mount larval third-instar brains using the ImageJ 3D Viewer.

A Dorsal view with the major brain regions reconstructed. The inset shows a magnified view of the MB. (B-D) Brain-wide expression of GFP using e*lav*-Gal4.

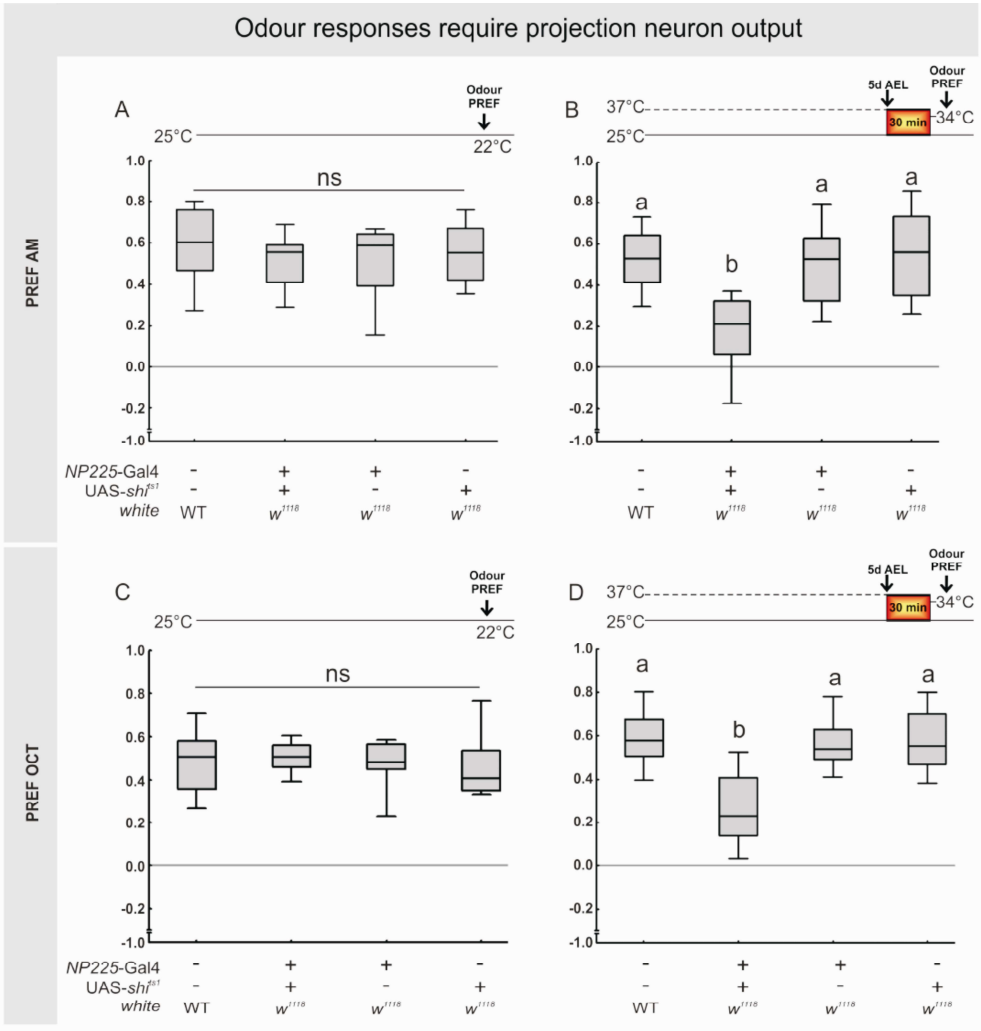
B Whole brain.

C, D Details of the brain seen in B.

E-H Mushroom body expression of GFP using *mb247*-Gal4 (E, F) with whole brain (E) and a magnified view of the mushroom body (F). (G, H) Mushroom body expression of GFP using *D52H*-Gal4 showing (G) both mushroom bodies and (H) a magnified view of a single mushroom body.

I-O Projection neuron expression of GFP in whole mounts using (I-L) *GH146*-Gal4 or (M-O) *NP225*-Gal4 as drivers for GFP expression. Additionally to projection neuron staining, a mushroom body extrinsic neuron (►) shows strong GFP immunoreactivity as well. (I, M) Whole brain. (K, L and N, O) Magnification of projection neurons and extrinsic mushroom body neurons. Optic lobe Anlagen (*), antennal lobe (AL), inner antennocerebral tract (iACT), projection neuron (PN), mushroom body (MB), calyx (Cx), peduncle (P), medial lobe (ML)vertical lobe (VL), lateral horn (LH), ventral nerve cord (VNC).

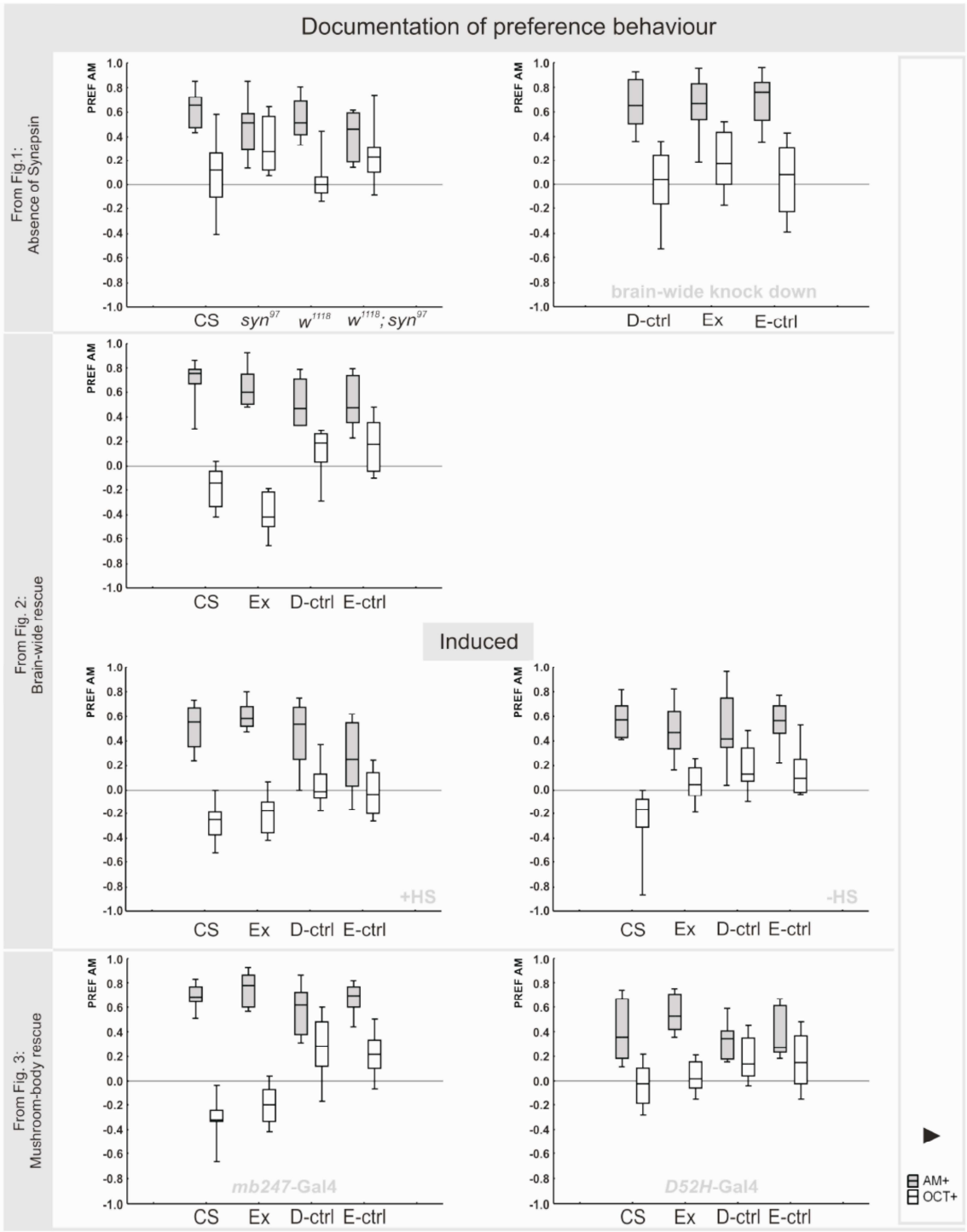
Scale bars: 50 µm in B, E, I, M; 25 µm in C, D, F-H, K, L, N, O.

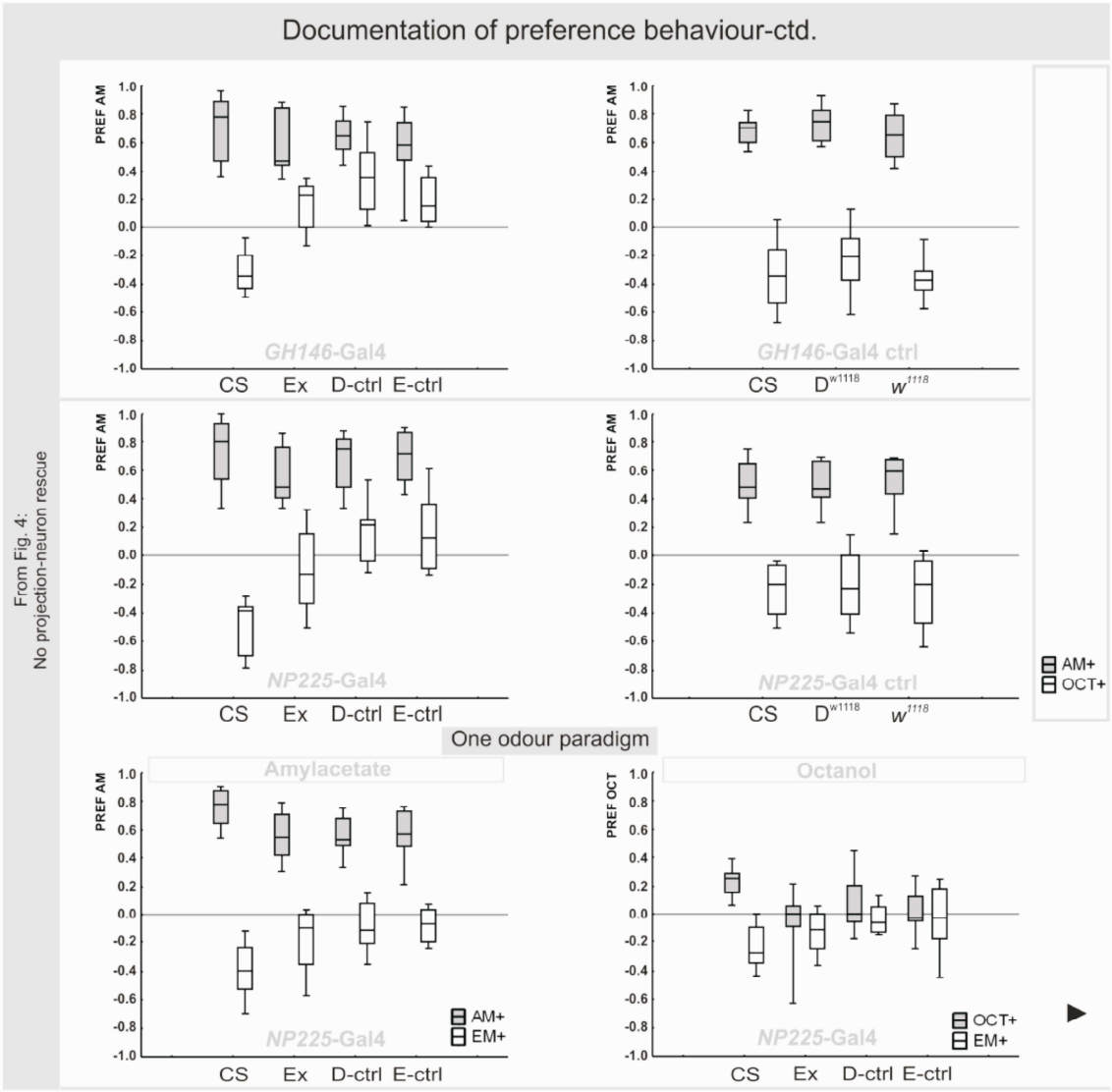


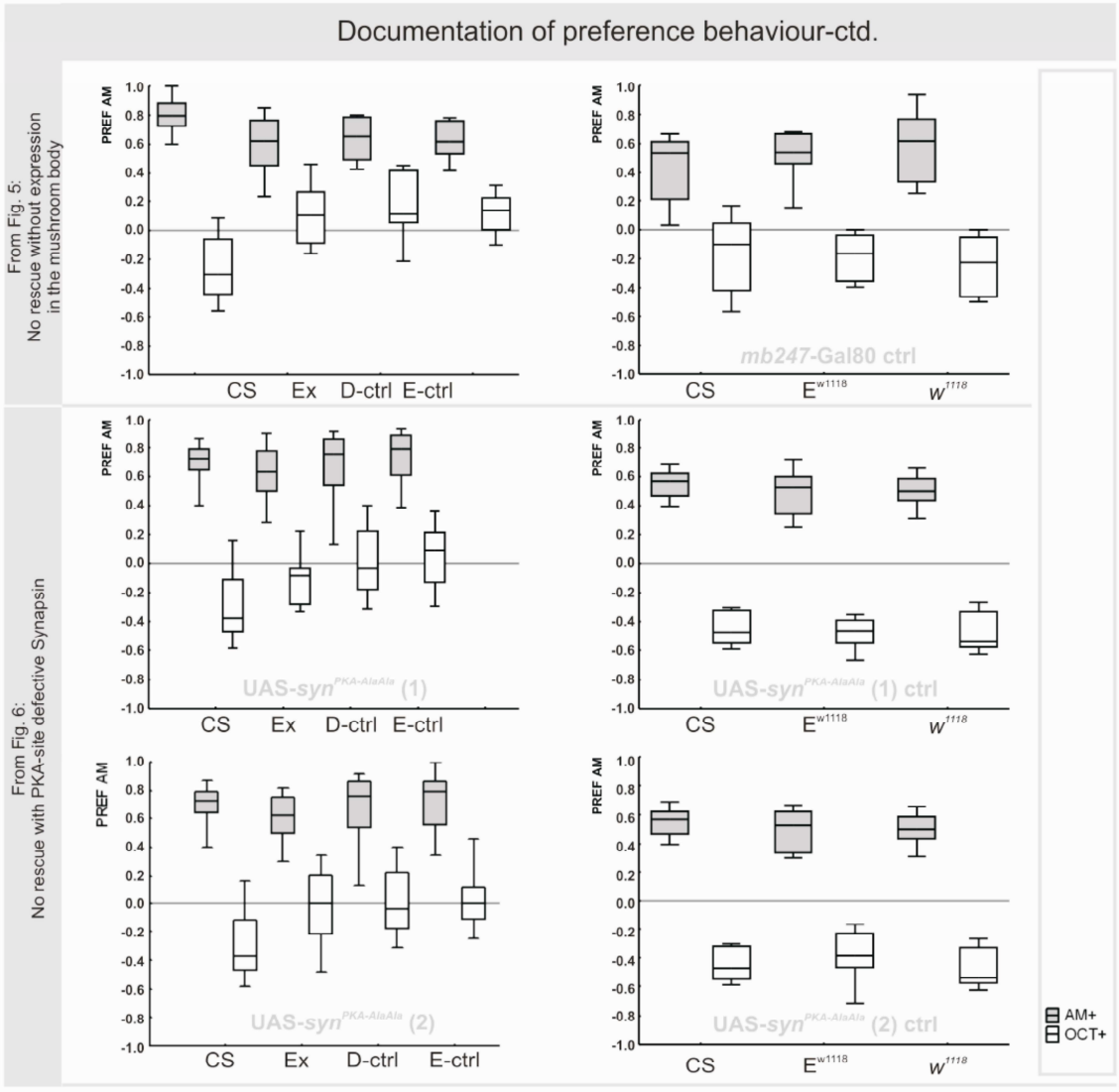
Supplementary Figure 2

Blocking synaptic output from projection neurons massively reduces odor preferences

The following genotypes were generated: for the experimental group we crossed *NP225*-Gal4 to UAS-*shi*^{ts1}, yielding double heterozygous larvae (; *NP225*-Gal4/ +; UAS-*shi*^{ts1}/ +); for the driver control we crossed *NP225*-Gal4 to no-transgene carrying flies yielding single-heterozygous (; *NP225*-Gal4 / + ;); for the effector control we crossed UAS-*shi*^{ts1} to no-transgene carrying flies yielding ; ; UAS-*shi*^{ts1}/ + animals. Experimentally naive larvae were incubated in their food vials for 30 min on 37 °C in a water bath. To test their ability to detect odors, we determined their PREF values if given a choice between either paraffin-diluted AM versus paraffin, or between undiluted OCT versus an empty container. These odor preference tests were performed either at 34 °C (restrictive temperature) or at room temperature (22 °C). *NP225*-Gal4/ UAS-*shi*^{ts1} larvae do not differ from controls when AM Preference (A; KW-test: P= 0.58; H= 1.94; df= 3; N= 16 for all genotypes) or OCT Preference (C; KW-test: P= 0.57; H= 2.00; df= 3; N= 16 for all genotypes) was measured at 22 °C. However, when synaptic output of projection neurons is blocked at restrictive temperature, odor preferences of *NP225*-Gal4/ UAS-*shi*^{ts1} are significantly lower than of control larvae, both for AM (B; KW-test: P< 0.05; H= 28.36; df= 3; N= 20 for all genotypes; *NP225*-Gal4/ UAS-*shi*^{ts1} versus wild-type CS: MW: P< 0.05/ 3; U= 29; N= sample size as above ; *NP225*-Gal4/ UAS-*shi*^{ts1} versus projection-neuron DRIVER control: MW: P< 0.05/ 3; U= 53; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus EFFECTOR control: MW: P< 0.05/ 3; U= 45; sample sizes as above) and for OCT (D; KW-test: P< 0.05; H= 27.45; df= 3; N= 20 for all genotypes; *NP225*-Gal4/ UAS-*shi*^{ts1} versus wild-type CS: MW: P< 0.05/ 3; U= 37; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus projection-neuron DRIVER control: MW: P< 0.05/ 3; U= 50; sample size as above; *NP225*-Gal4/ UAS-*shi*^{ts1} versus EFFECTOR control: MW: P< 0.05/ 3; U= 46; sample sizes as above).
All other details as in the legend of Fig. 1.


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


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



Supplementary Figure 3

Odor preferences, separated by training regimen.

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

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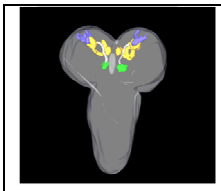
Movie Legends

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

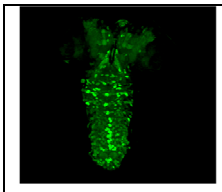
(available: http://learnmem.cshlp.org/content/18/5/332/suppl/DC1)



Movie S1:

Drosophila larval brain with the major brain regions reconstructed, related to Fig. 1.

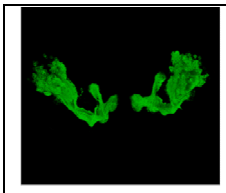
Shown are antennal lobes (green), projection neurons (white), the mushroom bodies (yellow), and the Kennyon cell bodies (blue). The light grey shade sketches the rest of the larval brain. Based on a brain from a larva obtained by crossing *GH146*-Gal4; *mb247*-Gal4 to UAS-*GFP*. The 3D representation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



Movie S2:

Gal4 expressing cells in elav-Gal4 monitored by UAS-GFP (green), related to Fig. 2.

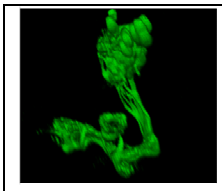
The larval brain shows GFP expression throughout all neuropil regions, with strong expression in the mushroom bodies.



Movie S3:

Gal4 expressing cells in mb247-Gal4 monitored by UAS-GFP (green), related to Fig. 3.

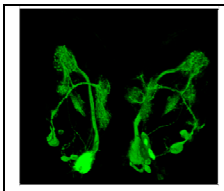
View on the larval mushroom body. In terms of expression pattern, *mb247*-Gal4 leads to GFP-expression in all basic compartments of the larval mushroom body, i.e. in calyx, peduncle and lobes.



Movie S4:

Gal4 expressing cells in D52H-Gal4 monitored by UAS-GFP (green), related to Fig. 3.

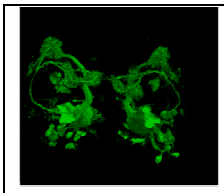
View on a single mushroom body. Expression is found in only very few mushroom body neurons (~7 mushroom body neurons per brain hemisphere). Notably, although only so few mushroom body neurons are covered, GFP expression reveals all basic compartments of the larval mushroom bodies; in particular the mushroom body input regions (the calyx) seems to be covered fairly well.



Movie S5:

Gal4 expressing cells in GH146-Gal4 monitored by UAS-GFP (green), related to Fig. 4.

View on the projection neurons in the larval brain. When the *GH146*-Gal4 driver is used to express GFP, additionally to the expression in the projection neurons, a single mushroom body-extrinsic neuron per hemisphere is GFP-positive.



Movie S6:

Gal4 expressing cells in NP225-Gal4 monitored by UAS-*GFP (green), related to Fig. 4.*

Same as Movie S5 but using *NP225*-Gal4 as another projection-neuron Gal4 strain.

I Olfactory associative learning in *Drosophila* larva

Cover Illustration
Timo Saumweber

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Behavioral/Systems/Cognitive

Behavioral and Synaptic Plasticity Are Impaired upon Lack of the Synaptic Protein SAP47

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 0270-6474/11/313508-11$15.00/0

4. Behavioral and synaptic plasticity are impaired upon lack of the synaptic protein SAP47

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Key words:

Learning, *Drosophila* larva, SAP47, Memory, Olfaction, RNAi

Abstract

The synapse associated protein of 47 kDa (SAP47) is a member of a phylogenetically conserved gene family of hitherto unknown function. In Drosophila, SAP47 is encoded by a single gene (*Sap47*) and is expressed throughout all synaptic regions of the wild-type larval brain; specifically, electron microscopy reveals anti-SAP47 immuno-gold labeling within 30 nm of presynaptic vesicles. To analyze SAP47 function, we use the viable and fertile deletion mutant $Sap47^{156}$, which suffers from a 1.7 kb deletion in the regulatory region and the first exon. SAP47 cannot be detected by either immuno-blotting or immuno-histochemistry in $Sap47^{156}$ mutants. These mutants exhibit normal sensory detection of odorants and tastants as well as normal motor performance and basic neurotransmission at the neuromuscular junction. However, short-term plasticity at this synapse is distorted. Interestingly, $Sap47^{156}$ mutant larvae also show a 50 % reduction in odorant-tastant associative learning ability; a similar associative impairment is observed in a second deletion allele ($Sap47^{201}$) and upon reduction of SAP47 levels using RNA-interference. In turn, transgenically restoring SAP47 in $Sap47^{156}$ mutant larvae rescues the defect in associative function. This report thus is the first to suggest a function for SAP47. It specifically argues that SAP47 is required for proper behavioral and synaptic plasticity in flies- and potentially its homologues in other species.

Introduction

The relationship between brain and behavior is the core topic of neuroscience. Given the multitude of events associated with any given behavior, it seems reasonable to address this issue in the form of its first time-derivative, i.e. to ask which change in the brain is necessary and sufficient for a given change in behavior. In this sense, studying associative learning and memory, rather than being particularly difficult, may be tackling the relation between brain and behavior in a particularly accessible form. Within this context, we focus on the role of a specific synaptic protein (the Synapse-associated-protein of 47 kDa [SAP47]; Reichmuth et al., 1995; Funk et al., 2004; Hofbauer et al., 2009) for associative function and synaptic physiology in larval *Drosophila*. This seems timely, given the importance of synaptic processes for learning in general (e.g. Lechner and Byrne, 1998; Pittenger and Kandel, 2003) and in larval *Drosophila* in particular (e.g. Michels et al., 2005).

The *Sap47* gene of *Drosophila* (Fig. 1A) was identified as a synaptic protein by a monoclonal antibody screen from a hybridoma library raised against *Drosophila* brain (Hofbauer et al., 2009). It codes for a protein with a highly between nematode, fly, fish and human conserved BSD domain (Reichmuth et al., 1995; Funk et al., 2004). The BSD domain is localized in a conserved central region and found in BTF2-like transcription factors, SAP47 homologue proteins, and DOS2-like proteins. The domain is characterized by three predicted α helices and a conserved phenylalanine-tryptophan amino acid pair (Doerks et al., 2002). SAP47 is abundant in synaptic terminals (Reichmuth et al., 1995; Funk et al., 2004), but a role in synaptic function and / or behavior has not been determined, in any species.

Here we test for an association of SAP47 with synaptic vesicles using immuno-gold-labelling in electron microscopy and describe the expression pattern of SAP47 in the brain of larval *Drosophila* by confocal immuno-histochemistry. To analyze the function of SAP47, we use the fully viable and fertile deletion mutant $Sap47^{156}$ (Funk et al., 2004). After extensive outcrossing of this mutant with the wild-type strain Canton-S (henceforth called WT), we test for the genomic status of WT and $Sap47^{156}$ by PCR, and for a possible residual expression of SAP47 in $Sap47^{156}$ mutants. We then ask whether basic synaptic transmission at the neuromuscular junction as well as short-term synaptic plasticity at this synapse may be distorted, and whether sensory and motor abilities are intact in larvae lacking SAP47. We then move on to test whether $Sap47^{156}$ mutants are impaired in an odorant-reward associative conditioning task (see Review by Gerber and Stocker, 2007), and whether knocking down SAP47 by RNAi mimics this impairment (Brand and Perrimon, 1993; Smith et al., 2000; Kalidas and Smith, 2002). We finally test whether the associative defect of $Sap47^{156}$ mutant

larvae can be rescued by restoring SAP47. We will conclude that SAP47 functions in associative behavioural and short-term synaptic plasticity, without, however, contributing to basic synaptic transmission or to task-relevant sensory-motor function.

Materials and Methods

We use third-instar feeding-stage larvae collected 5 days after egg laying. Flies are kept in mass culture and are maintained at 25° C, 60- 70 % relative humidity and a 14/ 10 hour light/ dark cycle. Electrophysiology and behavioral experiments are performed blind with respect to genotype and treatment condition; these are decoded only after the experiments.

Single-larvae PCR

To confirm the deletion status of the *Sap47*^{156} mutant (Funk et al., 2004) single-larva PCR is performed according to Gloor et al., (1993) (Fig. 1A, B and Suppl. Fig. 10A): the primer I binding site (5′ GAG AAG AGC TCG ACT TTC CAG 3′) is upstream of the deletion, the binding site of primer II (5′ CTT CGC TCT CTT GGA CTC G 3′) is within the deletion and the binding site of primer III (5′ CCT ATC CAC TCA GTT TGA GGG 3′) is downstream of the deletion. The PCR product of primer pair I / II should generate a 644 nt fragment only in WT, whereas primer pair I / III should produce a 582 nt product only in Sap47156 mutants because an elongation time is chosen that is too short to amplify the predicted 2309 nt WT fragment.

Probing for expression of long isoforms of SAP47

Given that the predicted long isoforms of SAP47 cannot readily be detected on a Western blot, we probed for the expression of long isoforms of Sap47 on the cDNA level. Total RNA was isolated from WT larvae by homogenizing 200 larvae in 1 ml TRIzol (Invitrogen, Karlsruhe, Germany) followed by 5 min incubation at room temperature. After adding 200 µl chloroform the samples were centrifuged (12.000 g) and supernatant was selected for an isopropanol precipitation. Then, RNA was resuspended in 100 µl DEPC-water. cDNA was produced using oligo-dT-primer with the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany). PCR specific for long Sap47 isoforms was subsequently performed using the following primers: (A) 5′-CTC CGC AAG GGC GCA GGA-3′ (forward-primer); (B) 5′- TTC AGT GAT GAT CTT GGG CAC CAG-3′ (reverse-primer); (C) 5′- CCC AGC TCT TTG CCG GC- 3′ (reverse-primer). PCR was performed using standard protocols, followed by electrophoresis in 0.8 – 1.0 % agarose gels.

Fly strains

We compare WT to the deletion mutant *Sap47*^{156}. The *Sap47*^{156} strain carries a deletion generated by imprecise P-element excision; it is characterized by a 1727 bp deletion, which removes part of the promoter region, the first exon and a small part of the first intron of the *Sap47* gene (Fig. 1A and Suppl. Fig. 10) (Funk et al., 2004). This strain is outcrossed to WT for nine generations to remove marker genes and to effectively adjust genetic background, which may otherwise distort results (DeBelle and Heisenberg, 1996; Diegelmann et al., 2006a). The second mutant *Sap47* allele used is *Sap47*^{201}, also generated by P-element jumpout and suffering a ~ 5.8 kb deletion containing the promoter region, the first exon and a larger part of the first intron; this strain still is in the *w*^{1118} mutant background. The status of the *white* gene is inconsequential for associative function in our paradigm (Fig. 5A, A´).

For a knock-down of the SAP47 protein we use the widely-expressing *elav*-Gal4 driver strain (*elav*-Gal4 [X]: Robinow and White, 1988; called c155 in Lin and Goodman, 1994;) from the Bloomington stock centre. These *elav*-Gal4 flies are crossed to UAS-RNAi-SAP47-effector flies ([VII10], the construct consists of a ca. 1.1 kb fragment of *Sap47* cDNA [exons 1, 3- 7] inserted in sense and anitsense orientation; for more detail see Funk et al., 2004) to yield animals that carry both transgenes heterozygously; therefore, in these animals SAP47 expression should be reduced pan-neuronally. Genetic controls are heterozygous for either the driver or the effector transgene, respectively.

For pan-neural rescue expression of SAP47, we combine the driver strain *elav*-Gal4 [X] (c155) into the *Sap47*^{156} mutant background by classical genetics. As effector strains for the rescue experiments we use either UAS-*Sap47-RF*; *Sap47*^{156} [III] or UAS-*Sap47-RA*; *Sap47*^{156} [II; III]. The UAS-*Sap47-RF*; *Sap47*^{156} [III] strain was generated on the basis of Wegener, 2008 (loc. cit. Fig. S2 UAS-*Sap47-1*; *Sap47*^{156}; the coding region corresponds to Flybase [http://flybase.bio.indiana.edu]: Transcript Dmel\ *Sap47-RF*: FlyBase ID: FBtr0301655; for the predicted amino acid sequence, see Suppl. Fig. 10). To generate the experimental genotype for rescue experiments, we cross the driver strain (*elav*-Gal4; *Sap47*^{156}) to the UAS-*Sap47-RF*; *Sap47*^{156} effector strain, yielding double heterozygous larvae in which expression of the 47 kDa isoform of SAP47 is restored. Genetic controls are in the *Sap47*^{156} mutant background and are heterozygous for either the driver or the effector transgene, respectively.

For expressing the full length isoform of SAP47, the UAS-*Sap47-RA*; *Sap47*^{156} [II; III] effector strain was generated. The full length cDNA clone LD36546 ([http://flybase.bio.indiana.edu]: Transcript Dmel\ *Sap47-RA*; FlyBase ID: FBcI0175830; for the predicted amino acid sequence, see Suppl. Fig. 10) from the *Drosophila* Genomics

Resource Center (CGB, Indiana University, Bloomington, USA) was used to amplify the full length cDNA by PCR with primers containing a NotI and KpnI restriction site. The used primer pair was: Primer 1: 5´-ATA AGA ATG CGG CCG CCG CAG TTG TTG TTT CC-3´ and Primer 2: 5´-GAG CGG TAC CGG TTT CGA ATA GTT TTG TAT TTT GTT TGG-3´. The resulting PCR fragment was excised as a 2822 bp NotI/ KpnI fragment, ligated into NotI/ KpnI-cut pUAST (Brand and Perrimon, 1993) and transformed into recombination-deficient SURE2 super competent cells (Stratagene, La Jolla, USA). Germ-line transformation then was performed into a w^{1118} strain (Bestgene, Chino Hills, USA). The resulting effector strain was combined into $Sap47^{156}$ mutant background by classical genetics. To generate the experimental genotype for the rescue experiments, we cross the driver strain (*elav*-Gal4; $Sap47^{156}$) to the UAS-*Sap47-RA*; $Sap47^{156}$ effector strain, yielding double hetrozygous larvae in which expression of the full length isoform of SAP47 of about 70 kDa is restored. Genetic controls are also in the $Sap47^{156}$ mutant background, and are heterozygous for either the driver or the effector transgene, respectively.

Please note that all transgenic fly strains used are in the *white* mutant background (w^{1118}) in order to keep track of the transgenes. Therefore, genetic controls are established by crossing the respective transgenic strains to a w^{1118} strain (in the case of the RNAi experiment) or a w^{1118}; $Sap47^{156}$ strain (in the case of the rescue experiments). To see whether the w^{1118} mutation may have an effect, either on associative learning (Diegelmann et al., 2006a; Yarali et al., 2009b) or on SAP47 expression, we compare w^{1118} versus WT larvae in odorant-reward associative ability as well as by Western blot analysis (Fig. 5A, A´).

Immunocytochemistry: Electronmicroscopy

For immuno-gold localisation of SAP47, preparations of the nerve-muscle synapse are made in ice-cold HL3 ringer (Stewart et al., 1994), fixed in 4 % paraformaldehyde in 0.1 cacodylate buffer (pH 7.2) for 90 min on ice, washed three times for 30 min in this buffer, incubated in 10 mM ammonium chloride for 15 min, and washed two times for 15 min in H_2O. After dehydration in ascending ethanol series, 15 min at -20° C for each step, the tissue is incubated for 1 h in a 1+1 mixture of LR-White (Polysciences Europe GmbH, Eppelheim, Germany) and ethanol at -20° C, followed by two 1-h incubation periods in pure LR-White at -20° C, before the preparations are stored at 4° C for 3 days to achieve complete penetration of the resin. Polymerization is allowed to proceed for five days at 41° C. After verification of section plane from 2 µm sections in the light microscope, ultrathin (70 nm) sections are cut and transferred to copper grids; grids are then washed 5 min on a drop of PBS at pH 7.2,

blocked for 5 min on a drop of 1 % BSA in PBS and incubated for 24 h at 4° C on a drop of the mouse monoclonal antibody nc46 (Hofbauer et al., 2009; see also Funk et al., 2004), diluted 1:25 in PBS. After storage of the grids for 1 h at 37° C in a moist chamber to increase antibody affinity, they are washed thoroughly in PBS and incubated for 1 h on a drop of 1:10 diluted anti-mouse IgG conjugated to 12 nm gold particles (Dianova, Hamburg, Germany) and subjected to a final wash.

Immunocytochemistry: Neuromuscular junction (NMJ)

Third instar larvae are dissected in calcium-free saline (Stewart et al., 1996) and fixed in 4 % paraformaldehyde/ phosphate buffer for 30 min at room temperature. After three 10-min washes in PBST, filets are blocked with 1.5 % normal horse serum diluted in PBST for 30 min at room temperature. The used primary monoclonal anti-SAP47 mouse antibody (nc46, 1:10 in PBST) is added and all filets are kept overnight at 4° C. As secondary antibody, a rabbit anti-mouse Ig conjugated with Alexa Fluor® 488 (1:500 in PBST) (Molecular Probes, Invitro Detection Technologies, Karlsruhe, Germany) is used; to label cell membranes, a TexasRed-coupled rabbit anti-HRP antibody (1:250) (Jackson labs; Maine; USA) is added to the incubation solution. Confocal data are acquired as image stacks of separate channels with a Leica TCS SP 1 laser scanning confocal microscope, combined and visualized with Leica TCS NT software. Final pictures are obtained as maximum intensity projections.

Immunocytochemistry: Whole-brain and cephalic sensory system preparation

Brains are dissected in Ringer solution on ice and incubated in fixative (2.5 % paraformaldehyde and 0.3 % Triton X-100 in 1xPBS) for two hours. After washing them three times in PBST (0.3 % Triton X-100 in 1xPBS), blocking is performed with 3 % normal goat serum (NGS) in 1xPBST for 1.5 hours. The primary monoclonal anti-SAP47 mouse antibody nc46 (1:10 in 3 % NGS-PBST) is added and all brains are incubated overnight at 4° C. Samples are washed six times in PBST for 10 min and incubated overnight with Alexa Fluor® 568 Phalloidin (1:200) (Molecular Probes; Lot 41A1-4; Eugene; Oregon; USA) for counterstaining of F-Actin, and the secondary antibody (goat anti-mouse Ig, conjugated with Alexa Fluor® 488 1:200 in PBST; Molecular Probes, Invitro Detection Technologies, Karlsruhe, Germany) at 4° C. Preparations of the cephalic sensory system are additionally stained with a rat anti-Elav antibody (Jackson labs; Maine; USA) as neuronal nucleus marker. As secondary antibody goat anti-rat Ig conjugated with Alexa Fluor® 647 1:200 (Molecular

Probes, Invitro Detection Technologies, Karlsruhe, Germany) is used. Preparations are examined under a Leica TCS SP 1 laser scanning confocal microscope, combined and visualized with Leica TCS NT software.

Western blotting

After homogenization of 10 larval brains in Laemmli buffer and electrophoretic separation (SDS-PAGE), proteins are transferred onto a nitrocellulose membrane (45 µm, Schleicher and Schuell, Dassel, Germany) by semi dry blotting (Khyse-Anderson, 1984) with a semi-dry electro-blotter (PEQLAB, Erlangen, Germany). After blotting, the membrane is transferred in 5 % powdered milk in TBST and washed for three times in TBST. Blots are probed with either the nc46 antibody, or with the mouse monoclonal antibody nb200; these antibodies recognize different epitopes of the protein within (nc46) and more C-terminal (nb200) of the deletion (Fig. 1G; Hofbauer et al., 2009; see also Funk et al., 2004), as well as with the mouse monoclonal ab49 antibody against the Cysteine String Protein (CSP) (corresponding to the DCSP1 antibody in Hofbauer et al., 2009; see also Arnold et al., 2004), marking a band at 32 kDa as loading control in all cases. Antibodies are used at the following dilutions in 1x TBST: nc46 [1:100], nb200 [1:50] and ab49 [1:400]. To detect antibody labelled proteins IgG-HRP conjugated goat anti-mouse (1:3750 in TBST) and ECL^{TM}-Western blotting detection reagents (Amersham, GE Healthcare, Ismaning, Germany) are applied.

Electrophysiology

Two-electrode voltage clamp (TEVC) recordings are performed on ventral longitudinal muscle 6 of male third-instar larvae in extracellular haemolymph-like solution HL3 containing 70 mM NaCl, 5 mM KCl, 20 mM $CaCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES and 1 mM$CaCl_2$. Recordings are made from cells with input resistances of at least 4 MΩ and initial membrane potentials between -50 and -70 mV. Intracellular electrodes with resistances of 10- 35 MΩ filled with 3 M KCl are used. The holding potential is -60 mV for evoked excitatory postsynaptic currents (eEPSCs) and -80 mV for miniature excitatory postsynaptic currents (mEPSCs). EPSCs are recorded at a stimulation frequency of 0.1 Hz. For the analysis of short-term plasticity, trains of 100 stimuli at 60 Hz are applied. Care is taken to ensure the recruitment of both motoneurons innervating muscle 6. Recordings are analyzed with pClamp10 (Axon Instruments). Experiments are carried out blind with respect to genotype.

Associative function

Larval learning experiments represent a modified version of the mass assay described in Neuser et al., (2005; for sketches see Fig. 2A, B). Notably, unless mentioned otherwise, we here use only one odorant, n-amyl acetate (AM, CAS: 628-63-7, purity: 99 %, Merck, Darmstadt, Germany) to simplify the paradigm (Selcho et al. 2009; Saumweber et al., 2011a). That is, we train groups of 30 larvae each and compare olfactory choice performance after either of two reciprocal training regimen: one group is exposed to the odorant AM in the presence of a positive reinforcer and to a no-odor situation without the reinforcer (AM+ / noAM); the second group is trained reciprocally, i.e. by unpaired presentations of odorant and reinforcer (AM / noAM+). Then, animals are tested for their choice between AM versus noAM. Associative learning is indicated by systematic differences in test performance between the reciprocal treatment conditions. The reciprocally trained groups were run alternately, which allows stringent pairing of data for the calculation of a performing index (PI; see below and discussion in Hendel et al., 2005). For a differential, two-odor version of our paradigm (Suppl. Fig. 5), we use 1-OCT (OCT, CAS: 111-87-5; purity: 99 %, Sigma-Aldrich, München, Germany) as second odor.

Petridishes (Sarstedt, Nümbrecht, Germany) with 85 mm inner diameter are filled with 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany) allowed to solidify, covered with their lids, and left untreated until the following day. As positive reinforcer we use 2 mol fructose (FRU, purity: 99 %) added to 1 l of agarose 10 min after boiling.

As an odorant we use amyl acetate diluted 1:1600 in paraffin oil which by itself is not behaviorally active (Saumweber et al., 2011a); OCT is used undiluted. Experiments are performed in red light under a fume hood at 21- 24° C. Just before the experiments, we replaced the regular lids of the Petri-dishes with lids perforated in the center by roughly 60 1-mm holes to improve aeration.

A spoonful of food medium containing larvae is taken from the food bottle and transferred to a glass vial. Thirty animals are collected, briefly washed in tap water and as a group transferred to the assay plates for the start of training. Each training trial lasts 5 minutes. Immediately before a trial, two custom-made Teflon containers for possible loading with odorant (5 mm inner diameter, lid with seven 0.5 mm holes) are placed onto the assay plate on opposite sides of the plate, 7 mm from the edges. Within each reciprocal training condition, for half of the cases we start with AM in the odorant containers, for the other with noAM. Consequently, for half of the cases we start with an agarose plate that had FRU added to the substrate, and for the other with a plate without FRU. Then, the lid is closed and the larvae are allowed to move for 5 min. The larvae are then transferred to a plate with the

alternative odorant condition and the respective other substrate for 5 min. This cycle is repeated three times. Fresh assay plates are used for each trial.

After this training, animals are tested for their odor choice. The larvae are placed in the middle of a fresh, pure agarose assay plate with a container of odorant on one side and an empty container on the other side (AM *versus* noAM); for half of the cases, AM is to the left, for the other half of the cases to the right. After 3 min, the number of animals on the "AM" or "noAM" side is counted. 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 noAM side, divided by the total number of larvae:

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

For all learning experiments, these PREF scores are documented in the Supplementary material. To determine whether these preferences are different depending on training regime, we took the paired data from alternately run, reciprocally trained groups and calculate a performing index ranging from –1 to 1 as:

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

After the data for one such PI value had been collected for one of the genotypes, the corresponding data for the other genotype are gathered, i.e. data from both genotypes are obtained alternately.

Detectability of odorant and reward

To test whether larvae are able to detect the to-be-associated stimuli, animals are tested for their preference between AM versus noAM as well as between FRU and noFRU. The assay for the detectabilty of the odorant is the same as described above for the test in equation (1), except that experimentally naive animals are used.

To test the ability to detect FRU, split Petri dishes are prepared with one side pure agarose and the other with fructose added to the agarose (Hendel et al., 2005). Larvae are positioned in the middle of the Petri dish; after 3 min, the number of animals on either side is determined for calculation of the FRU preference in a way corresponding to equation (1).

Sham training and tests for sequence effects

Associative training by necessity involves exposure to the odorant, the reward and handling. One may therefore argue that these aspects of training may induce motivational changes, fatigue, habituation, and / or adaptation, which may feign a learning deficit in the mutants if they were more – or less- susceptible to these kinds of effects than the wild-type. Therefore, the response to the odorant needs to be tested after so-called ´sham training´ (Michels et al., 2005; Knapek et al., 2010). Two types of sham training controls are run: Both consisted of the same treatments as in the learning experiment, except that either the reinforcer is omitted ($SHAM^{Odor\text{-}noReward}$), or the odorant is omitted ($SHAM^{Reward\text{-}noOdor}$). After either of these sham training regimen, the ability of the larvae to detect the odorant is tested as detailed above.

The learning experiment allowed for a post-hoc analysis of possible effects of the sequence of stimulus presentation on test performance: We compare the AM-preferences of those groups which share the same odorant-reward contingency, but differ in terms of the temporal pattern of the two types of trials (AM+ / noAM *versus* noAM / AM+ [Suppl. Fig. 1A]; noAM+ / AM *versus* AM / noAM+ [Suppl. Fig. 1B]). We do not find any effect of the timing of trial types, neither in WT (Suppl. Fig. 1) nor in $Sap47^{156}$ (not shown). The same kind of analysis is possible for sham training experiments: We compare the AM-preferences between that group of larvae exposed to AM during the first, third and fifth trial and to noAM during the other trials (AM / noAM) to the AM-preferences of that group that was exposed to AM during the second, fourth and last trial (noAM / AM) (Suppl. Fig. 1C). Similarily, we compare the AM-preferences between those groups that did or did not receive the reward during the first, third and fifth trial before test (Fru / noFRU *versus* noFRU / FRU; Suppl. Fig. 1D). In both cases, the sequence of stimulus presentation is without effect on test performance in WT (Suppl. Fig. 1) as well as in $Sap47^{156}$ (not shown).

Statistical analyses

In a conservative approach, we use non-parametric analyses throughout; comparisons of values against zero, i.e. chance level, are made with one-sample sign tests. All comparisons are significantly different from chance, unless mentioned otherwise. Comparisons between multiple or two genotypes are done with Kruskal-Wallis or Mann-Whitney U-tests, respectively. We correct the level of significance in cases where multiple comparisons are made by dividing the P-level of 0.05 by the number of comparisons made (Bonferroni-correction) to maintain an experiment-wide error rate of 5 %. Shared letters above boxes indicates that groups behave not significantly different whereas significant differences between groups are indicated by different letters above boxes. Data are displayed as box plots

with the middle line indicating the median and box boundaries and whiskers the 25, 75, 10 and 90 % quantiles, respectively. Analyses are carried out with Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA) on a PC.

Results

Ultra structural localization of SAP47

After confirming the genomic status of WT and the outcrossed *Sap47*^{156} mutant (Fig. 1A, B), we determined the ultra structural localization of SAP47 at presynaptic terminals of WT larval motor neurons (Fig. 1C). Of 1631 gold particles in sections from 18 different presynaptic terminals, 87.5 % were located within 30 nm of a synaptic vesicle (SV). Only 8.5 % of the particles did not have a vesicle within that distance. We considered 4 % of the gold particles detected outside boutons as unspecific background. Interestingly, SAP47 did not appear to be integral to the synaptic vesicle membrane because SAP47 was found in the soluble fraction of brain homogenate (Arnold et al., 2004, loc. cit. Fig. 2), and glycerol density gradient centrifugation separated SAP47 from known integral synaptic vesicle membrane proteins (such as CSP: Umbach et al., 1994; Arnold et al., 2004, loc. cit. Fig. 3).

Thus, SAP47 is associated with synaptic vesicles, but is not an integral part of the synaptic vesicle protein complement.

Sap47^{156} *is a protein-null mutant allele*

At neuromuscular junctions of muscle pair 6 / 7 and in preparations of the whole larval brain viewed under the confocal microscope, the Sap47156 strain did not show any SAP47 immunoreactivity, whereas in WT presynaptic terminals and the complete neuropil region, respectively, were strongly stained (Fig. 1D, E; used antibody: nc46). SAP47 was also expressed in the cephalic chemosensory system (DO, TO and the Bolwig organ) of WT third instar larvae (Fig. 1F). Furthermore, we could not detect any SAP47 signal on a Western blot for *Sap47*^{156}, whereas WT showed the expected (Reichmuth et al., 1995; Funk et al., 2004; Hofbauer et al., 2009) strong band at 47 kDa with both monoclonal antibodies used (Fig. 1G; nc46 with its epitope FSGLTNQFTS which was within the *Sap47*^{156} deletion; nb200 with its epitope QQAKHF which is downstream, C-terminal of the *Sap47*^{156} deletion [Hofbauer et al., 2009]). One of the heavier bands at ~62 kDa was seen only with the nc46 antibody; this band, however, is typically weaker and more variable in Western blots, potentially because of temporal and / or local specificity of expression (Funk et al., 2004; see also our Fig.s 1G, 6B, 7B).

Thus, also at the larval stage, *Sap47*^{156} qualifies as a protein-null mutant (for adult *Drosophila*: Funk et al., 2004).

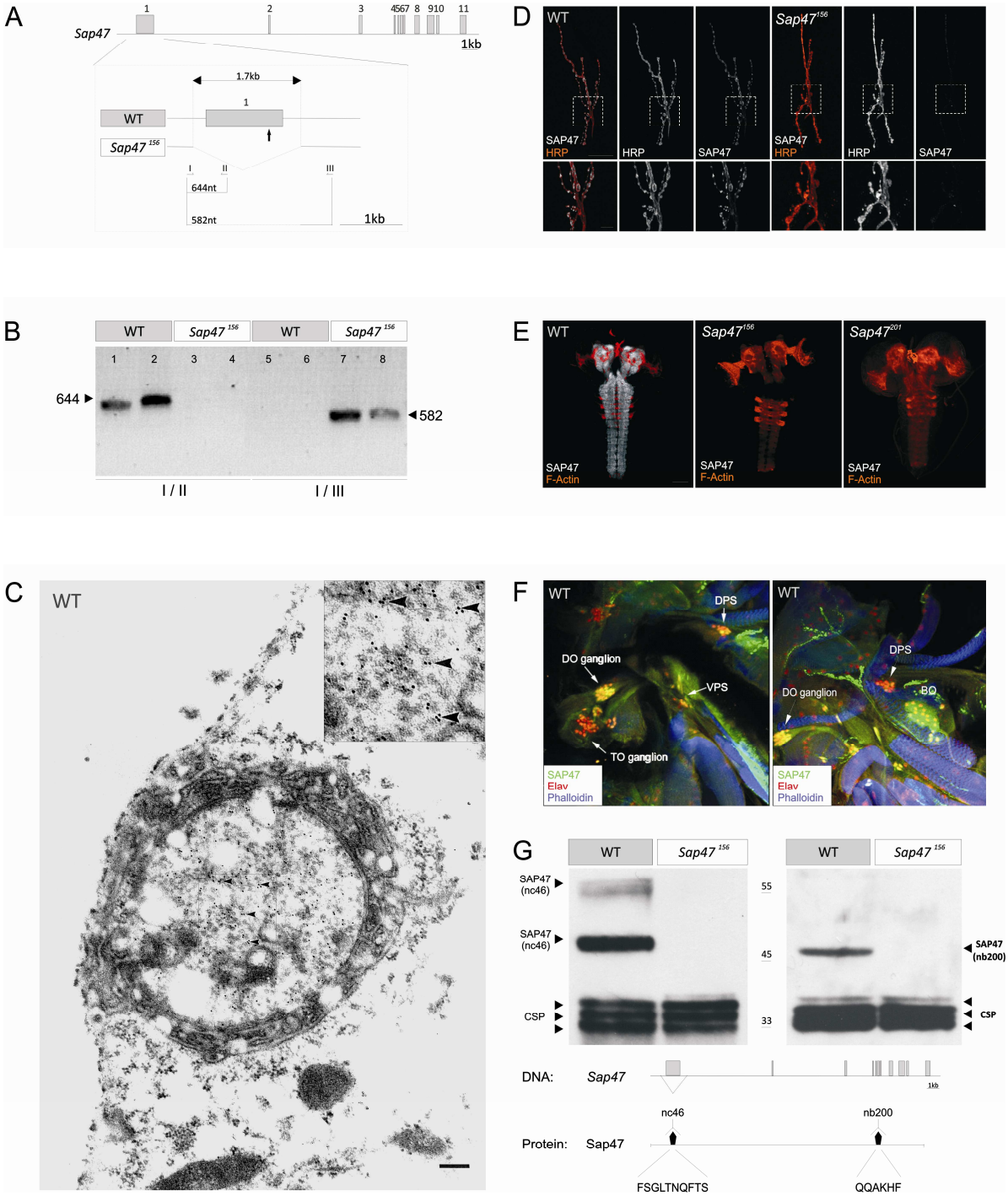


Figure 1:

Characterizing the Sap47 gene and the Sap47^{156} *mutant*

A: Gene structure
Shown are the exon-intron structure of the *Sap47* gene in wild-type (WT: exons grey). The deletion in *Sap47*^{156} is shown in higher magnification. Arrows and roman numerals indicate binding sites of primers used for PCR in (B).

B: PCR

PCR with primer pair I / II generates a 644 bp fragment only in WT, because there is no binding-site for primer II in the *Sap47*^{156} mutant; primer pair I / III generates a 582 bp fragment only in the *Sap47*^{156} mutant, because elongation time was chosen that is too short for amplification of the long WT fragment. Two samples were run for each condition.

C: Electron microscopy

Shown is a synaptic bouton with synaptic vesicles and immuno-localization of SAP47 in WT larvae using electron microscopy at the larval neuromuscular junction. Bound primary mouse anti-SAP47 antibodies were detected with gold-conjugated secondary anti-mouse antibodies. Gold particles (arrowheads) are localized close to synaptic vesicles. The inset represents the boxed area in higher magnification. Scale bar 200 nm.

D: Neuromuscular junction

At the neuromuscular junction (muscle pair 6 / 7), the *Sap47*^{156} strain does not show any anti-SAP47 immuno-reactivity, whereas in WT, synaptic boutons are stained (white). Preparations are double-labelled with a TexasRed-coupled anti-HRP antibody to label cell membranes (orange), and the nc46 antibody to label SAP47, and are viewed under a confocal microscope. Scale bar 20 µm. The lower panels represent an enlarged view of the boxed area. Scale bar 5 µm.

E: Whole mount larval brains

In both mutant strains (*Sap47*^{156} and *Sap47*^{201}), no anti-SAP47 immunoreactivity is detectable in whole mount preparations of the larval brain, whereas the neuropil regions in WT are strongly stained (antibody: nc46 [white]). For orientation, F-Actin is visualized with phalloidin staining (orange). Scale bar 50 µm.

F: Cephalic sensory systems

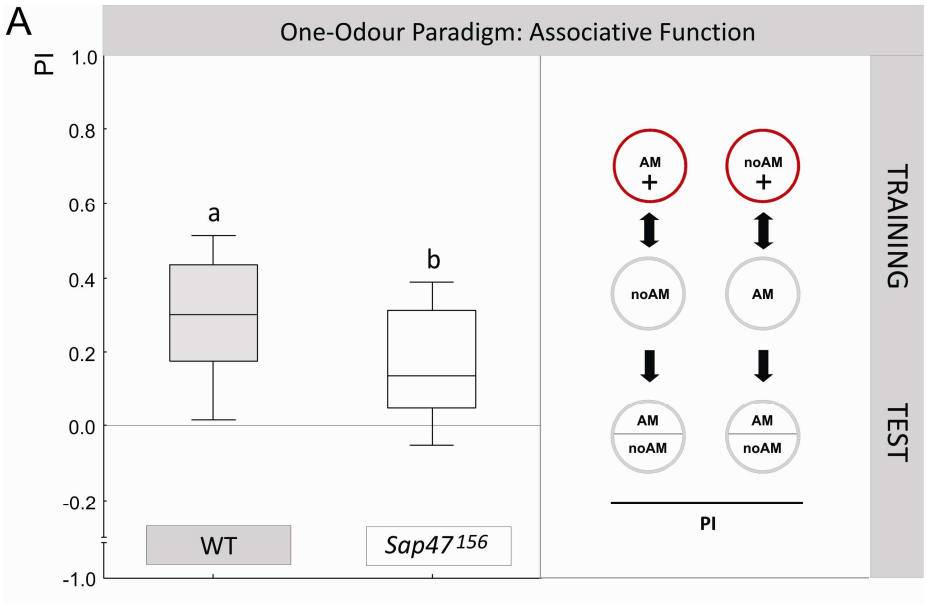
SAP47 immuno-reactivity is detectable in single confocal slices of the cephalic sensory systems of third instar WT larvae. DO, TO, the dorsal, posterior and ventral pharyngeal sense organs (DPS, PPS (not shown) and VPS) and the Bolwig organ are at least partially stained (antibody: nc46 [green]). For orientation, F-Actin is visualized with phalloidin (blue) and an anti-Elav antibody is used to stain neuronal nuclei (red). Note that in the DO ganglion SAP47 is found in cell nuclei.

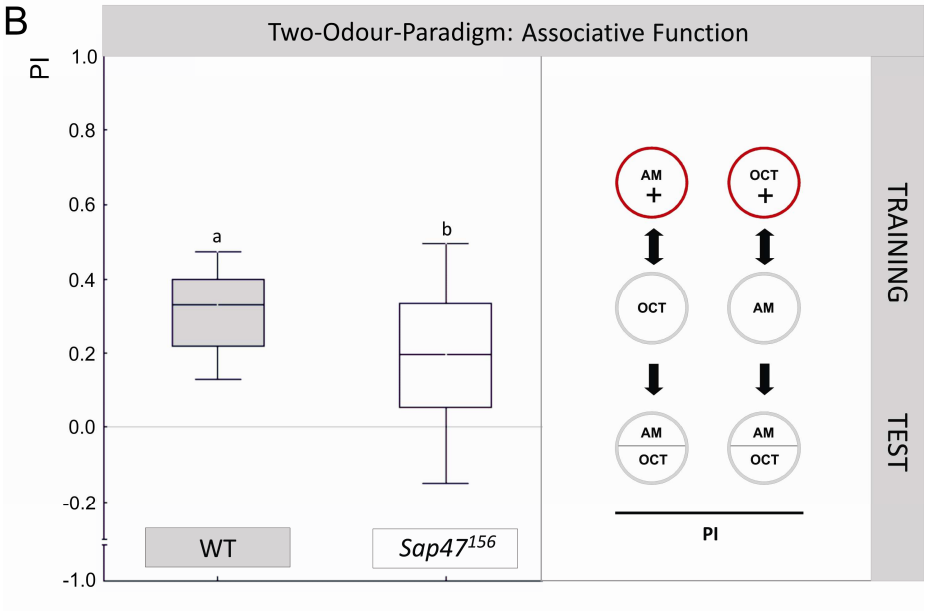
G: Western blot

There is no SAP47 signal detectable on Western blot in the *Sap47*^{156} mutant, whereas WT shows the expected (Reichmuth et al., 1995; Funk et al., 2004) strong band at 47 kDa with both monoclonal antibodies used (left: nc46, with its epitope FSGLTNQFTS, which is within the *Sap47*^{156} deletion; right: nb200, with its epitope QQAKHF, which is downstream/ C-terminal of the *Sap47*^{156} deletion [Hofbauer et al., 2009]). As loading control, a monoclonal antibody against the synaptic protein CSP is used (antibody: ab49).

Sap47^{156} *larvae are defective in associative function*

We next tested whether *Sap47*^{156} mutant larvae are impaired in associative function. In an odorant-reward associative learning experiment, we found significant performance indices for both *Sap47*^{156} (Fig. 2A; one-sample sign test: $P < 0.05 / 2$; $N = 35$) and WT (Fig. 2A; onesample sign tests: $P < 0.05 / 2$; $N = 35$), arguing that associative faculties in principle are available to both genotypes. However, associative function in Sap47156 was reduced to ~50 % of WT levels (Fig. 2A; $P < 0.05$, $U = 408.5$; sample sizes as above). The same defect was observed for a two-odor differential conditioning paradigm (Fig. 2B; see legend for statistics) as well as for another deletion allele, which is also a protein null mutant (Fig. 2C; see legend for statistics).


A
One-Odour Paradigm: Associative Function
PI
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-1.0
a
b
WT
Sap47156
AM +
noAM +
noAM
AM
AM noAM
AM noAM
PI
TRAINING
TEST


B
Two-Odour-Paradigm: Associative Function
PI
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-1.0
a
b
WT
Sap47156
AM +
OCT +
OCT
AM
AM OCT
AM OCT
PI
TRAINING
TEST

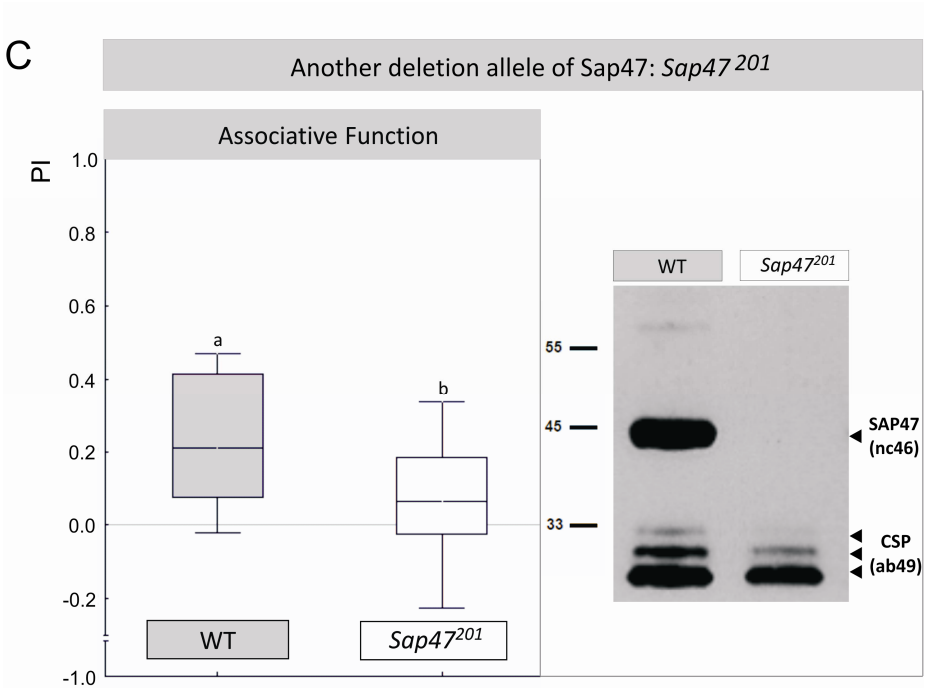


Figure 2:

Sap47^{156} mutants are impaired in associative function

A: *Sap47^{156}* mutants are impaired in associative function
WT and *Sap47^{156}* can learn, but performance indices in *Sap47^{156}* mutants are reduced by ~50 %. The inset illustrates the training regimen: For each genotype, one group is trained such that in the presence of amyl acetate animals are rewarded, whereas in the absence of amyl acetate they are not rewarded (AM+ / noAM); the second group is trained reciprocally (AM / noAM+). Note that in half of the cases the sequence of trials is as indicated (i.e. the respective AM-containing trials first), but in the other half of the cases is inverted; the sequence of training trials is without effect on test performance (Suppl. Fig. 1). The PI measures the extent to which both reciprocally trained groups differ in their AM-preference during the test, and thus provides a measure of associative learning; the PREF scores underlying all PI values are documented in Suppl. Fig. 2A. N= 35, 35.
Different lettering above plots signifies P< 0.05 in a Mann-Whitney-U-test. Box plots represent the median as the middle line, 25 and 75 % quantiles as box boundaries, as well as 10 and 90 % quantiles as whiskers.

B: Also in a differential, two-odor paradigm Sap47156 mutants are impaired in associative function
Performance indices of *Sap47^{156}* mutants are also reduced by ~50 % compared to WT in a differential, two-odor version of the learning paradigm. The inset illustrates the training regime. N= 35 in both cases (one-sample sign tests: P< 0.05 / 2 for both genotypes). Different lettering above plots signifies P< 0.05 (U= 414.5; sample sizes as above) in a Mann-Whitney- U-test. The PREF scores underlying all PI values are documented in Suppl. Fig. 2B.

C: Another deletion allele of Sap47 is also impaired in associative function
Left: Performance indices in the one-odor paradigm in *Sap47^{201}* mutants are reduced by ~50 % compared to WT. N= 16, 14, respectively (one-sample sign test: P=0.21 for *Sap47^{201}* and P< 0.05 / 2 for WT). Different lettering above plots signifies P< 0.05 (U= 61; sample sizes as above) in a Mann-Whitney-U-test. The PREF scores underlying all PI values are documented in Suppl. Fig. 2C.
Right: No signal is detectable on a Western blot of the Sap47201 mutant, whereas WT shows the expected band at 47 kDa. Anti-SAP47: nc46; anti-CSP: ab49 (loading control).

To find out whether reduced associative ability is secondary to sensory or motor impairments, we tested whether *Sap47^{156}* mutants and WT differ in their behavior towards the

to-be-associated stimuli. We did not find any difference between the genotypes in the behavior of experimentally naïve larvae to amyl acetate (Fig. 3A; P> 0.05; U= 827.0; N= 42, 44). Within each genotype, we found approach towards the odorant (Fig. 3A; one-sample sign tests: P< 0.05 / 2 in both cases; sample sizes as above). This argues that animals from both genotypes are able to detect amyl acetate, are attracted by it, and do not differ in those kinds of motor ability that are necessary to behaviorally express their attraction towards this odorant. With respect to the reinforcer, a trend towards lower response levels to the fructose reward in the Sap47156 mutants remained far from being statistically significant, although sample size and scatter seems permissive to pick up such an effect (Fig. 3B; P= 0.21; U= 1207.5; N= 53, 53; for two additional datasets confirming this lack-of-effect see Suppl. Fig. 7AB). Specifically, animals from both genotypes showed a clear preference for fructose (Fig. 3B; one-sample sign tests: P< 0.05 / 2 in both cases; sample sizes as above).

These behavioral control procedures are state-of-the-art in the neurogenetics of *Drosophila* learning. We have, however, recently argued that such testing of behavior in experimentally naïve animals is not sufficient to conclude that an apparent associative defect is indeed due to reduced associative faculties (Michels et al., 2005; Knapek et al., 2010). That is, any associative training procedure obviously requires exposure to the to-be-associated stimuli, i.e. to both the odorant and the reward. Odor exposure is often found to reduce odor preferences in larval *Drosophila* (Boyle and Cobb, 2005; see discussion in Colomb et al., 2007b and Gerber and Stocker, 2007). If in the $Sap47^{156}$ mutants such a decrease in preference would be particularly strong, this could feign an ‘associative' defect. Following Michels et al., (2005), we therefore run a ´sham-training´ control, which involved exactly the same treatment as during a learning experiment, except that the reward was omitted ($SHAM^{Odor\text{-}noReward}$). After such treatment, we found that both genotypes behaved towards the odorant in the same way (Fig. 3C; P> 0.05, U= 743.0, N= 39, 42), in that both genotypes were attracted to the odorant (Fig. 3C; one-sample sign tests: P< 0.05 / 2 in both cases; sample sizes as above). Regarding reward exposure, we in turn run a second kind of ‘sham training’ (Michels et al., 2005), which again mimicked the learning protocol, except that this time the odorants were omitted ($SHAM^{Reward\text{-}noOdor}$). Also after this kind of treatment, behavior towards the odorant did not differ between genotypes (Fig. 3D; P> 0.05; U= 1066.5, N= 50, 48), in that both genotypes were attracted by the odorant (Fig. 3D; one-sample sign tests: P< 0.05 / 2 in both cases; sample sizes as above).

Thus, $Sap47^{156}$ mutants have defects in associative function which do not seem to be due to any task-relevant sensory or motor impairment, or to an altered sensitivity to any nonassociative effect of odor exposure, reward exposure, satiety, or handling.

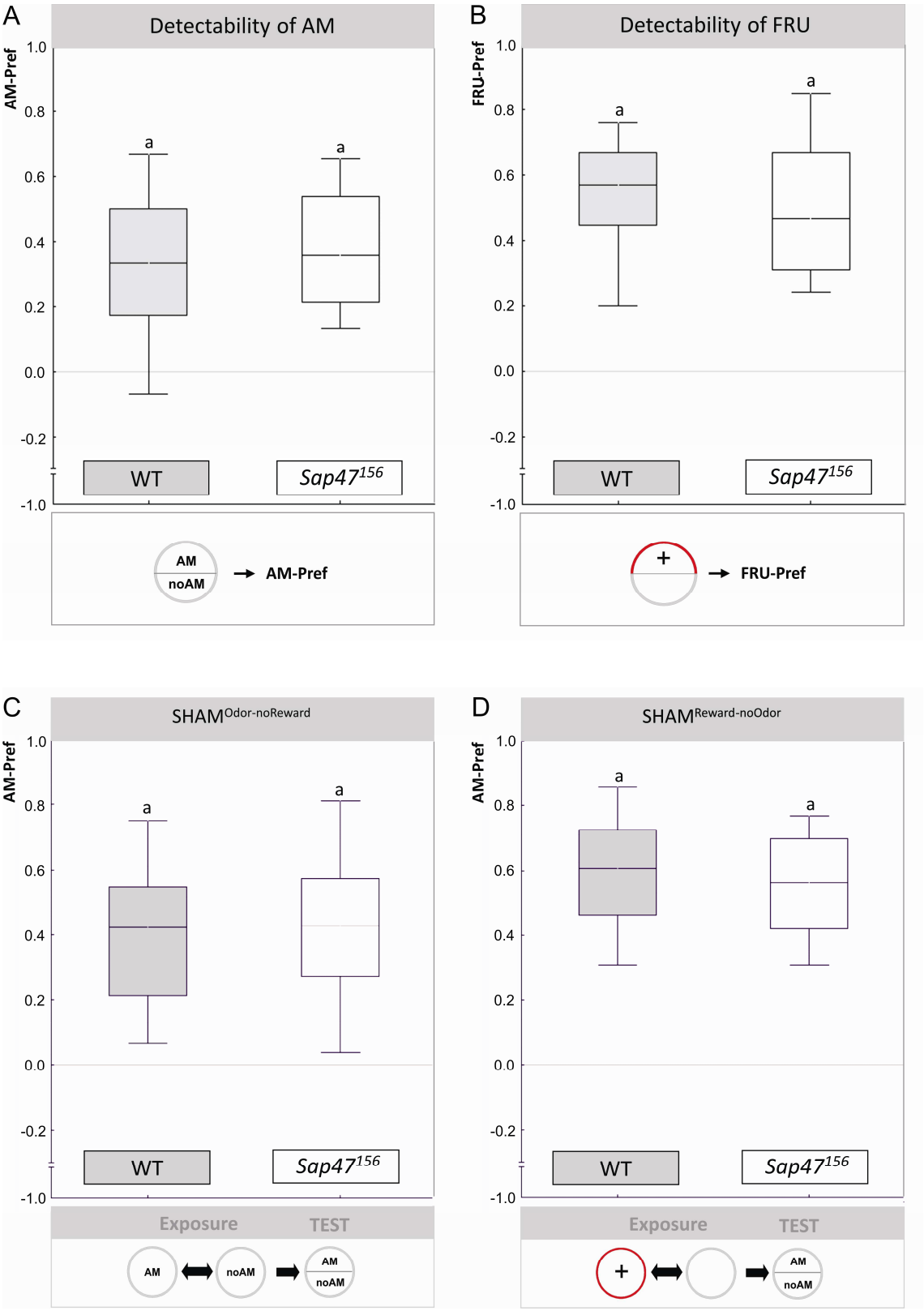


Figure 3:

Behavioral controls: Sensory and motor ability are normal in Sap47^{156}

In (**A**, **B**), the behavior of experimentally naïve animals towards the to-be-associated stimuli is compared between WT and *Sap47^{156}* mutants; in (**C**, **D**) behavior towards the odorant is compared

after animals had undergone “sham-training”. Insets in the figure illustrate the experimental regimen.
A: Behavior towards amyl acetate (AM) in experimentally naïve larvae: WT and *Sap47*^{156} show the same level of preference for amyl acetate. N= 42, 44.
B: Behavior towards the fructose reward (FRU) in experimentally naïve larvae: WT and *Sap47*^{156} show the same level of preference for fructose. N= 53, 53.
C: Behavior towards amyl acetate (AM) after sham-training (SHAM$^{Odor\text{-}noReward}$) which involves exposure to the odorant, but not to the reward. After such treatment, the *Sap47*^{156} mutants and the WT larvae show the same level of AM-preference. N= 39, 42.
D: Behavior towards amyl acetate (AM) after sham-training (SHAM$^{Reward\text{-}noOdor}$) which involves exposure to the reward, but not to the odorant. The *Sap47*^{156} mutants and the WT larvae show the same level of AM-preference also after this kind of treatment. N= 50, 48.

Shared lettering above plots signifies P> 0.05 in Mann-Whithney U-tests.

Synaptic transmission is intact but short-term plasticity in Sap47^{156} *mutant larvae is defect*

Consistent with normal locomotion, basal synaptic transmission at the neuromuscular junction is not altered in *Sap47*^{156}. That is, in voltage clamp recordings, both evoked and spontaneous miniature excitatory postsynaptic currents (eEPSCs and mEPSCs, respectively) were unaltered in *Sap47*^{156} mutants compared to WT (Fig. 4A, B). Furthermore, evoked and spontaneous synaptic potentials are unaltered in current-clamp recordings, too (Suppl. Fig. 9). However, genotypes differed in short-term plasticity during high-frequency stimulation (100 pulses at 60 Hz): *Sap47*^{156} mutants showed stronger depression of synaptic transmission after sustained stimulation as quantified by the steady-state amplitude, which was significantly lower in *Sap47*^{156} mutants than in WT (Fig. 4C; $P< 0.05$, $U= 64$; $N= 15, 15$).

Thus, we conclude that in *Sap47*^{156} mutant larvae basic synaptic transmission is intact, but short-term plasticity is distorted.

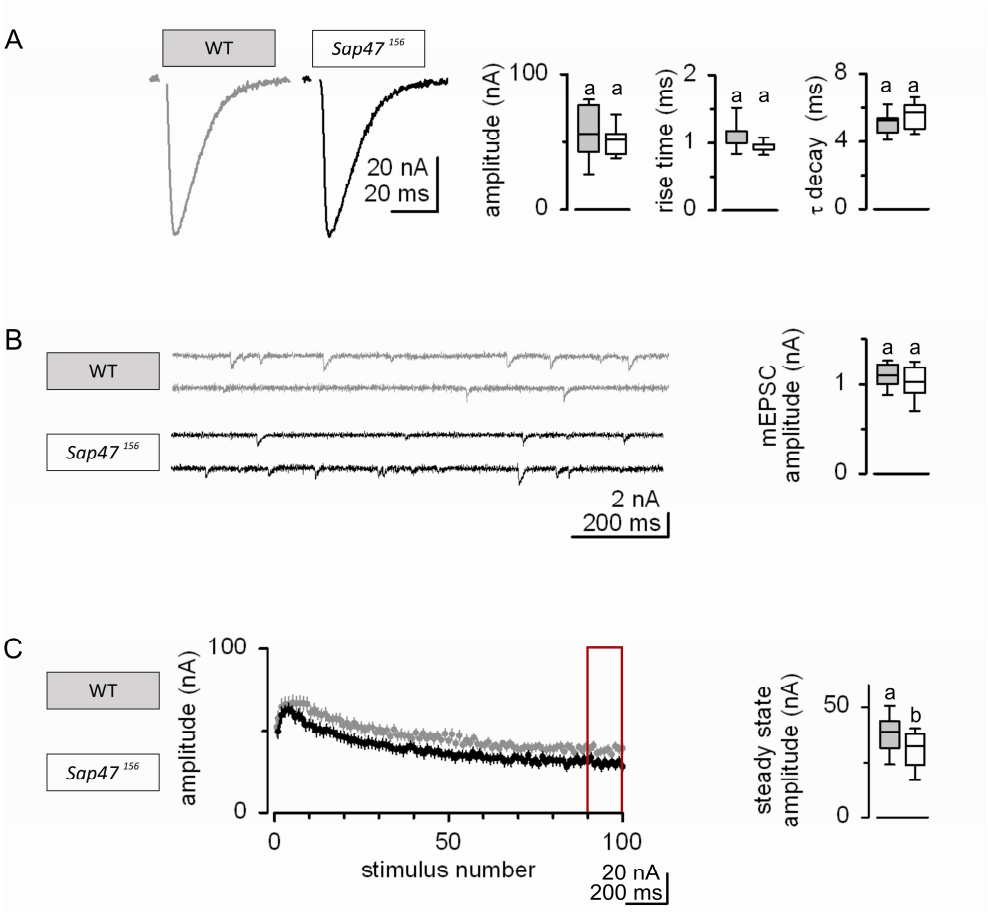


Figure 4:

Electrophysiology

A: Evoked excitatory postsynaptic currents
Representative traces of EPSCs evoked by 0.1 Hz nerve stimulation. The peak amplitude, 10 - 90 % risetime and decay time constants of EPSCs were not significantly altered in *Sap47*^{156} mutants (white, N= 8) compared to WT (grey, N= 9).

B: Spontaneous excitatory postsynaptic currents
Sample traces of spontaneous miniature EPSCs (mEPSCs). The amplitude of miniature EPSCs was not significantly different in *Sap47*^{156} mutants and WT.

C: Short-term depression quantified by steady-state amplitude
To the left, EPSC amplitudes (mean and SEM as error bars) evoked by 60 Hz stimulations (100 pulses) are displayed; the *Sap47*^{156} mutants showed stronger depression (white, N= 15) than WT (grey, N= 15). This is quantified for the boxed area, showing that the steady-state amplitude was significantly reduced in *Sap47*^{156} mutants compared to WT.
Shared lettering above plots signifies P> 0.05, different lettering P< 0.05 in Mann-Whitney U-tests.

RNAi-mediated knock-down of SAP47 phenocopies the defect in associative function

To independently test for the requirement of SAP47 for associative function, we knocked down SAP47 by RNA-interference (Smith et al., 2000; Kalidas and Smith, 2002). We used a UAS-RNAi-SAP47 fly strain as the effector strain and *elav*-Gal4 as the driver strain. As these transgenic Drosophila strains are in the *white* mutant background (w^{1118}; this is necessary to monitor for a possible loss of the transgenic constructs), we first tested for SAP47 expression in WT and w^{1118} on a Western blot as well as for associative function in these two strains. In both respects, the w^{1118} mutation was without phenotype (Fig. 5A, A´): SAP47 levels appeared normal (Fig. 5A) and both genotypes showed associative performance indices (Fig. 5A´; one-sample sign tests: $P< 0.05 / 2$; N= 16, 16) at equal levels (Fig. 5A´; P= 0.66, U= 109; sample sizes as above; see also Yarali et al., 2009b). Therefore, data obtained with transgenic strains in our paradigm can be interpreted without reference to *white* function.

After confirming the effectivity of the RNAi-mediated knock-down of SAP47 on a Western blot (Fig. 5B), we thus could move on to test for the effect of this knock-down on associative function. Given that the transgenic driver and effector control strains showed equal levels SAP47 as well as of associative function (Suppl. Fig. 8A; P= 0.79; U= 153; N= 19, 17), behavioral data were pooled for subsequent analyses. Compared to WT, performance indices in the knock-down group were reduced by ~50 % (Fig. 5B´; $P< 0.05 / 2$, U= 199; N= 32, 19) as was the case when compared to controls (Fig. 5B´; $P< 0.05 / 2$, U= 186; N= 19, 36; the Kruskal-Wallis Anova across all three groups yielded: $P< 0.05$; H= 8.58; df= 2; sample sizes as above).

We therefore conclude that an RNAi-mediated reduction of SAP47 causes an impairement in associative function similar to the one seen in the $Sap47^{156}$ null mutant.

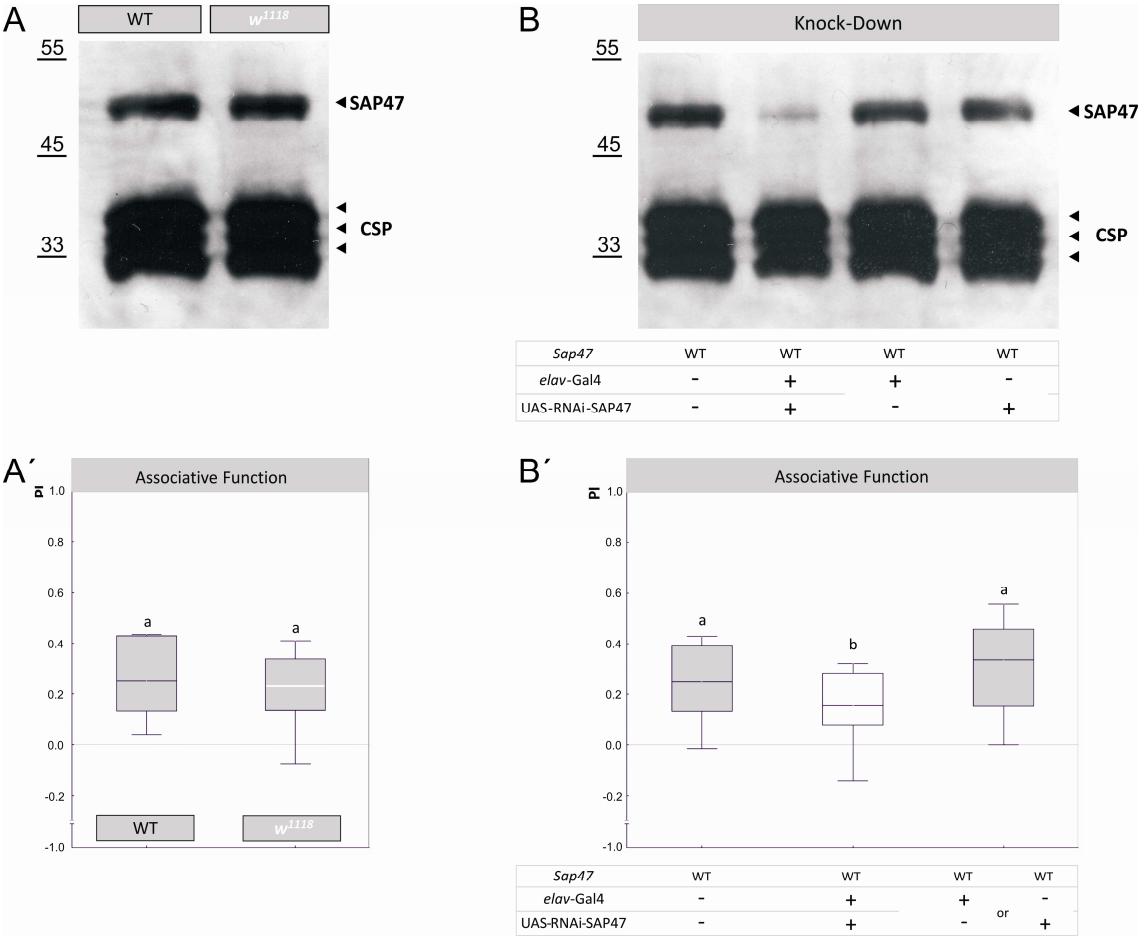


Figure 5:

SAP47 Knock-Down

A: Western Blot of WT and *white* mutants
Given that all strains are in the w^{1118} mutant background (see text), a test of SAP47 expression in w^{1118} is warranted, which shows no difference in SAP47 expression to WT. Antibodies used are the monoclonal nc46 for SAP47 detection and, as loading control, the monoclonal ab49, the latter labelling CSP, another presynaptic protein.

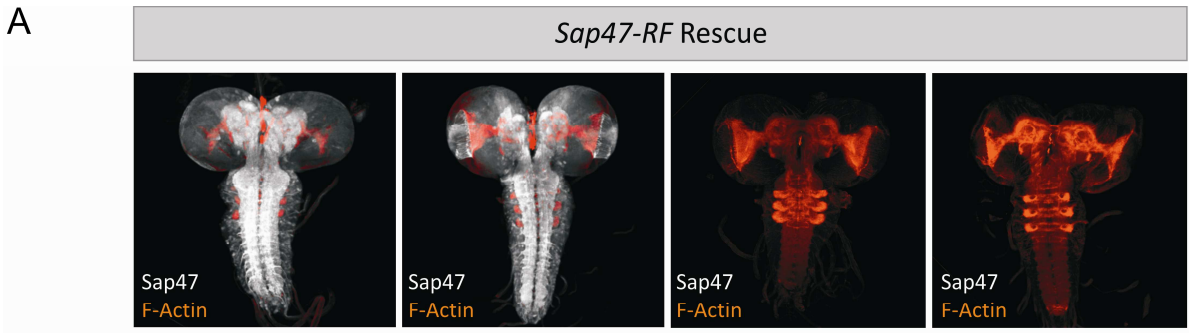
A´: *white* mutants are not impaired in associative function
WT and w^{1118} perform equally well in the associative learning paradigm. The PREF scores underlying PI values are documented in Suppl. Fig. 3. N= 16, 16. Shared lettering above plots signifies P > 0.05 in a Mann-Whitney U-test.

B: Western Blot of SAP47 Knock-Down
The *elav*-Gal4 driver-control and the UAS-RNAi-SAP47 effector-control strains show no difference to WT in terms of SAP47 expression level, but the knock-down larvae show an obvious reduction.

B´: SAP47 knock-down larvae are impaired in associative function
Associative function is reduced to ~50 % of control levels upon expression of an RNA-interference mRNA, using *elav*-Gal4 as driver- and UAS-RNAi-SAP47 as effector-strain. Both controls perform equally well (see Suppl. Fig. 8A) and are therefore pooled. Notably, this RNAi-induced reduction of associative function is as severe as the one seen upon a total lack of SAP47 in the $Sap47^{156}$ mutant (Fig. 2). The PREF scores underlying the PI values are documented in Suppl. Fig. 4. N= 32, 19, 36.
Different lettering above plots signifies P< 0.05/ 2 in Mann-Whitney-U-tests.

The associative defect in the Sap47^{156} *mutant is rescued by transgenic SAP47 expression*

For a rescue of the defect in associative function of the Sap47156 mutant, we used the driver strain *elav*-Gal4; *Sap47*^{156} crossed to UAS-*Sap47-RF*; *Sap47*^{156} as effector strain to transgenically restore expression of the 47 kDa PF isoform of the protein broadly throughout the larval brain (Fig. 6A, B). Because no significant difference in associative function was found between the driver and effector control (Suppl. Fig. 8B; P= 0.59; U= 2188; N= 67, 69) these were pooled for subsequent analysis. It turned out that rescue larvae were significantly better in associative function than the control larvae in the *Sap47*^{156} mutant background (Fig. 6C: P< 0.05 / 2, U= 3668; N= 69, 136), but rescue larvae did not quite reach WT levels in associative performance indices (Fig. 6C; P< 0.05 / 2; U= 1655; N= 68, 69) (the Kruskal-Wallis test across all three genotypes yielded: P< 0.05; H= 32.49; df= 2; sample sizes as above). This suggests that transgenic expression of the 47 kDa RF isoform of SAP47 partially rescues the *Sap47*^{156} mutant learning defect.



Sap47^{156}	WT	*Sap47*^{156}	*Sap47*^{156}	*Sap47*^{156}
elav-Gal4	-	+	+	-
UAS-*Sap47-RF*	-	+	-	+

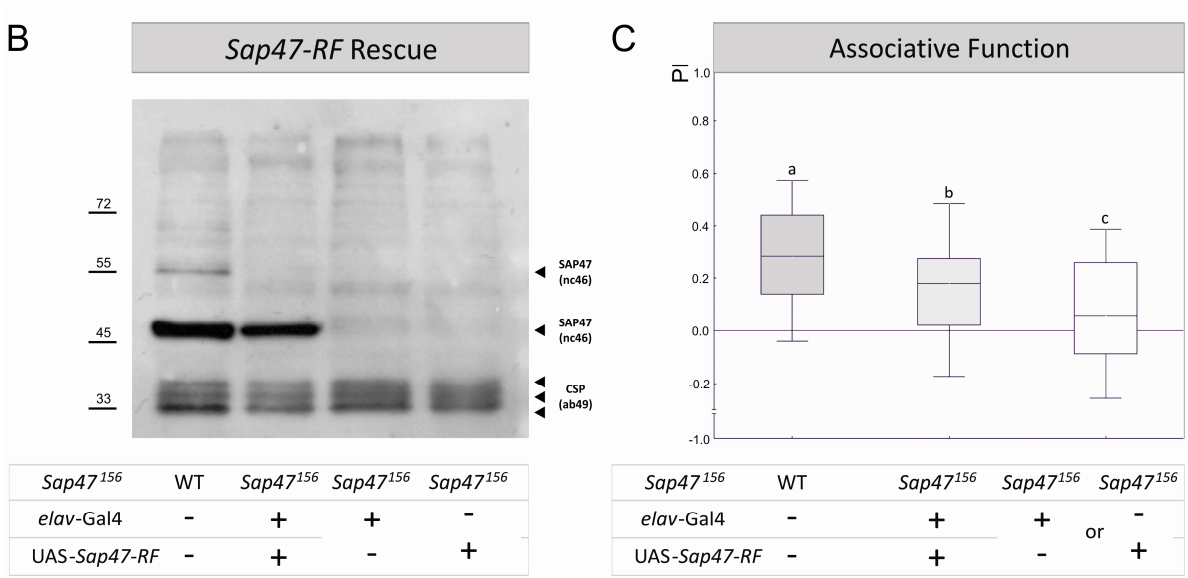


Figure 6:

SAP47 Rescue with 47 kDa isoform

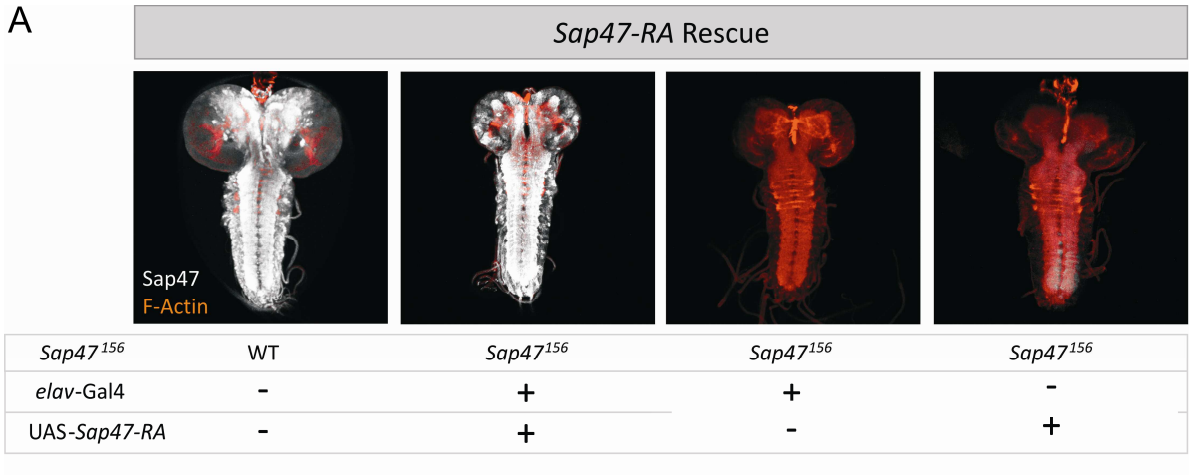
A: Whole mounts
In contrast to WT and recue larvae, the *elav*-Gal4; *Sap47*^{156} driver-control and the UAS-*Sap47-RF*; *Sap47*^{156} effector-control strains show no SAP47 expression. Phalloidin is used to visualize F-actin (orange).

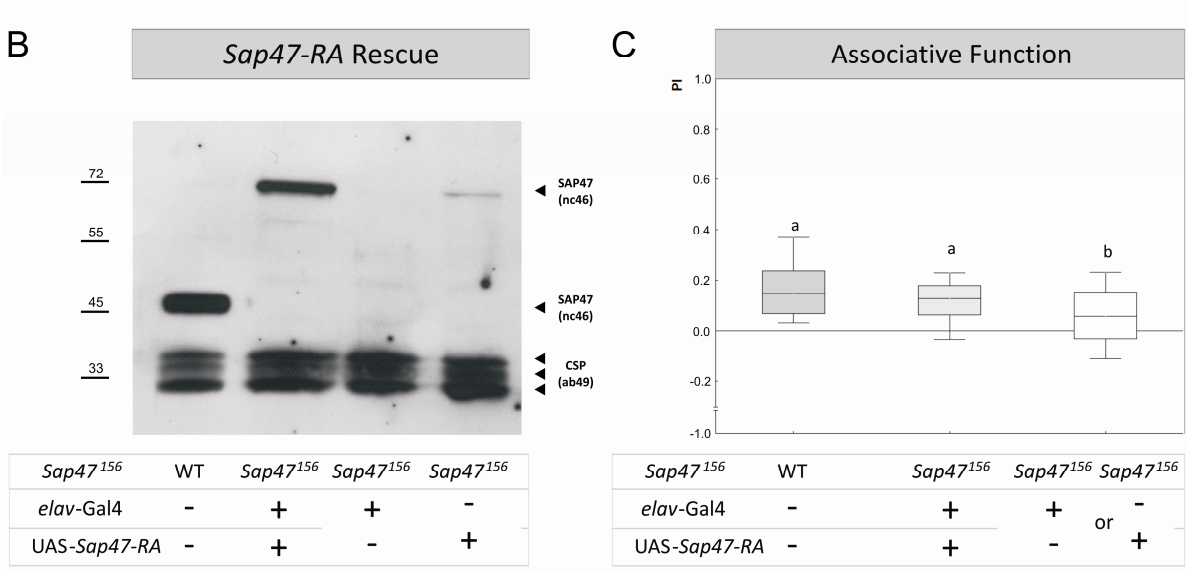
B: Western Blot
In contrast to WT, the *elav*-Gal4; *Sap47*^{156} driver-control and the UAS-*Sap47-RF*; *Sap47*^{156} effector-control strains show no SAP47 expression; the rescue larvae show an obvious SAP47 band of 47 kDa, as is to be expected from the coding region used for the UAS-*Sap47-RF* transgene; a higher isoform is only detectable in WT. Anti-SAP47: nc46; anti-CSP: ab49 (loading control).

C: Expression of the PF isoform of SAP47 partially rescues impairment in associative function
Associative function is reduced to ~50 % of control level (WT) in genetic controls, using *elav*-Gal4; *Sap47*^{156} as driver- and UAS-*Sap47-RF*; *Sap47*^{156} as effector-strain. Both controls perform equally well and are therefore pooled as genetic controls (Suppl. Fig. 8B). The experimental group shows higher associative performance compared to genetic controls, but does not reach wild-type level. N= 68, 69, 136. Different lettering above plots signifies P < 0.05/ 2 in Mann-Whitney U-tests. The PREF scores underlying all PI values are documented in Suppl. Fig. 5.

To see whether rescue expression of the full length cDNA of SAP47 would yield a full rescue of associative function, we crossed *elav*-Gal4; *Sap47*^{156} as driver strain to the UAS-*Sap47- RA*; *Sap47*^{156} effector strain. Expression of the full-length PA isoform of SAP47 was restored throughout the larval brain (Fig. 7A, B). Again, no significant difference in associative function was found between driver and effector control (Suppl. Fig. 8C; P= 0.27; U= 686; N= 40, 40), so these were pooled for subsequent analysis. Larvae expressing SAP47-PA performed significantly better than control larvae in the *Sap47*^{156} mutant background (Fig. 7C: P< 0.05 / 2, U= 1155; N= 40, 80). Indeed, these rescue larvae reached WT levels of associative function (Fig. 7C P= 0.18; U= 661; N= 40, 40) (the Kruskal-Wallis test across all three genotypes yielded: P< 0.05; H= 16.5; df= 2; sample sizes as above). Please note a

tendency for over-all low associative performance indices in this experiment; this is within the normal range of variation of behavioral experiments and underlines the necessity to train and test all genotypes to be compared statistically in parallel, as was done throughout this study. We further note a weak leaky expression in the effector control detectable on the western blot (Fig. 7B). This expression is at the caudal tip of the ventral nerve cord (right most panel in Fig. 7A), a region not previously implicated in learning and memory, and indeed is inconsequential for associaitive function (see right most plot in Fig. 7C, and the trend for lower associative performance indices in the effector than in the driver control: Suppl. Fig. 8C). In any event, given that the full length PA-isoform cannot be detected in Western blots of WT larvae, potentially because of local restriction of expression, we wondered whether longer isoforms of SAP47 are expressed in WT lavae at all. This is indeed the case, as suggested by PCR (Fig. 7D, E).





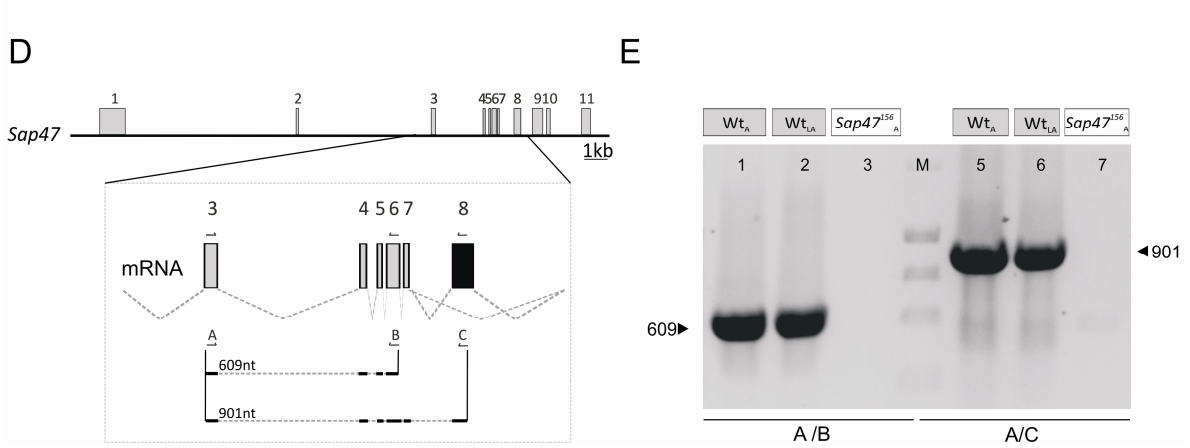


Figure 7:

SAP47 full length Rescue

A: Whole mounts
In contrast to WT and rescue larvae, the *elav*-Gal4; *Sap47*^{156} driver-control shows no SAP47 expression whereas there is a weak leaky expression detectable at the caudal tip of the ventral nerve cord of the UAS-*Sap47-RA*; *Sap47*^{156} effector-control strain. Phalloidin is used to visualize F-actin (orange).

B: Western Blot
The WT shows the prominent band at 47 kDa, whereas the rescue strain shows the band of the full length PA isoform of SAP47 of about 70 kDa, as to be expected from the coding region used for the UAS-*Sap47-RA* element. No SAP47 signal is detectable in the driver-control, but a weak leaky expression is seen in the UAS-*Sap47-RA*; *Sap47*^{156} effector-control strain, corresponding to the expression seen in the whole mount at the caudal tip of the ventral nerve cord (A). Anti-SAP47: nc46; anti-CSP: ab49 (loading control).

C: Expression of the full length isoform of SAP47 fully rescues associative function
Driver and effector-control are impaired in associative function to ~50 % of control level (WT), using *elav*-Gal4; *Sap47*^{156} as driver- and UAS-*Sap47-RA*; *Sap47*^{156} as effector-control. Both controls perform equally well and are therefore pooled as genetic controls (Suppl. Fig. 8C). Larvae expressing the full length cDNA of SAP47 perform as good as WT. N= 40, 40, 80. Different lettering above plots signifies $P < 0.05 / 2$ in Mann-Whitney U-tests. The PREF scores underlying all PI values are documented in Suppl. Fig. 6.

D: Gene structure of SAP47 in WT larvae
Shown is the gene structure of Sap47 with exons and introns in wild-type (exons grey, see also Fig. 1A). Eight transcripts are annotated, five longer and 3 shorter transcripts (Flybase: http://flybase.bio.indiana.edu). All of the longer transcripts contain exon 8, whereas this exon is spliced out in all of the short transcripts, indicated by black colour in the magnification of the mRNA from exon 3 to exon 8. This situation allows differentiating the long from the short transcripts. The arrows and letters indicate binding sites of primers used for PCR in (E) and size of the expected PCR products of 609 nt using primer pair A and B and 901 nt using primer pair A and C.

E: PCR
After isolation of total RNA from 100 WT adult flies (WT_A), 200 WT third instar larvae (WT_{LA}), and 100 adult *SAP47*^{156} flies (*SAP47*$^{156}{}_A$) and producing Sap47 cDNAs, the primer pair A / B generates a 609 nt fragment only in WT. Also using primer pair A / C, a 901 nt fragment is generated in only WT, and notably in both larvae and adults, indicating that long isoforms of SAP47 are expressed. Note that, on the protein level, these longer isoforms are often hard to detect (see Western blot in B).

We therefore conclude SAP47 functions in associative behavioral plasticity.

Discussion

We report that the SAP47 protein is widely expressed in the neuropil regions of the larval brain (Fig. 1E). The protein is associated with synaptic vesicles (Fig. 1C), but is not an integral part of the synaptic vesicle membrane (Mastrogiacomo et al., 1994; Umbach et al., 1994; Arnold et al., 2004, loc. cit. Fig. 3). The *Sap47*^{156} mutation leads to a total absence of the SAP47 protein in larvae (Fig. 1 D, E, G) (for adult Drosophila: Funk et al., 2004). Lack of SAP47 entails a ~50 % reduction in odorant-reward associative ability as compared to WT (Fig. 2A); both strains had been adjusted for genetic background by extensive outcrossing. Importantly, this associative defect appears to be specific on the behavioral level because sensory and motor capacity regarding the to-be-associated stimuli is intact, be it in terms of naïve responsiveness, or in terms of the ability to behave towards the learned odorant at the moment of test (Fig. 3). To independently verify this impairment, we tested *Sap47*^{156} larvae also in a two-odor version of the conditioning paradigm (AM *versus* OCT) and found a similar reduction in associative function (Fig. 2B). Also, another deletion allele (*Sap47*^{201}) shows the same phenotype in associative function as *Sap47*^{156} (Fig 2C). When SAP47 was transgenically knocked-down by RNAi (Fig. 5B, B´), we found a similar ~50 % reduction of associative function as by the *Sap47*^{156} or the *Sap47*^{201} deletion (Fig. 2). Together, and considering the outcrossing regimen for the *Sap47*^{156} mutant, it seems reasonable to attribute the learning defect upon deletions in the *Sap47* gene to a lack of the SAP47 protein, rather than to spurious differences in genetic background (see also below). We note that the associative defect in all cases (*Sap47*^{156}, *Sap47*^{201}, in RNAi knockdown larvae, as well as in the genetic controls in both rescue experiments) is partial, arguing that there are SAP47-independent mechanisms to support associative function in our paradigm. Alternatively, there could be hitherto unknown *Sap47*–like genes in the fly genome; however, we do not see any SAP47 protein in the mutants, with neither antibody used (Fig. 1D, E, G; 6A, B; SAP47 expression in the effector control of Fig. 7A, B is due to weak and mnemonically inconsequential leaky expression from the used effector construct), and the *Drosophila* genome does not contain any obvious SAP47 sequence homolog. In this regard, SAP47 is similar to synapsin, the lack of which also entails a reduction of associative performance indices in our paradigm by ~50 % (Michels et al., 2005). Both proteins are also similar regarding their association with synaptic vesicles, but not being integral part of the vesicular protein complement (Hilfiker et al., 1999; Arnold et al., 2004). Whether and how these proteins interact and whether their learning phenotypes are additive remains to be investigated. Maybe most importantly, transgenic expression of the full length SAP47 protein

from the *elav*-Gal4 driver fully rescues associative function in the $Sap47^{156}$ mutant (Fig. 7), providing compelling evidence for a function of SAP47 in behavioral associative plasticity. With respect to the physiological mechanism of SAP47 function, we analyzed basic transmission as well as short-term plasticity at the larval neuromuscular junction, the only cellular site in *Drosophila* that at present is routinely amenable to such analyses. The pronounced synaptic depression during sustained bursts of neuronal activity is consistent with a hypothesis that SAP47 contributes to the recruitment of vesicles to the release site (Hallermann et al., 2010). Although the plasticity processes that underlie odorant-taste learning and memory likely happen within the central brain (Gerber and Stocker, 2007), previous extrapolations between behavioral and synaptic plasticity at the neuromuscular synapses have been surprisingly successful (e.g. regarding the cAMP-PKA cascade: Kidokoro et al., 2004; Ueda and Wu, 2009). Thus, the distortion of short-term plasticity observed here may well be the cause for impaired associative function on the behavioral level (Abbott and Regehr, 2004; Rothman et al., 2009).

To summarize, this study is the first to identify a behavioral and physiological function of the phylogenetically conserved SAP47 protein: Our results indicate that SAP47 is required for normal short-term synaptic plasticity at the neuromuscular junction as well as for normal levels of associative behavioral plasticity. Given that molecular determinants of behavioral and synaptic plasticity in invertebrates have repeatedly turned out to be shared with mammals (Pittenger and Kandel, 2003, Davis, 2005; Keene and Waddell, 2007), this may be an inspiring finding.

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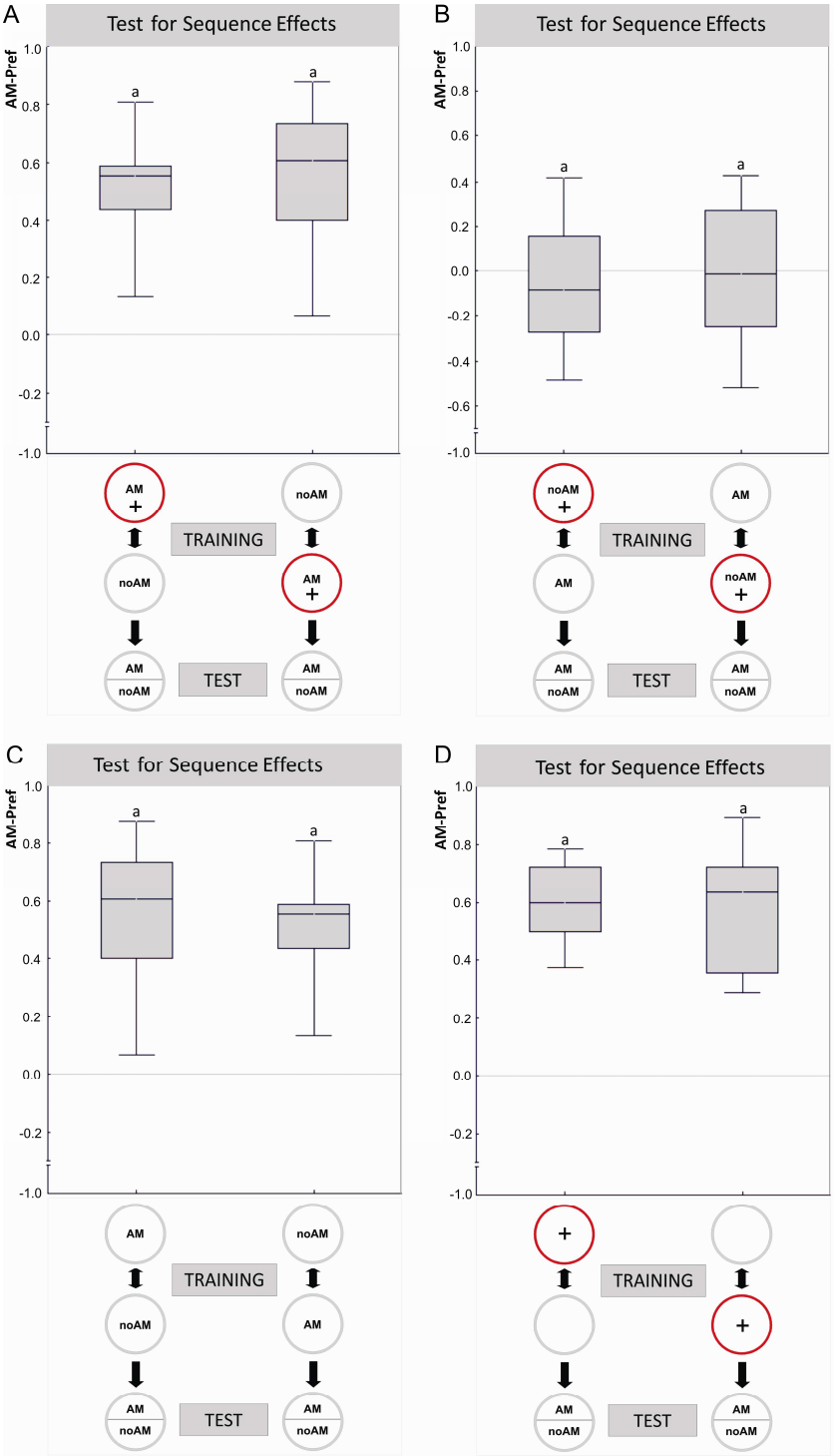
Supplementary Material



Supplementary Figure 1:

Testing for sequence effects

A: Testing for sequence effects after training: AM rewarded
Analysis of odor preferences from the learning experiment displayed in Figure 2A. For those groups sharing the same odorant-reward contingency, the sequence of training trials is without effect on odor preference at test. The same pattern of results is found in *Sap47*^{156} (not shown). N= 17, 18.

B: Testing for sequence effects after training: AM not rewarded
Analysis of the odor preferences from the learning experiment displayed in Figure 2A. For those groups sharing the same odorant-reward contingency, the sequence of training trials is without effect on odor preference at test. The same pattern of results is found in *Sap47*^{156} (not shown). N=

17, 18.
Insets illustrate the experimental regimen. Shared lettering above plots signifies P > 0.05 in Mann-Whitney U-tests.

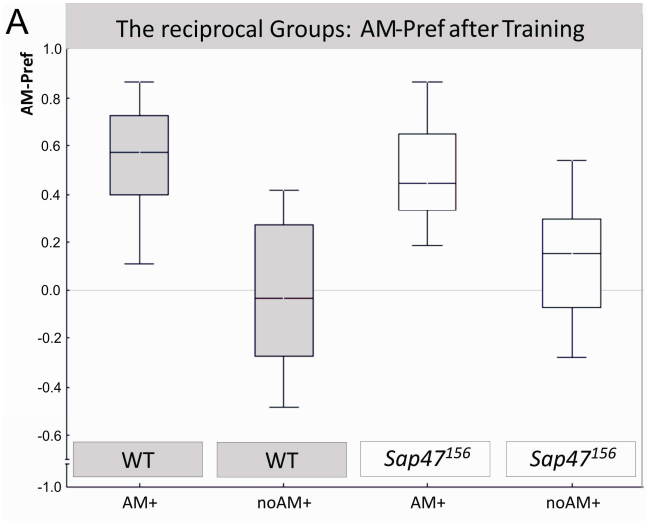
C: Testing for effects of the sequence of odor exposure on odor preference

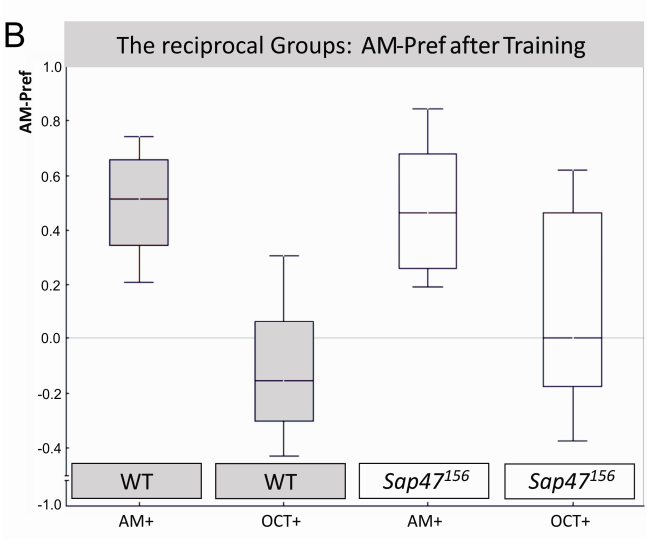
AM preferences after odor exposure (Fig. 3C) are shown separately for those groups differing in the temporal pattern of odor exposure. There is no significant difference between the AM-preferences of these groups and therefore there is no effect of the sequence of odor exposure on test performance. N= 20, 22. The same pattern of results is found in *Sap47*^{156} (not shown).

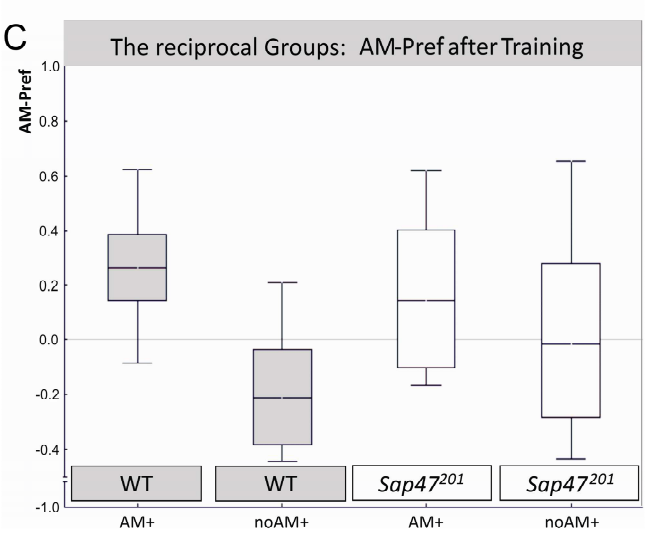
D: Testing for effects of the sequence of reward exposure on odor preference

AM preferences after reward exposure (Fig. 3D) are shown separately for those groups differing in the temporal pattern of reward exposure. There is no significant difference between the AM-preferences of these groups and therefore no effect of the sequence of reward exposure on test performance. The same pattern of results is found in *Sap47*^{156} (not shown). N= 23, 25.

Insets in the figure illustrate the experimental regimen. Shared lettering above plots signifies P > 0.05 in Mann-Whitney U-tests.







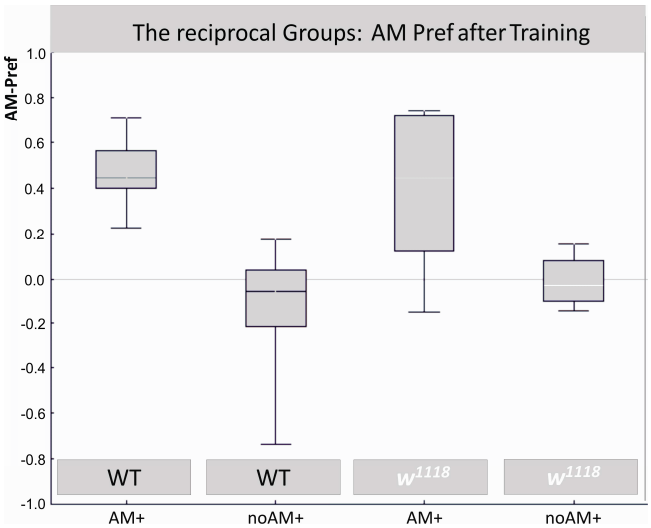
Supplementary Figure 2:

AM-preferences after training

A: WT *versus* *Sap47*^{156}: one-odor paradigm
For documentation, the odor preferences from the learning experiment featuring WT and *Sap47*^{156} (Fig. 2A) are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (noAM+). N= 35 in all cases.

B: WT *versus* *Sap47*^{156}: two-odor paradigm
For documentation, the odor preferences from the learning experiment in Fig. 2B are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (OCT+). N= 35 in all cases.

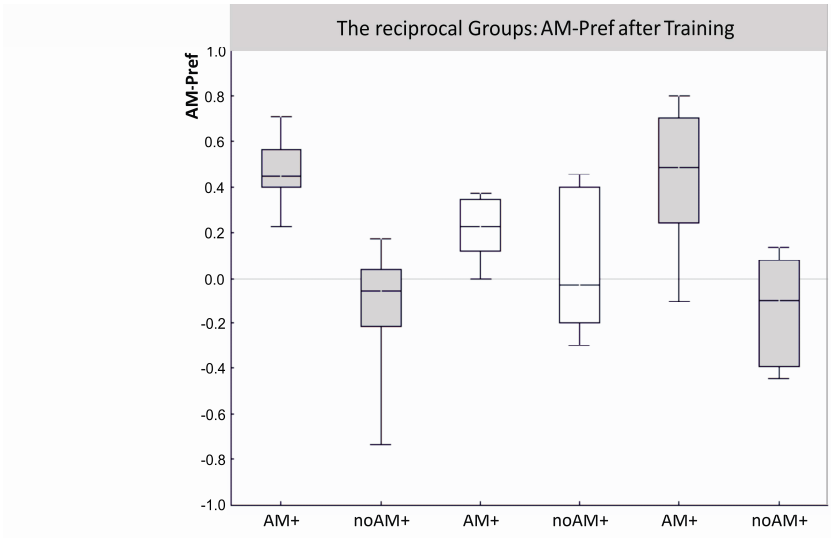
C: WT *versus* *Sap47*^{201}: one-odor paradigm
For documentation, the odor preferences from the learning experiment in Fig. 2C are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (noAM+). N= 16, 16, 14, 14.
Note that the *Sap47*^{201} mutant is in the *w*^{1118} background; *white*-status does not affect behavior in the present paradigm (Fig. 5A´).



Supplementary Figure 3:

AM-preferences after training

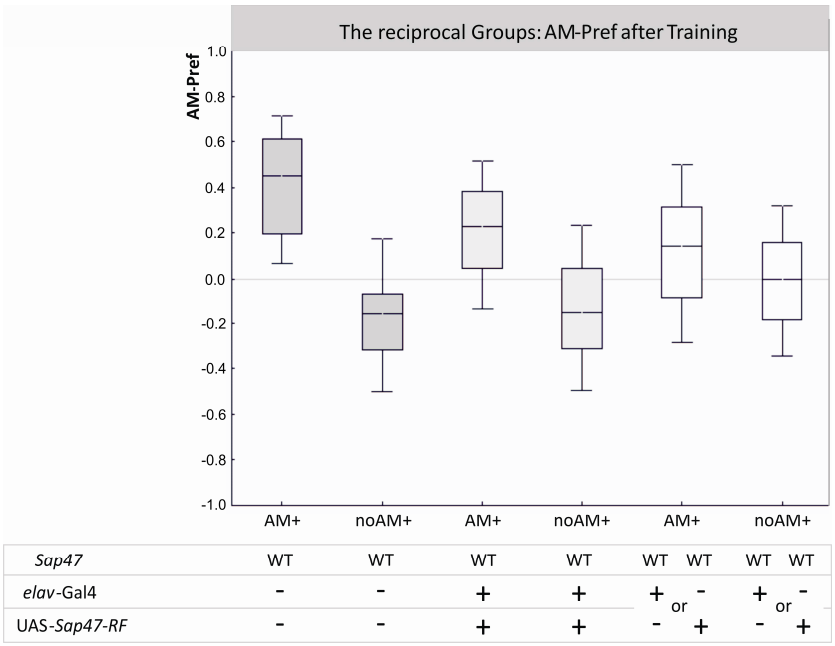
For documentation, the odor preferences from the learning experiment featuring WT and w^{1118} (Fig. 5A´) are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (noAM+). N= 16 in all cases.



Supplementary Figure 4:

AM-preferences after training

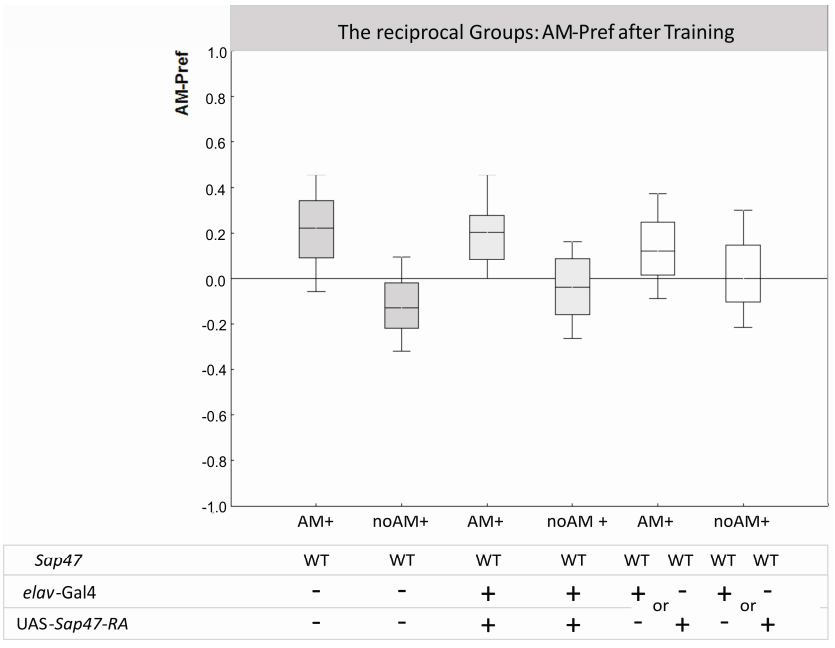
For documentation, the odor preferences from the knock-down experiment (Fig. 5B') are shown, for the cases when AM was rewarded during training (AM+) versus the reciprocally trained groups (noAM+). N= 32, 32, 19, 19, 36, 36.



Supplementary Figure 5:

AM-preferences after training

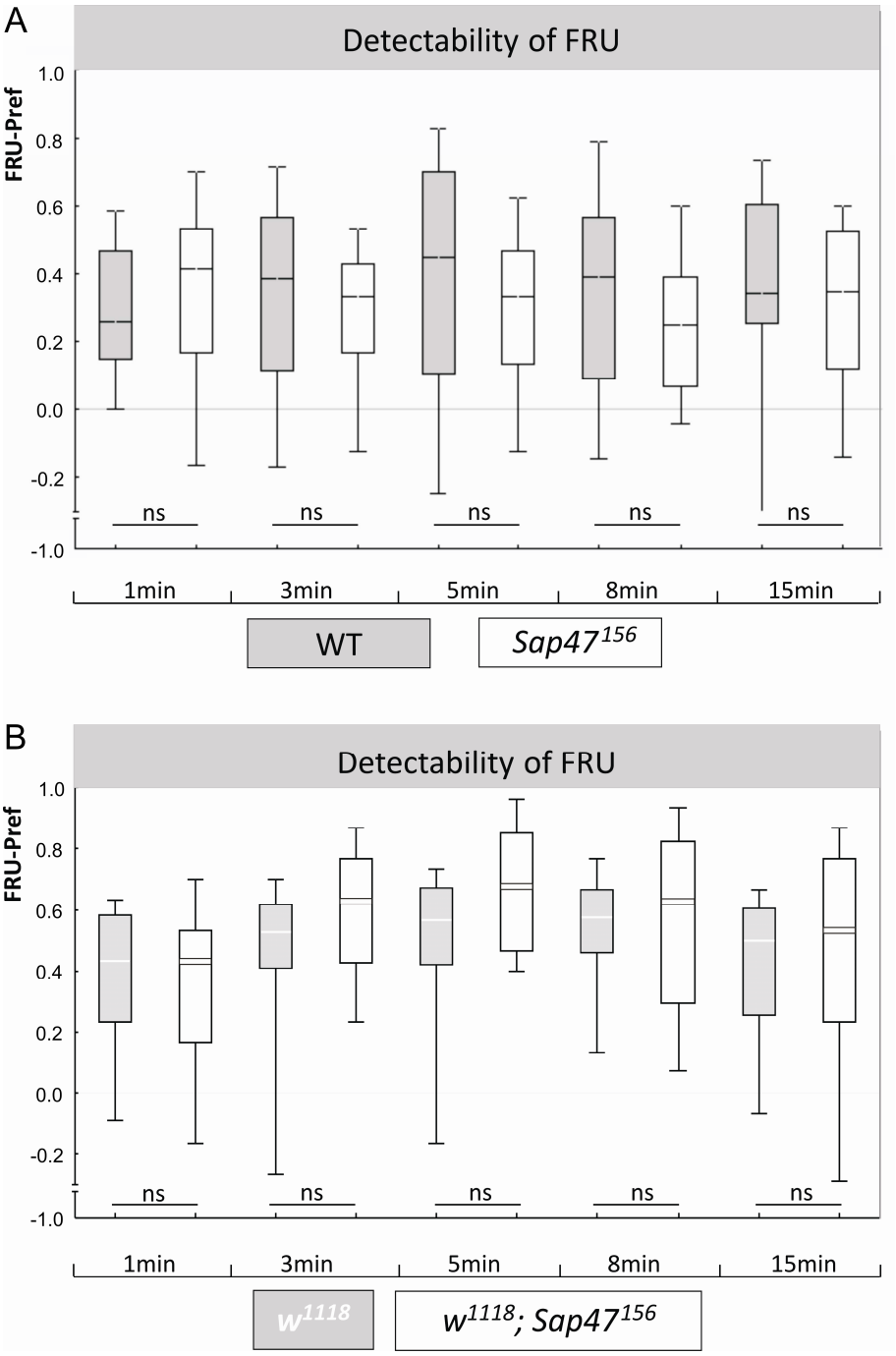
For documentation, the odor preferences from the rescue learning experiment using the 47 kDa PF isoform of SAP47 (Fig. 6C) are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (noAM+). N= 68, 68, 69, 69, 136, 136.



Supplementary Figure 6:

AM-preferences after training

For documentation, the odor preferences from the rescue learning experiment using the full length PA isoform of SAP47 (Fig. 7C) are shown, for the cases when AM was rewarded during training (AM+) *versus* the reciprocally trained groups (noAM+). N= 40, 40, 40, 40, 80, 80.



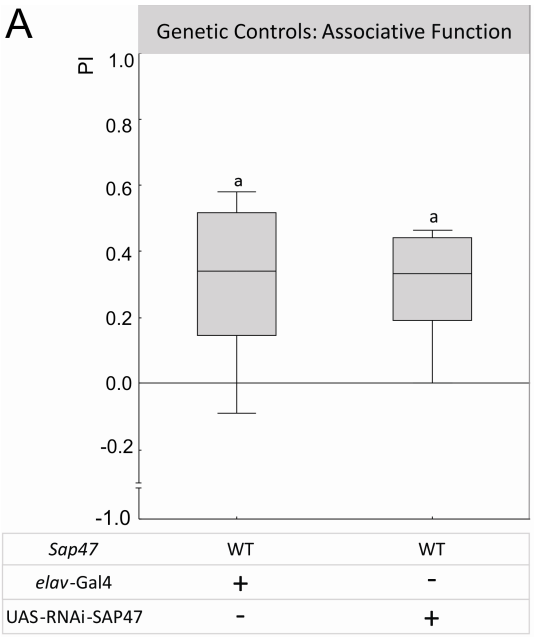
Supplementary Figure 7:

Detectability of reward

A: Comparing WT *versus* *Sap47*^{156} mutant
Fructose preferences are not significantly different between WT and *Sap47*^{156} mutants when allowing 1, 3, 5, 8 or 15 minutes for choice. ns: P> 0.05 / 5 (Mann-Whitney-U-tests). N= 40, 39.

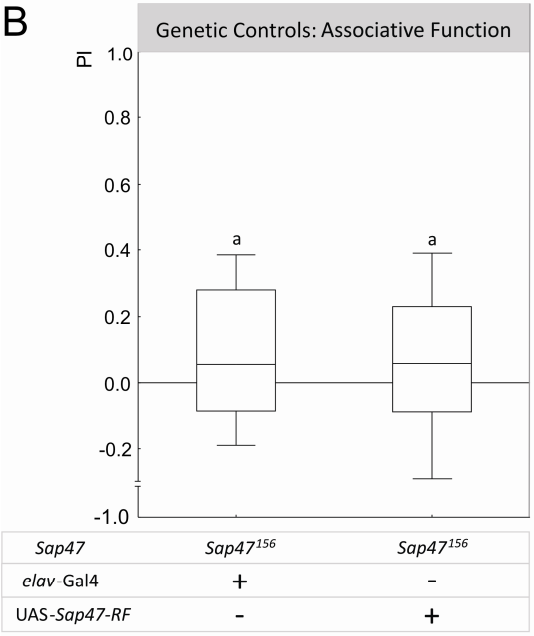
B: Comparing *w*^{1118} versus *w*^{1118}*; Sap47*^{156} double-mutants
Fructose preferences between *w1118 versus w1118; Sap47156* double-mutants are not significantly different at any tested time point (1, 3, 5, 8 or 15 minutes). ns: P> 0.05 / 5 (Mann-Whitney-U-tests). N= 32, 29.

A



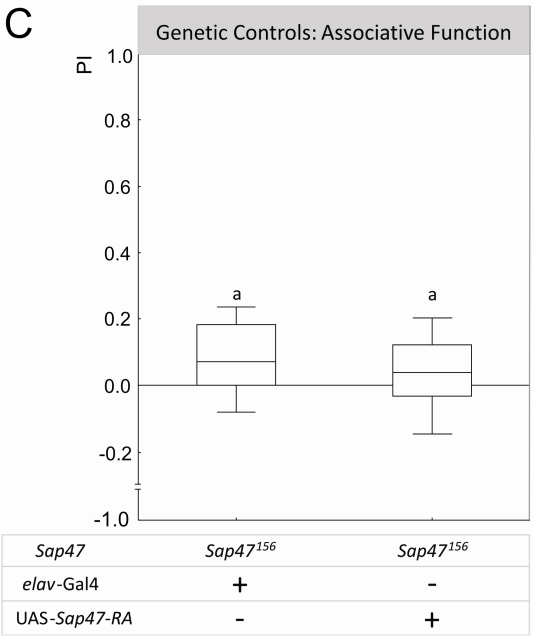
Sap47	WT	WT
elav-Gal4	+	-
UAS-RNAi-SAP47	-	+

B



Sap47	$Sap47^{156}$	$Sap47^{156}$
elav-Gal4	+	-
UAS-*Sap47-RF*	-	+

C



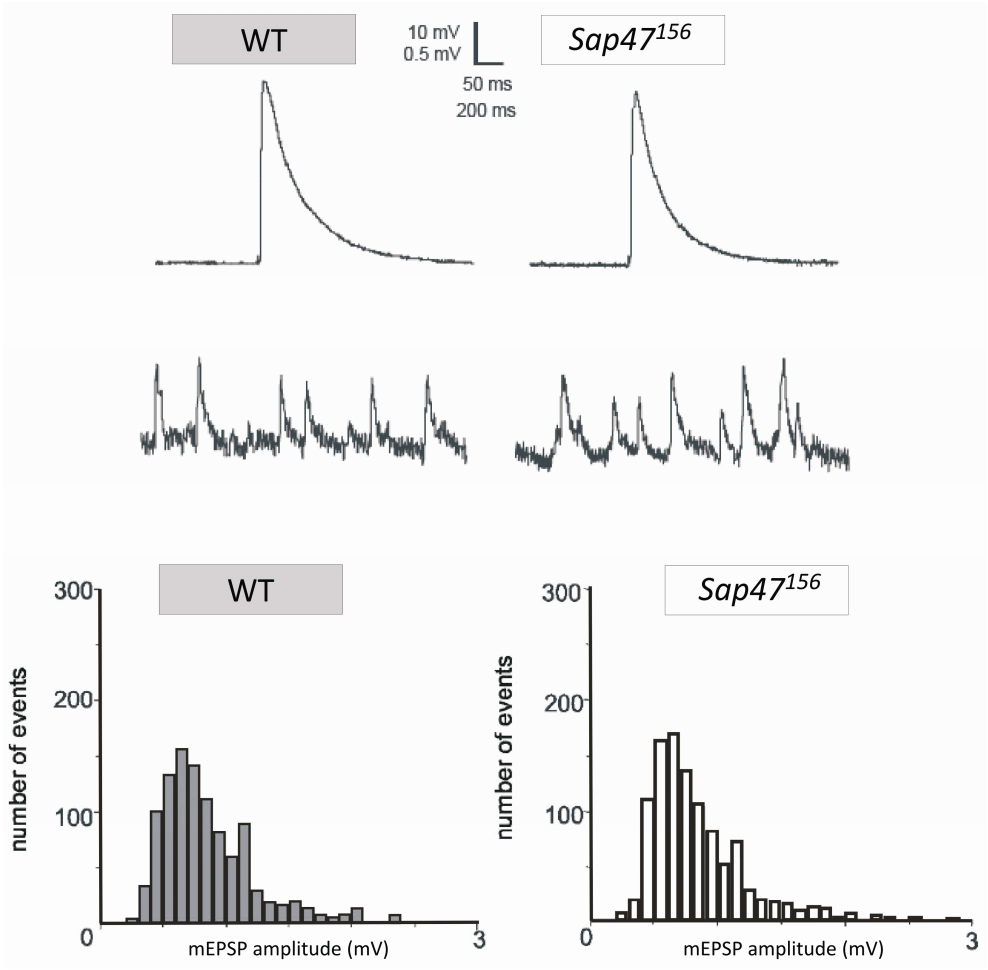
Sap47	$Sap47^{156}$	$Sap47^{156}$
elav-Gal4	+	-
UAS-*Sap47-RA*	-	+

Suppl. Fig. 8:

A: Associative function of controls in the knock-down experiment
No significant difference in associative function is found between driver- and effector-control (*elav*-Gal4 and UAS-RNAi-SAP47). Data for these strains are therefore pooled as genetic controls. Shared lettering above plots signifies P > 0.05 in a Mann-Whitney U-test. N= 19, 17.

B: Associative function of controls in the rescue experiment using the 47 kDa RF isoform of SAP47
No significant difference in associative function is found between driver- and effector-control (*elav*-Gal4; $Sap47^{156}$ and UAS-*Sap47-RF;* $Sap47^{156}$). Data of these strains are therefore pooled as genetic controls. Shared lettering above plots signifies P > 0.05 in a Mann-Whitney U-test. N= 67, 69.

C: Associative function of controls in the rescue experiment using the full length RA isoform of SAP47
No significant difference in associative function is found between driver- and effector-control (*elav*-Gal4; $Sap47^{156}$ and UAS-*Sap47-RA;* $Sap47^{156}$). Data of these strains are therefore pooled as genetic controls. Shared lettering above plots signifies P > 0.05 in a Mann-Whitney U-test. N= 40, 40.



Supplementary Figure 9:

Typical eEPSPs (upper traces), sample mEPSPs (middle traces) and distribution of mEPSP amplitudes

Current-clamp recordings at muscle 6/ 7 upon low-frequency nerve stimulation.
mEPSP amplitude-frequency histograms of all recorded events
(WT: 1027 events from 10 animals and *Sap47*^{156}: 1042 events from 10 animals) reveal no difference in the amplitude distribution of mEPSPs between WT (grey) and *Sap47*^{156} (*white*).

Supplementary Figure 10:

Genomic and protein level information

A: Genomic *Sap47* sequence
Shown is the first part of the *Sap47* genomic sequence of WT and *Sap47*^{156} mutant. The deletion (orange) in *Sap47*^{156} spans 1727 base pairs and affects the promoter region, the first exon (dark blue) and part of the first intron. Primer sequences used for PCR (Fig. 1B) in red. We respresent the epitope-coding region for the antibody nc46 in light blue.

B: Predicted amino acid sequence of the SAP47-PF protein
Shown is the predicted amino acid sequence of the SAP47-PF protein isoform coded by *Sap47-RF* in WT; the epitopes for the anti-Sap47 antibodies nc46 and nb200 are colour coded in blue.

C: Predicted amino acid sequence of the SAP47-PA protein
Shown is the predicted amino acid sequence of the SAP47 protein isoform coded by *Sap47-RA* in WT; the epitopes for the anti-Sap47 antibodies nc46 and nb200 are colour coded in blue.

II Associative learning in *Drosophila*

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Genetic Distortion of the Balance between Punishment and Relief Learning in *Drosophila*

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II Genetic distortion of the balance between punishment and relief learning in **Drosophila**

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Running title
Genetic distortion of punishment-relief balance

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Abstract

An experience with electric shock can support two opposing kinds of behavioral effects: Stimuli that precede shock during training are subsequently avoided as predictors for *punishment*, whereas stimuli that follow shock during training are later on approached, as they predict *relief*. We show here for the fruit fly *Drosophila* that upon loss of *white* function the balance between these two kinds of learning is distorted in favour of punishment learning: *white*^{1118} mutants show stronger punishment learning and weaker relief learning as compared to wild-type flies. Thus, *white*^{1118} mutants establish overall more 'negative' memories for the shock experience. This only concerns the mnemonic effects of the shock; the immediate, reflexive responsiveness to shock remains unaltered. Also, learning about reward is unaffected, both in adult and larval *Drosophila*. Prompted by the proposed function of the White protein as transporter for biogenic amine precursors, we probe the brains of *white*^{1118} mutants for the amounts of biogenic amines (octopamine, tyramine, dopamine and serotonin) using high performance liquid chromatography coupled to mass spectrometry. Using this method, we find, however, no difference between *white*^{1118} mutants and wild-type for any of the probed amines. In any event, analyses of how the *white*^{1118} mutation affects the balance between punishment and relief learning should provide a study case of how heritable distortions of such balance can come about. Finally, the effects of the *white*^{1118} mutation should be considered as a source of confound when using *white* as 'marker gene'in behavior-genetic analyses of any sort.

Introduction

The first mutant animal ever described as such was a white-eyed *Drosophila* fruit fly (Morgan et al., 1915) which consequently was called *white*. Subsequent analyses revealed that the gene is located on the first chromosome and codes for a 'half-size ATP-binding cassette transporter' (O'Hare et al., 1984). Heterodimers of the White protein with two other such transporters, Scarlet (Tearle et al., 1989) and Brown (Dreesen et al., 1988), respectively, pump tryptophan and guanine into cells. In *Drosophila* retinal pigment cells, these are precursors for the pigments (Sullivian and Sullivian, 1975), whose lack makes the eyes appear unpigmented (i.e. white).

Given its historical primacy and conspicuous phenotype, the *white* gene has become one of the most widely used tools in *Drosophila* genetics. In particular $white^{1118}$, which is a null allele of the *white* gene resulting from spontaneous deletion of a part of *white* (Hazelrigg et al., 1984), is employed as a 'marker' to keep track of transgenic constructs (see Discussion). Given the extensive use of such transgenes in *Drosophila* research, the effects of alterations in *white* function on behavior may be critical. These effects are manifold: Ectopic, ubiquitous over-expression of White induces male-to-male courtship (Zhang and Odenwald, 1995; Hing and Carlson, 1996; Nilsson et al., 2000; An et al., 2000), and loss of *white* function (in the $white^{1118}$ mutant) suppresses male-male aggression (Hoyer et al., 2008). Furthermore, $white^{1118}$ mutant flies are impaired in heat-reinforced place-learning, whereas in associative odor-shock learning, they perform better than wild-type (Diegelmann et al., 2006a). How can the *white* gene affect such a broad spectrum of behavioral phenotypes? We note that in neurons, tryptophan, one cargo of the White transporter, is converted to serotonin, a notorious modulator of behavior (e.g. circadian rhythmicity, sleep [Yuan et al., 2005; Yuan et al., 2006], aggression [Dierick et al., 2007], learning [Sitaraman et al., 2008]). Also, White's other cargo, guanine, is converted to '6H-tetrahydrobiopterin', a cofactor for the synthesis of serotonin, dopamine, and nitric oxide (reviewed by Koshimura et al. [2000]). Dopamine, apart from signalling aversive reinforcement (Schwaerzel et al., 2003; Riemensperger et al., 2005; Schroll et al., 2006), affects arousal (Andretic et al., 2005) and 'decision making' (Zhang et al., 2007). Last but not least, nitric oxide is an atypical neurotransmitter in the synapses of the olfactory, visual and mechanosensory system, as well as at the neuromuscular junction (reviewed by Bicker [2001]). Thus, roles of White in behavior may conceivably come about by its effects on serotonin, dopamine, and/ or nitric oxide signalling.

Here, following up on Diegelmann et al. (2006a), we analyse how loss of *white* function in the *white*^{1118} mutant affects olfactory associative learning. We do so with respect to two opposing kinds of memory which are established upon painful experience: In wild-type flies, those odors that precede an electric shock are learned as predictors for *punishment* and are subsequently avoided (punishment learning), whereas those odors that follow shock are learned as signals for *relief* and are subsequently approached (relief learning) (Tanimoto et al., 2004; Yarali et al., In press). In addition, we test whether *white*^{1118} mutants are altered in associating an odor with a sugar reward. In order to offer an explanation for behavioral alterations, we provide an analysis of the brain-levels of biogenic amines (octopamine, tyramine, dopamine, serotonin) using high pressure liquid chromatography coupled to mass spectrometry.

Materials and Methods

Flies

Drosophila melanogaster are reared as mass culture at 25 °C, 60- 70 % relative humidity, under a 14: 10 h light: dark cycle. The Canton-Special wild-type strain is used as a control for the White-null *white*^{1118} strain, which has been back-crossed to this wild-type strain for more than six generations to adjust genetic background (Hazelrigg et al., 1984; also see Diegelmann et al., 2006a; Hoyer et al., 2008).

Adult behavior

One day prior to experiments, 1- 4 day-old flies are collected in fresh food vials and kept over-night at 18 °C and 60- 70 % relative humidity. For sugar reward learning, flies are starved over-night for 18- 20 h at 25 °C and 60- 70 % relative humidity in vials equipped with moist tissue and a moist filter paper. The experimental setup is as described by Schwaerzel et al. (2003). Flies are trained and tested in groups of 100- 150. Training takes place under dim red light which does not allow flies to see; tests are in complete darkness. As odorants, 90 µl benzaldehyde (BA) and 340 µl 3-octanol (OCT) (both from Fluka, Steinheim, Germany) are applied in 1 cm-deep Teflon containers of 5 and 14 mm diameters, respectively.

For electric shock-reinforced learning (Fig. 1A), flies receive 6 training cycles. Each cycle starts by loading the flies into the experimental setup (0:00 min). From 4:00 min on, a control odor is presented for 15 s. From 7:30 min on, electric shock is applied as 4 pulses of 100 V; each pulse is 1.2 s-long and is followed by the next with an onset-to-onset interval of 5

s. In different groups, a to-be-learned odor is presented at different times relative to this shock; thus, the interval between the to-be-learned odor and the shock (the inter-stimulus interval: ISI) is varied between groups. Negative ISIs indicate first-odor-then-shock presentation; positive ISIs mean first-shock-then-odor presentation. At 12:00 min, flies are transferred out of the setup into food vials, where they stay for 16 min until the next training cycle starts. At the end of the sixth training cycle, after the usual 16 min break, flies are loaded back into the setup. After a 5 min accommodation period, they are transferred to a T-maze, where they can choose between the two odors that they have encountered during training. After 2 min, the arms of the maze are closed and flies on each side are counted. A preference index (PREF) is calculated as:

$$(1) \qquad PREF = (\#Learned_{odor} - \#Control_{odor}) \times 100 / \#Total$$

In this equation, # indicates the number of flies found in the respective maze-arm. For each ISI, two subgroups of flies are trained and tested in parallel (Fig. 1A): For one of these, 3-octanol (OCT) is the control odor and benzaldehyde (BA) is to be learned; the second group is trained reciprocally, that is the roles of these two odors are switched. A learning index (LI) is calculated based on the PREF values from the two reciprocal measurements:

$$(2) \qquad LI = (PREF_{BA} + PREF_{OCT}) / 2$$

Subscripts of PREF indicate the learned odor in the respective subgroups of flies. Positive LIs indicate conditioned approach to the learned odor; negative values reflect conditioned avoidance.

To test for the immediate, reflexive shock response, flies are transferred to the choice point of a T-maze, 5 min after being loaded into the setup. 10 s later, one of the maze arms is electrified with four 1.2-s long pulses of 100 V shock with 5 s inter-pulse intervals. 10 s after the onset of the last pulse, arms of the maze are closed and flies on each side are counted. A preference index for the electrified arm (PREFShock) is calculated as:

$$(3) \qquad PREFShock = (\#Electrified\ arm - \#Non\text{-}electrified\ arm) \times 100 / \#Total$$

Again, # indicates the number of flies found in the respective maze-arm. Negative PREFShock values indicate avoidance of the shock.

Sugar reward learning requires a different set of training parameters to yield substantial learning scores; specifically, it uses two training cycles (Fig. 2A). Each cycle starts by loading the flies into the setup (0:00 min). 1 min later, flies are transferred to a tube lined with a filter paper soaked the previous day with 2 ml of 2 M sucrose solution and dried over-night. This tube is scented with the to-be-learned odor. After 45 s, the odor is removed, and after 15 further seconds flies are taken out of the tube. After a 1 min waiting period, flies are transferred into another tube lined with a filter paper which was soaked with pure water the previous day and also dried over-night. This second tube is scented with a control odor. After 45 s, this odor is removed and 15 s later, flies are taken out of the tube. The next training cycle then starts immediately. For half of the cases, training trials start with the to-be-learned odor and sugar; in the other half, control odor is given precedence. Once the training is completed, after a 3 min waiting period, flies are transferred to the choice point of a T-maze between the two odors. After 2 min, the arms of the maze are closed, flies on each side are counted and a preference index (PREF) is calculated according to Equation 1. As detailed above, two groups are trained reciprocally (Fig. 2A) and a learning index (LI) is calculated based on their PREF values according to Equation 2.

Larval behavior

Larval learning experiments follow the mass assay described in Neuser et al. (2005). Larvae, aged 5-days after egg-laying, are assayed in groups of 30, under a fume hood at 24- 28 °C, in regular day-light. One day before the experiments, Petri dishes (Sarstedt, Nümbrecht, Germany) with 85 mm inner diameter are filled with 1 % agarose (electrophoresis grade; Roth, Karlsruhe, Germany), allowed to solidify, then covered with their lids, and left untreated until the following day. As sugar reward, 2 M fructose (FRU, purity: 99 %; Sigma, Steinheim, Germany) is added to the agarose 10 min after boiling. During the experiments, the regular lids of the Petri dishes are replaced by lids perforated in the center by ~60 1-mm holes to improve aeration. The odor N-amylacetate (AM; Merck, Darmstadt, Germany) is diluted 1:1600 in paraffin oil (Merck, Darmstadt, Germany) and is applied in custom-made Teflon containers placed in the Petri dish on opposite sides, 7 mm from the edges; these containers are of 5 mm inner diameter and are closed with a lid with seven 0.5-mm holes.

To start training, 30 larvae are collected from food medium, briefly washed in tap water to then as a group be transferred into a Petri dish filled with sugar-added agarose, and with two containers filled with AM (Fig. 3A). Larvae are left to crawl in this Petri dish for 5 min, and then are transferred into another Petri dish filled with agarose only, and with two empty containers. Also in this Petri dish, larvae remain for 5 min. We repeat this training

cycle three times, each time using fresh Petri dishes. At the end of training, we place the larvae in the middle of a fresh Petri dish, filled with only agarose, and with one container of AM on one side and one empty container on the other side (sidedness is alternated for every other set of larvae). After 3 min, the number of animals on each side is counted. For each group of larvae thus trained (i.e. 'AM + / Empty' as in this example; note that in half of the cases training is in reversed order, i.e. 'Empty/ AM+'), another group of larvae are trained reciprocally as 'Empty + / AM' (or, in half of the cases as 'AM/ Empty+'; Fig. 3A). A learning index (LI) is then calculated as detailed above for adult learning.

Quantification of biogenic amine amounts

We quantify the amounts of octopamine, tyramine, dopamine and serotonin in the fruit fly brain using High Performance Liquid Chromatography, coupled to a tandem Mass Spectrometer (HPLC-MS/MS). For the non-specialist reader, we first explain the principle of HPLC-MS/MS and the quantification method to put the present method into context of other previously used methods (see Discussion). Then, we present the technical particulars.

Principle of method: Extracts of fruit fly brain homogenate are loaded onto a liquid chromatography column that contains silica particles coated with C18 hydrocarbon chains. Biogenic amines along with other organic molecules are retained by the column material. By increasing the proportion of the organic solvent in the aqueous mobile phase, molecules are gradually separated and eluted before they enter the MS. Retention of the molecules on the column depends largely on their lipophilcity, i.e. polar, hydrophilic compounds elute early while hydrophobic molecules elute late. Hence, molecules of interest reach the MS at different and characteristic Retention Times (RT). As the molecules enter the MS, they become ionized through protonation (i.e. become positively charged). Molecule ions characterized by their specific mass-per-charge (*m/z*) ratios are physically separated by the first MS. Next, selected molecular ions are broken by collision induced dissociation (CID) into a series of compound-specific fragments which are then physically separated by a second MS that also records the ion intensities of the derived fragments. In the Multi Reaction Monitoring mode (MRM), even several molecules co-eluting from the HPLC column (i.e. molecules with the same RT) can be sorted and analyzed within some hundreds of milliseconds. Hence, molecules are specifically identified and quantified according to their RT, the *m/z* value of the molecular ion and the *m/z* value(s) of one or several fragment-ions. In pilot experiments all these values (RT, CID-energy, *m/z* values) can be obtained by analyzing authentic reference compounds. Moreover, the technique allows the use of internal standards labeled with stable isotopes that are added to the tissue prior to extraction. These

standards display the same physic-chemical properties as the target molecules and only differ by their mass. Hence, compound losses occurring during sample preparation and processing are proportional for standard and target molecules. To quantify e.g. the amount of serotonin, a known amount of deuterated serotonin ([D4]serotonin) is added to the brain homogenate. Labeled and endogenous serotonin then are simultaneously extracted and purified by HPLC. The endogenous 'light' serotonin and the heavier [D4]serotonin can be separated by the MS according to their different *m/z* values and the intensities of the ions can be determined. The ratio of the ion intensities should be equal to the ratio of the amounts initially present in the sample and, hence, the amount of endogenous serotonin in the un-extracted sample can be calculated. To validate the method, for example with respect to serotonin, we initially prepare a series of samples; each sample contains 5 ng of [D4]serotonin and a certain known amount of unlabelled, light serotonin, varying between 5 pg and 1000 pg. The amount of serotonin in each sample is then determined as described above. A plot of the measured amount against the known actual amount results in a linear function; for serotonin such a plot is shown in Fig. 6A-A' (for the other amines, see the Suppl. Fig.s). When isotopically labeled standards are used, the slope of the linear fit is usually one, as in the case of octopamine (Suppl. Fig. 1A-A'). Sometimes, however, the ionization and fragmentation efficiencies differ between the isotopically labelled standard and the unlabelled, light molecule, resulting in a slope that is different from one; in such cases, a *correction factor* is employed to compensate (e.g. as in the case of serotonin [Fig. 6A- A'], tyramine [Suppl. Fig. 2A-A'] and dopamine [Suppl. Fig. 3A- 3A']).

Chemicals: [D3]octopamine and [D4]serotonin are from Medical Isotopes (Pelham, USA); [D2]tyramine and [D3]dopamine are obtained using acid catalyzed isotope exchange between dopamine/ tyramine and deuterated water (Pajak and Kańska, 2006). Unlabeled octopamine, tyramine, dopamine and serotonin are purchased as hydrochloride salts from Sigma-Aldrich (Munich, Germany).

Sample preparation: Each sample contains 5 female and 5 male brains (2- 3 days-old) from either *white*^{1118} mutant or Canton Special wild-type flies. Brains are dissected in ice-cold ringer and directly placed into 50 µl of ice-cold 50 mM citrate-acetate buffer (pH 4.5), which in addition contains 5 ng of each internal standard. Once 10 brains are collected (which takes ~ 30 min) they are homogenized in this solution on ice with a Teflon pestle. After centrifugation at 14000 rpm for 5 min at room temperature, 10 µl of the supernatant is analyzed by HPLC-MS/MS.

HPLC-MS/MS conditions: An Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Waters Micromass Quattro Premier triple quadrupole

mass spectrometer (Milford, MA, USA) is used. Liquid chromatography is performed using an Agilent Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 µm particle size; Agilent Technologies, Waldbronn, Germany). The column is eluted with a linear mobile phase gradient (0.6 ml/ min flow rate) starting from water containing 0.1% formic acid at 0 min to acetonitrile: water: formic acid mixture (50: 50: 0.1, v/ v/ v) at 10 min.

For MS, ionization is achieved using electrospray in the positive ionization mode (ESI+) with a capillary voltage of 2.5 kV. The temperature of the source block is set at 120 °C and nitrogen is used as desolvation and cone gas with a flow of 800 l/ h at 350 °C and 50 l/ h, respectively. In order to establish the appropriate conditions for the individual compounds and their respective deuterated analogues, standard solutions are directly infused into the mass spectrometer and the cone voltage is adjusted to maximize the intensity of the protonated molecular species. Collision-induced dissociation of each compound is performed using Argon as collision gas with a flow rate of 0.3 ml /min and a pressure of 3.0 x 10^{-3} mBar; collision energy (eV) is adjusted to optimize the signal for the most abundant fragment ions, which are subsequently used for Multiple Reaction Monitoring (MRM) analysis with a dwell time of 100 ms for each reaction. The MRM transitions and conditions for the measurement are given in Table 1.

Statistics

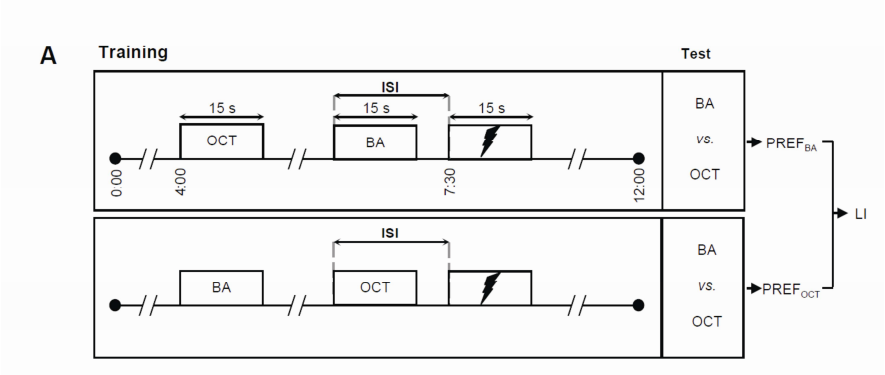
All data are analysed using non-parametric statistics and are reported as box plots, showing the median as the midline and 10, 90 and 25, 75 % as whiskers and box boundaries, respectively. For comparing values of each group to zero, we use one-sample sign tests. To compare values between two groups, we use a Mann-Whitney U-test. When multiple tests are performed within a single experiment, we adjust the experiment-wide error-rate to 5 % by Bonferroni correction; that is, we divide the critical $P< 0.05$ by the number of tests. For example, if 8 such comparisons are made, we report the P-level as $P< 0.05/ 8$. To compare more than two groups with each other, we use Kruskal-Wallis tests. Sample sizes are mentioned within the figures. All statistical analyses are performed on a PC using Statistica (Statsoft, Tulsa, Oklahoma, USA).

Results

white-function and olfactory associative learning

Regarding wild-type Control flies, conditioned behavior depends on the relative timing of odor and shock (red dispays in Fig. 1B: Kruskal-Wallis test: Control flies: H= 168.96, d.f.= 7, $P< 0.05$): If during training the odor is presented either long before (Fig. 1B: One-sample sign test: Control: ISI= -150 s: $P> 0.05/ 8$) or long after shock (Fig. 1B: One-sample sign tests: Control: ISI= 70 s and 200 s: $P> 0.05/ 8$ each) flies do not show any conditioned behavior. If the odor had shortly preceded or overlapped with shock during training, it is avoided in the test (punishment learning) (Fig. 1B: One-sample sign tests: Control: ISI= -45 s, -15 s and 0 s: $P< 0.05/ 8$ each). Contrarily, if the odor had shortly followed shock during training, wild-type flies later on approach it (relief learning) (Fig. 1B: One-sample sign tests: Control: ISI= 20 s, 40 s: $P< 0.05/ 8$ each). These results conform to the previous reports of Tanimoto et al. (2004) and Yarali et al. (In press).

Next, we compare *white*^{1118} mutants' learning to the wild-type. For very long ISIs, which do not support learning in the wild-type to begin with, we find no difference between the two genotypes (Fig 1B: U-tests: ISI= -150 s: U= 28.00; ISI= 70 s: U= 70.00; ISI= 200 s: U= 58.00; $P> 0.05/ 8$ each). In contrast, using short ISIs, which do support learning in the wild-type flies, loss of *white* function does have an effect: Namely, regardless of the sequence of the odor and the shock during training, the learning scores of the *white*^{1118} mutants are shifted 'southward' that is, towards stronger conditioned avoidance (Fig 1B: U-tests: ISI= -15 s: U= 183.00; ISI= 0 s: U= 745.00; ISI= 20 s: U= 157.00; ISI= 40 s: U= 226.00; $P< 0.05/ 8$ each; note however that for the -45 s ISI, U= 239.00, $P= 0.32$). Thus, the 'take home message' from the shock episode overall is more negative for the *white*^{1118} mutants than for wild-type flies.



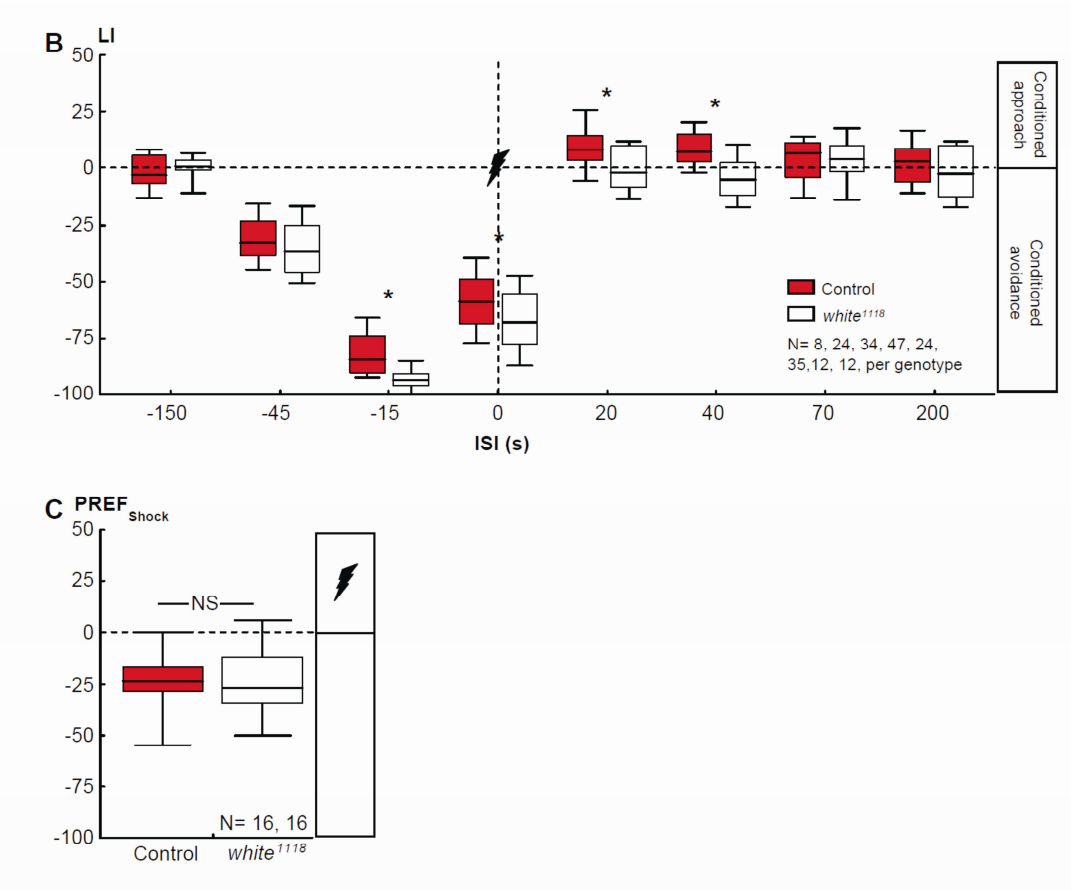


Figure 1:

Memory of shock overall more 'negative' for white^{1118} mutants

A Adult flies were trained with two odors and pulses of electric shock. Between the groups, we varied the interval between the as-yet-to-be-learned odor and the shock (interstimulus interval; ISI). Negative ISIs indicate odor-then-shock presentation; positive values reflect shock-then-odor presentation. For each ISI, two subgroups were trained reciprocally, that is, with switched roles for the odors 3-octanol (OCT) and benzaldehyde (BA). After training, each reciprocal group was allowed to choose between the two odors; based on their odor preferences (PREFs), we calculated a learning index (LI). Positive LIs indicate conditioned approach, and negative values mean conditioned avoidance.

B For wild type control flies, the ''sign'' of conditioned behavior depended on the ISI: If, during training, the odor had shortly preceded or overlapped with shock (ISI = -45, -15, or 0 s), control flies later on avoided it. If, during training, the odor had closely followed shock (ISI = 20 or 40 s), control flies later approached it. If the two events were too far apart in time (-150, 70, or 200 s), flies showed no signs of conditioned behavior. Concerning the *white^{1118}* mutants, scores overall were shifted ''southward'', that is, toward stronger conditioned avoidance. Sample sizes for the very long ISIs are lower because Tanimoto et al. (2004) and Yarali et al. (2008) showed that for such very long ISIs, the learning indices are zero in the wild type. In other words, expecting any kind of nonzero score for ISIs longer than 1 minute between odor and shock seems unlikely, in any genotype, such that differences between genotypes are unlikely, too. Therefore, a lack of difference for the long ISIs, although based on a small sample size, likely is real. * $P < 0.05/8$, while comparing between genotypes (i.e., Bonferroni correction; see Methods for details). Box plots represent the median as the midline; 25 and 75% as the box boundaries and 10 and 90% as the whiskers.

C Control and *white^{1118}* mutant flies avoided shock indistinguishably well. NS, $P > 0.05$. Box plots are as in (B).

Is this effect indeed specific for shock-related memories, or is it that the *white*^{1118} mutants regard the shock experience itself as more aversive? That is, is the effectiveness of shock as reinforcer, or its capacity to release avoidance behavior altered? We find that wild-type Control flies and *white*^{1118} mutants avoid shock to the same extent (Fig 1C: U-test: U= 123.5, $P> 0.05$; One-sample sign test: for the pooled data set: $P< 0.05$). Furthermore, loss of *white* function leaves olfactory discrimination ability in principle intact, as odor-reward learning remains unaffected: After odor-sugar training (Fig. 2A), learning scores do not differ between genotypes (Fig. 2B: U-test: U= 82.00, $P> 0.05$); when pooled, they reflect conditioned approach (One-sample sign test: for the pooled data set: $P< 0.05$).

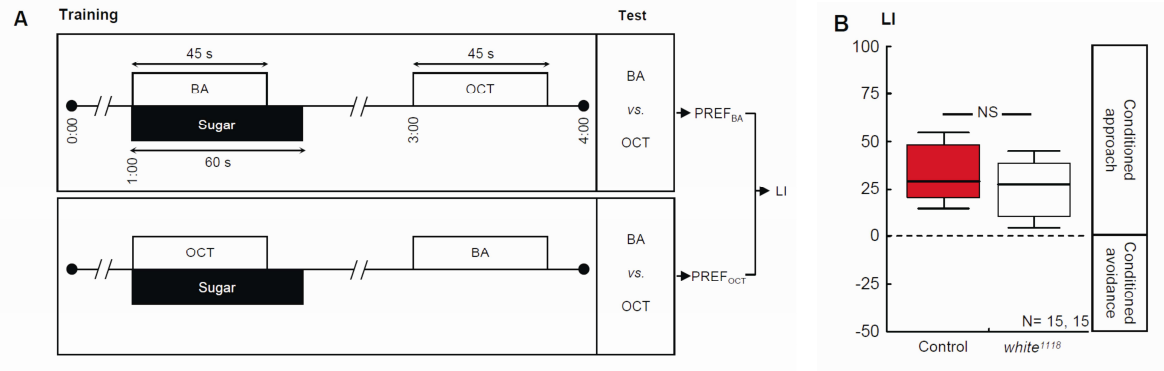


Figure 2:

Loss of white function does not affect olfactory reward learning in adult Drosophila

A: Adult flies are successively exposed to a to-be-learned odor in the presence of sugar and to a control odor without any sugar. Two subgroups are trained reciprocally, that is with switched-roles for the odors 3-octanol (OCT) and benzaldehyde (BA). Both subgroups are then given the choice between the two odors; a learning index (LI) is calculated based on their odor preferences (PREF). Positive values indicate conditioned approach towards the learned odor.

B: Control flies and *white*^{1118} mutants perform equally well after such reward learning. Details are as in 1C.

Also *white*^{1118} mutant larvae are not different from wild-type with respect to odor-sugar learning (Fig. 3B: U-test: U= 71.00, P> 0.05).

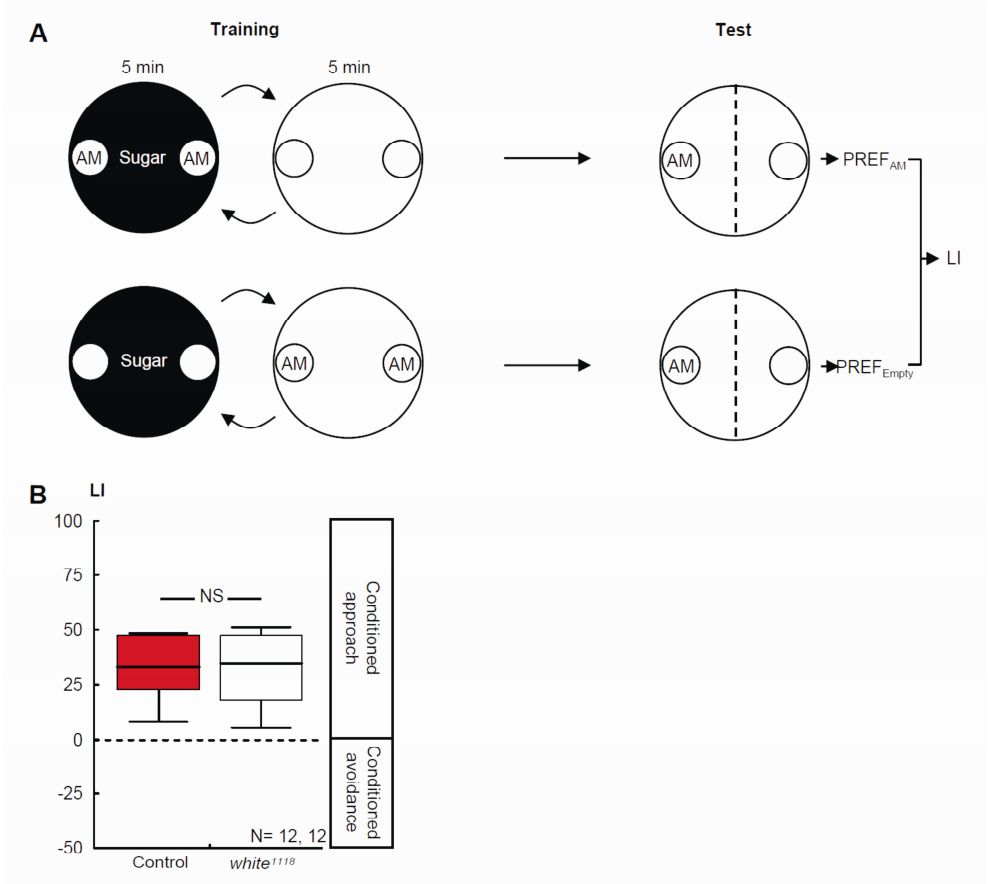


Figure 3:

Loss of white function does not affect olfactory reward learning in larval Drosophila

A: Larvae are successively exposed to the odor N-amylacetate (AM) in the presence of sugar and to a no-odor situation (Empty) without any sugar. Another group of larvae is trained reciprocally. Both groups are then tested for their response to AM; a learning index (LI) is calculated based on their AM preferences (PREF). Positive values indicate appetitive learning.

B: Control larvae and *white*^{1118} mutant larvae perform equally well in such reward learning. Details are as in 1C.

No effect of the loss of white *function on whole-brain amounts of biogenic amines*

Next, we probe the *white*^{1118} mutants' brains for abnormalities in the levels of the biogenic amines (octopamine, tyramine, dopamine and serotonin). This is because the White protein provides neurons with the precursor for serotonin as well as the precursor for a cofactor of serotonin- and dopamine-synthesis (see Introduction for details). Indeed, Sitaraman et al. (2008) have recently reported lower whole-head levels of serotonin and dopamine in *white*^{1118} mutants as compared to wild type flies.

Table 1. Multireaction monitoring mode transitions and conditions for the measurement of biogenic amines.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)
Octopamine	154	119	10	20
[D3]octopamine	157	121		
Tyramine	138	103	14	20
[D2]tyramine	140	105		
Dopamine	154	119	16	20
[D3]dopamine	157	121		
Serotonin	177	160	16	24
[D4]serotonin	181	164		

m/z values of precursor ions (protonated molecular ions) and specific product ions (fragment ions), cone ionization voltage, and energy for collision induced dissociation (fragmentation) are provided.

Using high performance liquid chromatography, coupled to mass spectrometry, we do not find a difference between *white*^{1118} mutants and wild-type Control flies in terms of the amounts of octopamine, tyramine, dopamine or serotonin in brain homogenates (Fig. 4: U-tests: octopamine: U= 16.00, *P*= 0.75; tyramine: U= 17.00, *P*= 0.87; dopamine: U= 16.00, *P*= 0.75; serotonin: U= 16.00, *P*= 0.75). As they stand, these data thus do not allow the effect of the loss of *white* function on learning to be attributed to an abnormality in the brain amounts of biogenic amines.

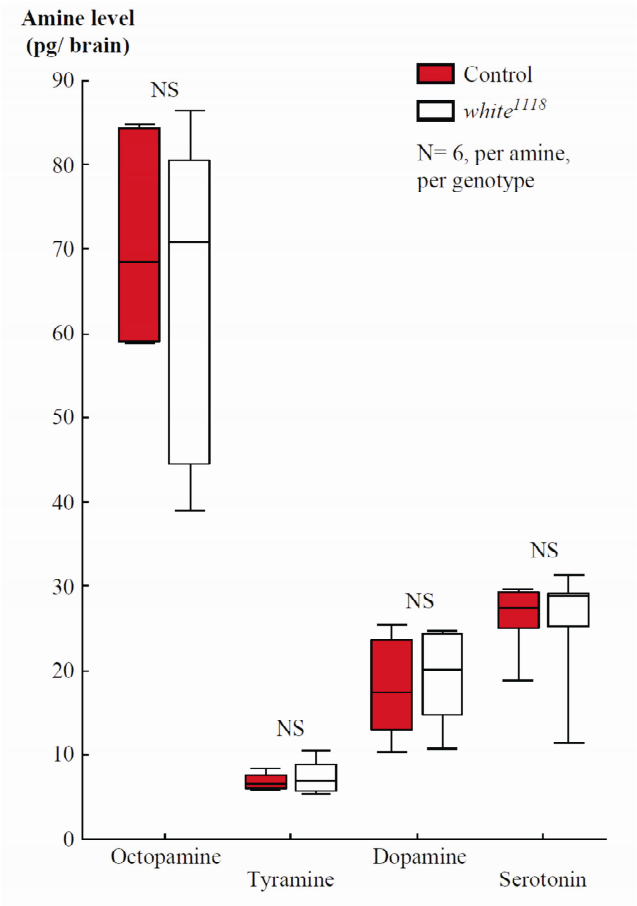


Figure 4:

Loss of white function does not affect the adult brain-amounts of biogenic amines

High performance liquid chromatography, coupled to mass spectrometry, reveals no difference between wild-type Controls and *white*^{1118} mutants in terms of the brain-amounts of octopamine, tyramine, dopamine or serotonin. From samples which include 10 brains, we report amine levels as pg per single brain. NS: *P*> 0.05. Box plots are as in 1B.

Discussion

We report an effect of the loss of *white* function on what fruit flies remember about a shock-episode (Fig. 1B). Namely, *white*^{1118} mutants, as compared to wild-type flies, build stronger aversive memories about the painful onset of shock (a finding in accord with the results from Diegelmann et al. [2006a]), and build weaker appetitive memories about its relieving offset. In other words, *white*^{1118} mutants remember the shock episode as overall more 'negative' than the wild-type flies. Importantly, the immediate aversiveness of shock remains unaltered for the *white*^{1118} mutants (Fig. 1C), arguing that it is indeed their memories of the shock episode, but not the shock itself, which appears more negative to them.

Keeping balance, losing it

As the case of the *white*^{1118} mutant shows, punishment and relief learning have common genetic determinants, keeping both processes in balance. This echoes Solomon and Corbit's (1974) theory of 'opponent processes', which suggests that a painful stimulus, in addition to its primary effect, also induces a state of relief upon its offset; the balance between these two opponent states is suggested to govern behavior towards painful stimuli as well as towards the stimuli associated with them. Distortion of the balance between these opponent processes in man are conceivably implicated in psychiatric conditions (anxiety: Vincent and Kukstas [1998]; addiction: Koob [2008]). Fruit flies seem to be an appropriate model to study the molecular and neuronal pivots of such balance, because comparable paradigms are available for assessing the behavioral consequences of both pain and relief. Importantly, the critical molecules may well be conserved from fly to man. Indeed, the human homolog of the *white* gene (i.e. *hW*, which has been mapped to chromosome 21q22.3) is implicated in mood and panic disorders (Straub et al., 1994; Croop et al., 1997; Nakamura et al., 1999).

white-*effect related to brain-levels of biogenic amines?*

In an attempt to account for the molecular mechanism by which the *white*^{1118} mutation exerts its effect, we probe for the brain-levels of the biogenic amines octopamine, tyramine, dopamine and serotonin. The amounts of these substances in the present analysis appear indistinguishable between *white*^{1118} mutants and wild-type (Fig. 4). This contrasts to the finding of Sitaraman et al. (2008), who report that *white*^{1118} mutants' heads contain less serotonin and less dopamine than the heads of wild-type flies.

In Fig. 5, we compare the present data on amine amounts to those previously reported. Obviously, the reported values substantially vary between studies. As a general remark, one

potential source of variability always is that in some cases mutations may cause phenotypes dependent on the genetic background (deBelle and Heisenberg, 1996). Second, sample preparation differs between studies in that homogenates from either whole heads or from only brains are assayed. This indeed can make a difference, even within a given study (Hardie and Hirsh, 2006; compare red triangles *vs.* red circles in Figure 5): Levels of e.g. dopamine are much higher in the head than in the brain, conceiveably because some dopamine is contained in the cuticle (Wright, 1987). Third, sample purification, detection and quantification differ across studies. Most studies cited in Fig. 5 couple HPLC to an electrochemical detector (HPLC-ECD), with two exceptions: (i) the present study, for all amines, employs HPLC tandem mass spectrometry; (ii) for measuring dopamine in un-purified head extracts, Sitaraman et al. (2008) use an enzyme immunoassay. Electrochemical detection has the drawback that oxidizable phenols/ catechols in the sample which co-migrate through the HPLC column with biogenic amines may accidentally yield electrochemical detector signals potentially resulting in over-estimations of amine levels. Therefore, methods relying on HPLC coupled to electrochemical detectors have to be carefully evaluated especially when un-purified samples from non-standard biological sources, potentially including unknown metabolites of the target trace-amount molecules, are analysed. A similar caveat may be raised concerning immunoassays: Since antibodies rarely display absolute specificity in particular for small molecules, cross reactivities with structurally related metabolites are often observed and may cause problems when un-purified samples are measured. In any event, both of these two methods do not employ isotopically labelled internal standards which help to compensate for variable extraction efficiencies, chemical degradation (i.e. autoxidaton) and losses during sample purification. Therefore, for trace analysis in particular of small molecules, coupled techniques in which the molecules of interest are first physically separated in a first dimension (i.e. by HPLC, gas chromatography or electrophoresis) and then specifically detected and quantified by mass spectrometry arguably seem preferable. Tandem mass spectrometry, as used in this study, adds two further dimensions of physical separation of molecules: i.e. the separation of the molecular ion in the first MS and the separation/ quantification of specific fragments ions in the second MS. In addition, the ionisation method and the collision energy employed further limit the type of molecules that can interfere with analysis, hence resulting in low background noise. Thus, apart from being highly specific, HPLC-MS/MS is also one of the most sensitive analytical methods available.

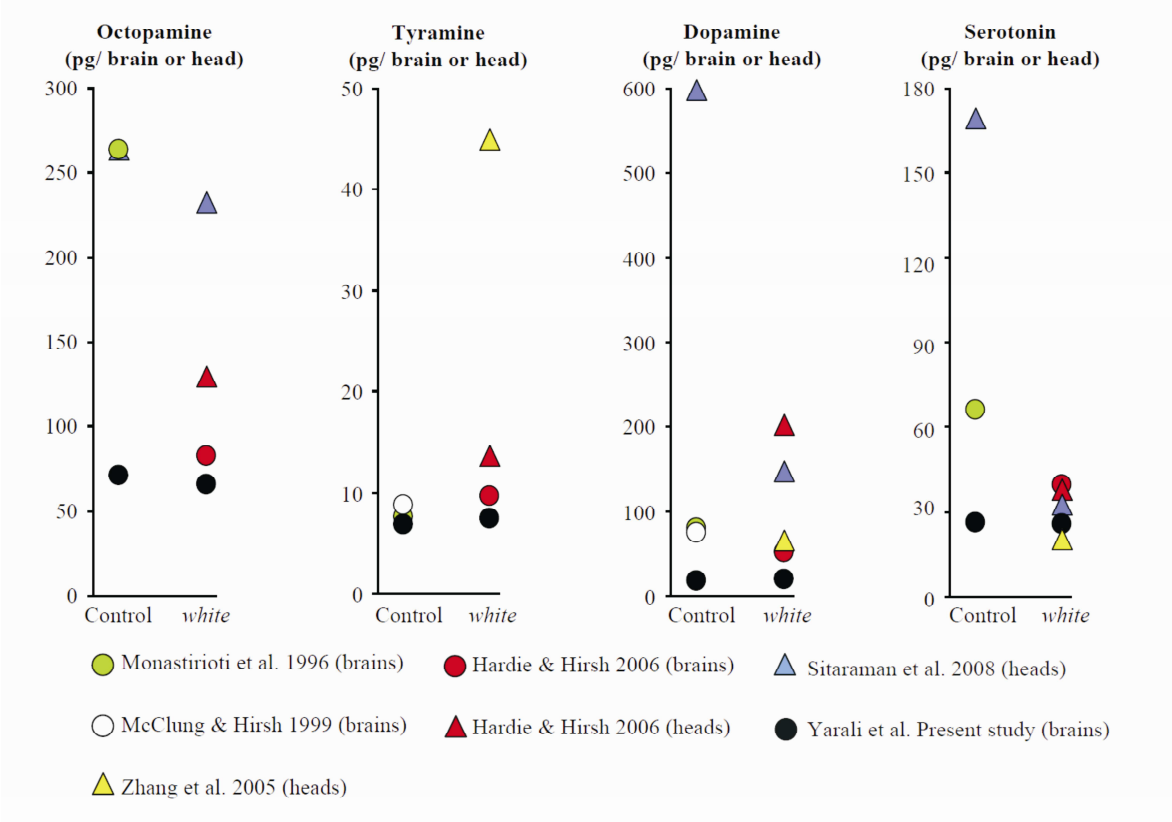


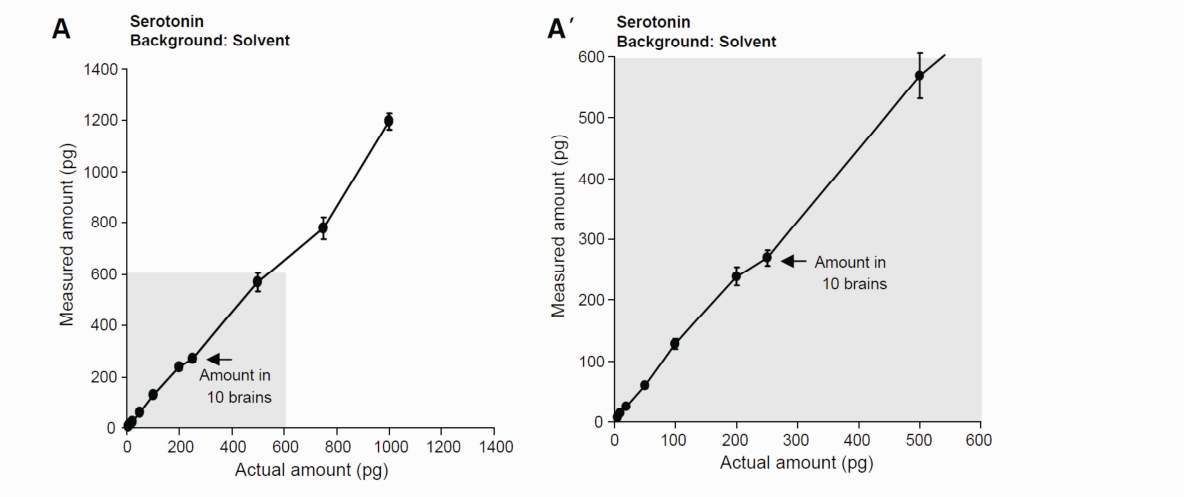
Figure 5:

Meta-analysis of amine amounts

We compare various HPLC-based studies (colour-coded) in terms of the biogenic amine amounts they find in whole-head (triangles) or only brain (circles) homogenates from wild-type Control or *white*^{1118} mutant flies. We plot mean values throughout in pg/ brain or head, to enable comparison between studies. Please note the different Y-axes for each amine.

With such methodology, the current study does not detect a difference between *white*^{1118} mutant and wild-type brains in terms of the biogenic amine levels. This contrasts to the finding of Sitaraman et al. (2008) that wild-type heads contain more dopamine and more serotonin than *white*^{1118} mutant heads. We take serotonin as a case to discuss whether such a between-genotype difference could in principle have been detected using the present method. As can be seen in Fig. 4, a number of independent reports, including the present one, agree upon the amount of serotonin per *white* mutant head/ brain. As compared to this 'consensus-level' of serotonin in the *white* mutant, Sitaraman et al. (2008) find 5- 6 fold more serotonin in wild-type heads. Could the present method have measured such a high serotonin amount? In Fig. 6A- A' the dynamic range of the present measurement, with respect to serotonin, can be seen. To reveal this dynamic range, we analysed by HPLC-MS/MS a series of samples each containing 5 ng of labelled [D4] serotonin and known amounts of unlabelled serotonin,

ranging from 5 pg to 1000 pg. We plot for each sample the measured serotonin amount against the actual, known amount; within a range of more than two orders of magnitude, these two amounts correspond well. Within this dynamic range, the total amount of serotonin in a homogenate of 10 brains as found in this study (Fig. 6A- A': black arrow) falls approximately in the middle, allowing to detect potential decreases as well as increases in serotonin levels. Specifically, it would in principle be possible to detect 4- fold higher serotonin levels than actually found in this study. This argument against a 'ceiling effect', obviously, is derived from measurements of serotonin over a solvent-'background'; does it apply for the experimental measurements of serotonin as well, i.e. for measurements over the brain-homogenate 'background'? In other words, is detection of serotonin within the brain homogenate possible with the same specificity as over the solvent 'background'? We compare chromatograms obtained over a solvent 'background' on the one hand (Fig. 6B) with the measurements over a brain-homogenate 'background' on the other hand (Fig. 6B'); both measurements have a reasonably good signal-to-noise ratio, arguing that the present method can detect serotonin equally well over either 'background'. These arguments also apply for octopamine, tyramine and dopamine (see Suppl. Fig.s).



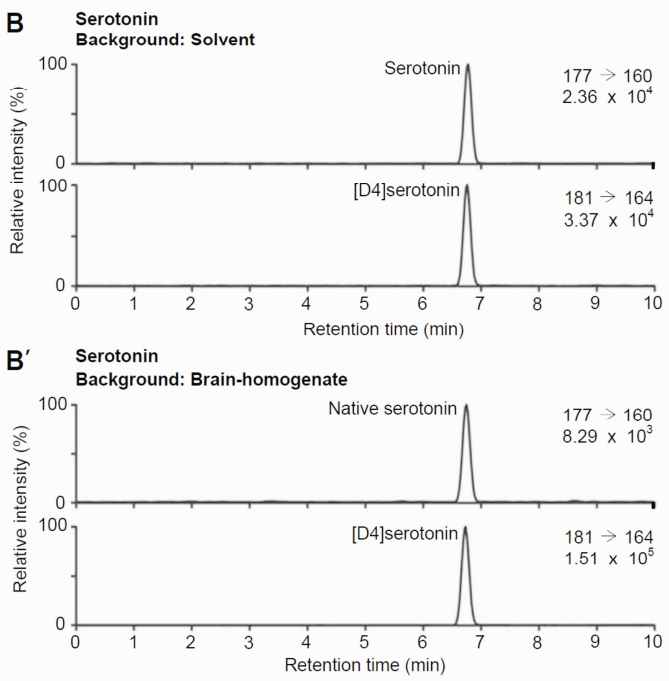


Figure 6:

A: Using HPLC-MS/MS we analyse a series of samples each containing 5 ng of [D4] serotonin and a known amount of unlabelled serotonin, ranging from 5 pg to 1000 pg. For each sample, we plot the measured amount of unlabelled serotonin against the actual, known amount. Mean ± SDs are obtained from three independent measurements. The black arrow marks the mean total amount of serotonin we find in a homogenate of 10 wild-type brains (i.e. we multiply the single-brain value from Fig. 4 with 10)

A': Close-up on the lower range of (A).

B: Example HPLC-MS/MS chromatograms for unlabelled serotonin (top) and labelled [D4]serotonin (bottom) over a solvent 'background'. As expected, their retention times are equal.

B': Example HPLC-MS/MS chromatograms obtained by analysing a homogenate of 10 wild-type brains, added with isotope-labelled serotonin (5 ng). Both unlabelled, native serotonin (top) and labelled [D4]serotonin (bottom) are clearly detectable. As expected, their retention times are the same. Note that the signal-to-noise ratio for the measurements over the solvent 'background' (B) does not apparently differ from the measurements over the brain-homogenate 'background'.

In turn, it may be that sample treatment in the current report unwittingly led to degradation of serotonin, such that over-all serotonin levels are too low to allow for between-genotype differences to be detected. As shown in Figure 6 A- A' (black arrow), a 5- fold decrease of serotonin levels would still be in the linear range of the current methodology. Thus, the assumption that the current report cannot detect between-genotype differences in serotonin levels because of a 'floor-effect' does not seem to be valid- unless one would assume that for to-be-identified reasons the degradation of serotonin were to happen in wild-

type, but not in the *white*^{1118} mutants. The same argument applies for the other amines as well (see Suppl. Fig.s).

With all these reasonings in mind, including the principle caveats of interpreting lack-of-difference results, we note that the present study does not find an abnormality of biogenic amine levels in the brains of *white*^{1118} mutants and hence cannot offer such variations to explain the effect of the *white*^{1118} mutation on shock-related learning. Obviously, this statement does not question the roles of amines for learning, as such roles have extensively been analyzed with genetic methods independent of *white* as well as by pharmacological intervention (fruit fly: Schwaerzel et al., 2003; Schroll et al., 2006; Sitaraman et al., 2008; honey bee: Hammer, 1993; Hammer and Menzel, 1998; Farooqui et al., 2003; Vergoz et al., 2007; cricket: Unoki et al., 2005; Unoki et al., 2006). In other words, both the mentioned amines and *white* can matter for learning, but these effects, based on the present data, may appear independent of each other.

A role for nitric oxide signalling?

Interestingly, guanine, which is transported into cells by the White-Brown heterodimer (Dreesen et al., 1988), is converted to '6H-tetrahydrobiopterin', which in turn is a cofactor for nitric oxide synthesis (reviewed by Koshimura et al. [2000]). Thus, effects of the *white* gene on nitric oxide signalling may explain its effects on learning. Indeed, nitric oxide may provide a retrograde signal at the output of the mushroom body Kenyon cells (Bicker and Hähnlein, 1995; Bicker et al., 1996), the suspected site of the odor-shock short-term memory trace (reviewed by Zars [2000], Heisenberg [2003], Gerber et al. [2004a] and Heisenberg and Gerber [2008]). Whether the effect of the *white*^{1118} mutation comes about via alterations in nitric oxide signalling remains to be tested.

Implications

Regardless of the underlying molecular mechanism, the behavioral effects of the *white* gene may in general concern *Drosophila* behavioral neurogeneticists. This is because a typical transgenic fly strain has a *white*^{1118} mutant genetic background and within the actual transgene carries a truncated so-called *mini-white* cDNA. This is done to ensure that a lack of insertion during the initial generation of the transgenic strain or loss of the transgene will reveal itself by white eye colour (this is why *white* is called a 'marker' gene). Thus, a confound in interpretation may arise when for example attempting to rescue a behavioral defect in a mutant X by transgenically expressing the cDNA of gene X using the Gal4- UAS system: In this case, the experimental flies not only transgenically express the potentially

rescuing gene, but they also bear both the Gal4 and the UAS transgenes and thus two copies of the *mini-white* cDNA. To the extent that loss of *white* function impairs the tested behavior, the experimental flies may indeed perform better than the controls, but conceivably not because of a rescue of gene X, but because two *mini-white* cDNAs rescue the $white^{1118}$ mutant phenotype better than one *mini-white* does in the genetic control strains (which carry either only the Gal4 or only the UAS construct). Thus, it would seem wise to probe for effects of *white* before launching a neurogenetic behavior analysis of any sort.

Conclusion

To summarize, we report that punishment learning (as induced by shock onset) is enhanced and relief learning (as induced by shock offset) is diminished in $white^{1118}$ mutants as compared to wild-type; thus, the balance between punishment learning and relief learning in the $white^{1118}$ mutant is distorted in favour of punishment learning. The molecular pivots of this distortion, in particular regarding the role of serotonin, however, remain controversial.

Acknowledgements

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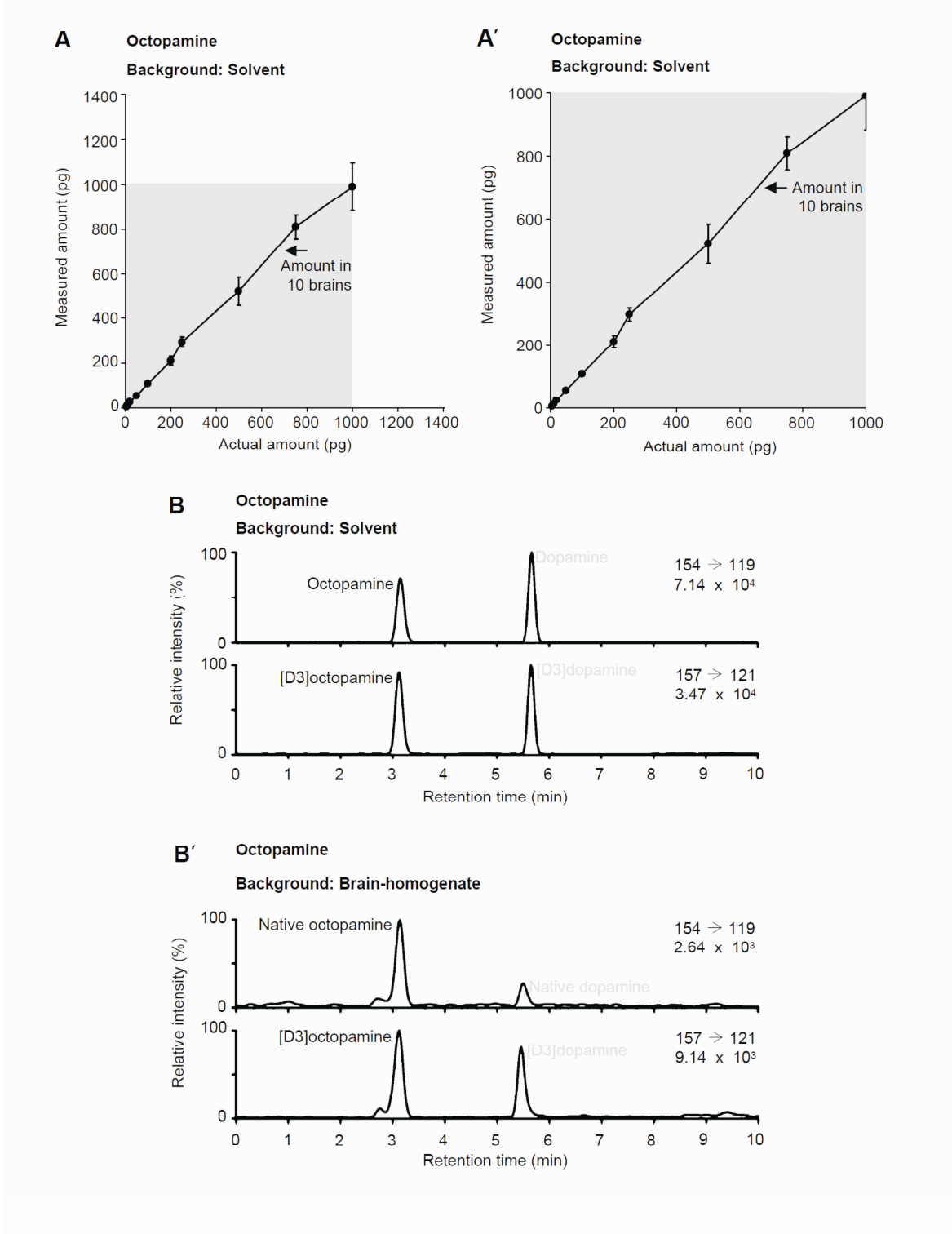
We are especially grateful to E. Münch for the generous support to A.Y. during the start-up phase of her PhD. The continuous support of the members of the Würzburg group, especially of M. Heisenberg, K. Oechsener and H. Kaderschabek, is much appreciated, just as the collegial discussions with T. Zars, University of Missouri-Columbia.

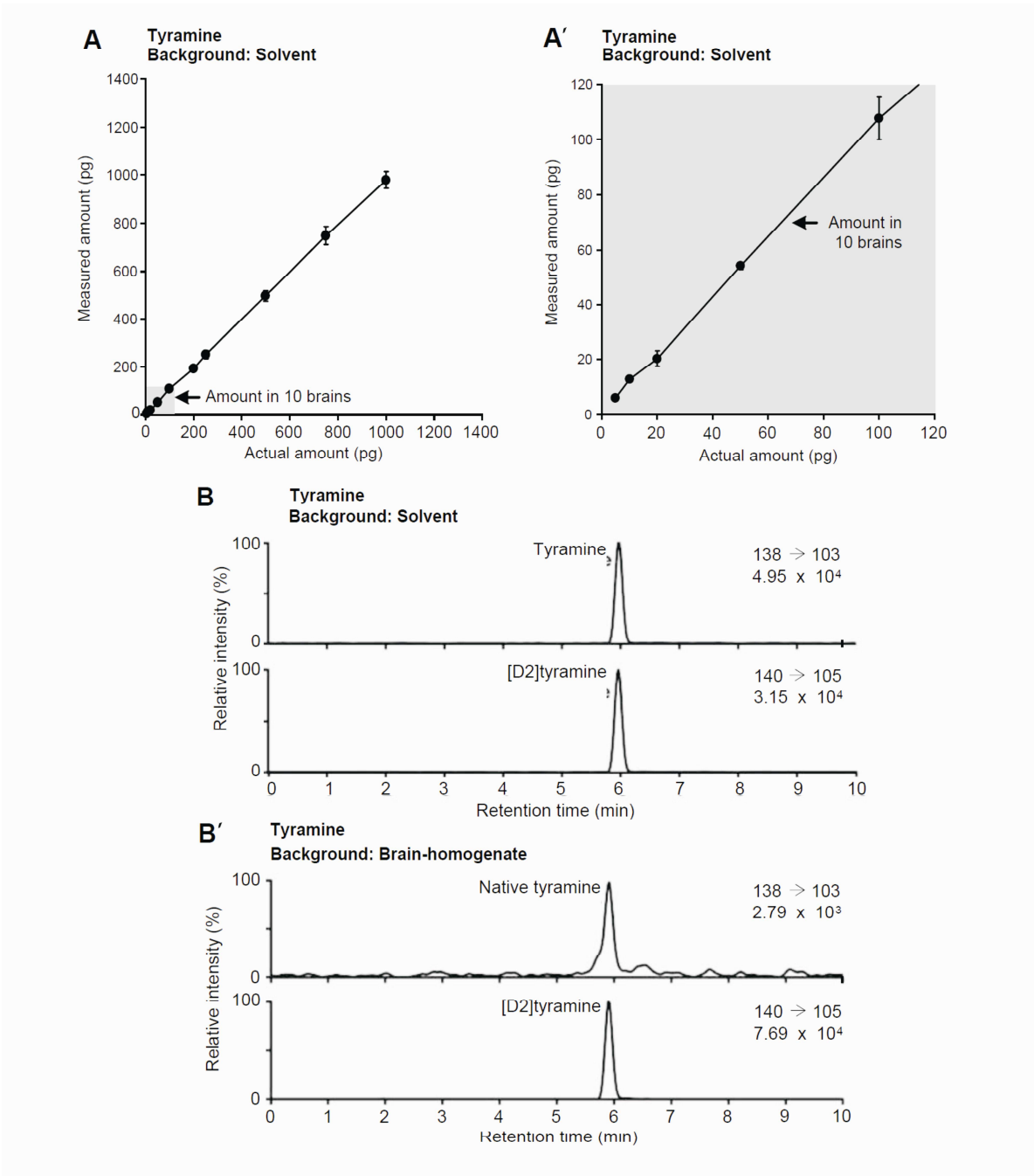
Supplementary Material

Assessment of the octopamine- (Suppl. Fig. 1), tyramine- (Suppl. Fig. 2) and dopamine- (Suppl. Fig. 3) measurements analogous to the one reported for serotonin in Fig. 6.

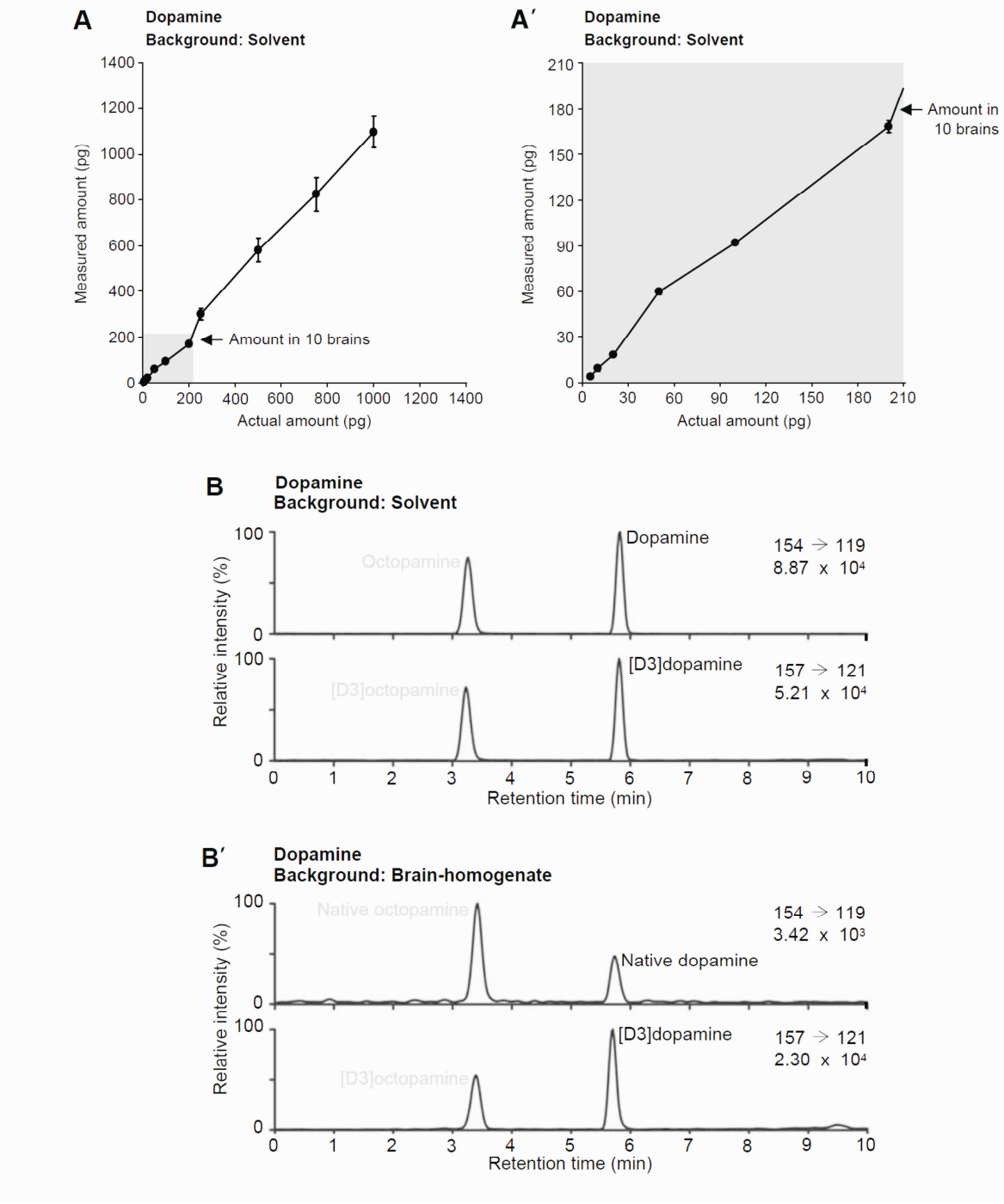
Supplementary Figure 1:



Supplementary Figure 2:



Supplementary Figure 3:



GENERAL DISCUSSION

What can we learn from a maggot?

To understand mechanisms of learning and memory one needs to address the behavioral, cellular and molecular level. I used the *Drosophila* larva as model organism because it is suitable in many regards. First larvae have a relatively simple nervous system (see Fig. 3 and 7), yet show a wide variety of behaviors including classical conditioning of odors with positive as well as negative reinforcers (Scherer et al., 2003; Hendel et al., 2005, Neuser et al., 2005; Michels et al., 2005; Niewalda et al., 2008; Selcho et al., 2009; Yarali et al., 2009b; Pauls et al., 2010a; Pauls et al., 2010b; Saumweber et al., 2011a; Saumweber et al., 2011b; Michels et al., 2011; Schleyer et al., *In Press*). On the behavioral level they combine the advantage of adult *Drosophila*, e.g. low cost of keeping and an immense number of progeny. On the cellular level, much is known in particular about their olfactory system. The involved cells and their connectivity share fundamental similarities with the adult fly and mammalians (Davis, 2004), although the number of involved cells is reduced (reviewed in Gerber et al., 2009). On the molecular level, the larva offers the whole genetic toolkit available for *Drosophila* with all its advantages - not matched in any higher organism. The fully sequenced fly genome (Adams et al., 2000) together with the Gal4/ UAS system (Brand and Perrimon. 1993) allow to express any gene, anywhere to any time. This gives access to identify single cells involved in the learning circuitry as well as to measure changes of their physiological properties after conditioning. These tools further allow straightforward analyses of protein function within the identified cells and figure out cascades in which they are involved (e.g. for adenylat cyclase see: Dudai et al., 1988; Abrams et al., 1998; Heisenberg, 2003; for Synapsin see: Michels et al., 2011 and 2005; Godenschwege et al., 2004; Hilfiker et al., 1999; Klagges et al., 1996 and for SAP47 see: Reichmuth et al., 1995; Funk et al., 2004; Hofbauer et al., 2009; Saumweber et al., 2011b).

The below discussion focusses on my two main projects (Innate attractiveness and associative learnability of odors can be dissociated in larval Drosophila [Saumweber et al., 2011a] and Behavioral and synaptic plasticity are impaired upon lack of the synaptic protein SAP47; [Saumweber et al., 2011b]) and offers a brief outlook concerning the other projects I contributed to.

Establishing a one-odor paradigm

I tackled the question what is learnt by *Drosophila* larvae during classical conditioning. Does the larva only have the possibility to associate an odor with a reinforcer when both these stimuli are presented together, or can they also learn something about the absence of a reinforcer, e.g. if odor and reinforcer are presented in an unpaired way. Therefore it was necessary to establish a new version of the paradigm introduced by Neuser et al., (2005) (chapter I.1, Saumweber et al., 2011a). Together with Jana Husse and Bertram Gerber I introduced the one-odor version of the paradigm (developed in Saumweber, 2007; also used in Selcho et al., 2009; Mishra et al., 2010; Chen et al., 2011) and hereby could reduce the complexity of the task. Only a single odor can be trained to be associated with a sugar as reinforcer. The new experimental design enables testing different odors for effectivity in sensing and learnability. We could show that 3-octanol, 1-octanol and amyl acetate result in positive naïve preferences as well as substantial associative performance indices, meaning that larvae can detect and learn these single odors. In contrast to Honjo and Furukubo-Tokunaga (2005 and 2009) for linalool experimentally naïve larvae behave not different from chance level and learning performance is rather low. Those authors found spontaneous preference towards linalool (as did Fishilevich et al., 2005) and strong training-dependent changes of linalool preferences. These effects are actually stronger than for most of the other 18 odors the authors had tested; also, it is reported that 3-octanol and 1-octanol are not learnt at all. As discussed in detail in chapter I.1 (Saumweber et al., 2011a; see also Gerber and Stocker, 2007) these discrepancies may reflect differences between wild type strains and/ or in case of the learning experiments substantial differences in the behavioral paradigms used. Profound differences are that they used a non-reciprocal experimental design, allowing for confounding non-associative effects as sensitization and habituation (see Gerber and Stocker, 2007 for discussion). There are further differences in time and kind of presentation of the odor-substrate combinations and also in the number of trained larvae making direct comparisons of our learning results to the ones of Honjo & Furukubo-Tokunaga (2005 and 2009) problematic. Importantly, our one odor reciprocal training procedure leads to substantial learning performance without confounding non-associative effects.

Innate attractiveness and associative learnability of odors

We analyzed if relative innate attractiveness and associative learnability can be dissociated, which is indeed the case for three of the four tested odor pairs (Saumweber et al., 2011a). The most dramatic example of dissociation between innate attractiveness and associative

learnability was found for linalool. As mentioned before larvae are naively not attracted by linalool, but larvae have the possibility to associate this odor with a sugar reward. Also, although for the odor pair 3-octanol and 1-octanol the relative innate preference are balanced, the associative performance indices are substantially higher for 3-octanol than for 1-octanol. A corresponding pattern of results is found for the odor pair amyl-acetate and 3-octanol. The possibility of such discrepancies between naive preferences and learnability of odors should be considered in odor-quality generalization experiments: Adjusting odor concentrations for equal naïve relative preference does not automatically make sure that learnability is equal. Therefore, these two odors may still be discriminated on the basis of intensity information. This could confound measures of odor qualitiy discrimination as well as odor quality generalization, and may in particular lead one astray when considering the capacities for odor-quality discrimination in single-receptor mutants (see also discussions in Mishra et al., 2010 and Chen et al., 2011).

Interestingly, the dissociation between innate attractiveness and associative learnability may shed some light on the particular anatomical feature of the insect olfactory pathway. Projection neurons have two target areas, the lateral horn where premotor neurons originate and the mushroom bodies where the odor-reward associative memory trace is established (Gerber et al., 2004a and see reviews by Heisenberg 2003; Gerber et al., 2009; for larva see also chapter I.4, Michels et al., 2011). This architecture of the motor system receiving two kinds of olfactory information, direct input via the lateral horn pathway, and indirect input via the mushroom body loop is common in most if not all insects. It seems likely that innate preference behavior is steered via the direct lateral horn pathway, whereas learnt behavior may require the read-out of the olfactory memory trace in the mushroom body loop (regarding the adult, see Heimbeck et al., 2001).

Learning about the absence of reward

After establishing this one-odor paradigm, it was possible to ask whether paired presentations of an odor with a reward increase whereas unpaired presentations of odor and reward decrease odor preferences after training. This analysis was in particular enabled by the possibility to determine the baseline of olfactory behavior after training, because the behavioral expression of odor-sugar memory can be blocked by presenting reward during the test (Gerber and Hendel, 2006). We could show that paired presentations of odor and reward increase odor preference abvove baseline, arguing that the trained odor is a predictor of reward after paired presentation; additionally, we could show that unpaired presentations of odor and reward

decrease odor preference below baseline, suggesting that the odor may predict the absence of reward after such unpaired presentation (for related reports in the bee: Bitterman et al., 1983; Hellstern et al., 1998). This poses a challenge to current models of how neurobiologically such learning comes about. The Rescorla Wagner model, created in 1972, is a mathematical model to account for the effects of classical conditioning. This model become one of the most influential models of learning, because it can generate clear and ordinal predictions and it has relatively few free independent variables. The change in associative strength is proportional to the difference between Vmax and the associative strength existing before the trial. This leads to the following equation (Rescorla and Wagner, 1972):

$$\Delta V_n \sim V_{max} - V_n$$

- V associative strength between a CS and a US
- ΔV_n: The change in associative strength
- Vmax: asymptotic value of V, characteristic for each combination of US and CS.
- n: number of training trials

This proportionality can be defined by two parameters: α and β:

$$\Delta V_n = \alpha \beta (V_{max} - V_n)$$

- α: salience of the CS
- β: rate parameter for the US (association value)

Animals learn about discrepancies between what is expected to happen and what actually happens. Only three factors are required for conditioning, namely contiguity, contingency, and prediction error (Schultz, 2006). In our larval odor-reward training, larvae crawl over a plate containing fructose with coincidental presentation of the odor that contiguity is given and contingency is complete because both stimuli, if occurring, occur together. A positive prediction error ensues during training when initially the reward is received in the presence of the odor and as training progresses, the odor becomes more and more predictive of the reward, and the prediction error is getting smaller until the learning process ceases. This seems obvious for paired stimuli presentation, but what happens during unpaired presentation? Interestingly in our one-odor version in the reciprocal group stimuli are presented in an unpaired way. Is there anything like absence prediction? We found that odor preferences after such kind of training are decreased below baseline. We therefore speculate that during a reward-only trial an association is formed between the 'experimental context' and the reward. There are many possibilities what 'experimental context' could be: the

artificial situation for the larvae getting out of the food vial, crawling over a pure agarose plate, being transferred by a brush etc. But importantly the context is the same in both kind of trials of the unpaired training regimen. In an ensuing odor-only trial within the same context, this context-reward association is activated and predicts the reward although no reward is actually present. This then leads to a negative prediction error, because less reward is experienced than is predicted. If at this moment of 'frustration' an odor is presented, the odor becomes a signal for no-reward, rather than remaining neutral, i.e. not being a signal for anything. This speculation about such an scenario requires now experimental scrutiny, including directly testing whether context-reward associations can be formed by the larvae, where in their brain these associations take place, and whether they can indeed account for learning by unpaired odor/ reward training - or not.

Potency of the one-odor paradigm

One further advantage of the one-odor paradigm is simplifying the kinds of behavioral control procedures which are necessary when investigating mutant larvae in associative odor-reward learning, because one has to control only for sensory ability concerning a single odor between a given mutant strain with the corresponding wild type strain (see Saumweber et al., 2011b and Michels et al., 2005).

It further enables analyzing the larval 'perceptual odor space' in generalization experiments (Chen et al., 2011), where one odor is trained, but another, not previously trained odor ist tested. For example, Mishra and colleagues (Mishra et al., 2010) took advantage of our one-odor paradigm by first adjusting learnability of two odors (3-octanol and 1-octen-3-ol). They then found that 3-octanol can be discriminated well from 1-octen-3-ol, if larvae had been trained discriminatively. On the other hand, no odor-specificity could be observed after non-discriminative training. Thus, for this odor pair there is both, strong discrimination and full generalization (Mishra et al., 2010). If the test involves a choice between these two odors, larvae showed conditioned preference for the rewarded odor only if training had been performed discriminatively, but not if training had not been performed discriminatively. In other words, for 3-octanol and 1-octen-3-ol only discrimination training confers an odor-specific memory trace, whereas one-odor training does not. This means that, at least for 3-octanol and 1-octen-3-ol, there is a degree of freedom in the olfactory system that allows enhancing or ignoring differences between odors flexibly, depending on the task (Mishra et al., 2010). Such kinds of comparisons are only possible since the development of our one odor version of larval learning paradigm.

The one-odor version was also inspiring regarding adult fly (e.g. Eschbach et al., 2011). They used the adult odor-shock paradigm (Quinn et al., 1974) to investigate how odor mixtures are perceived by flies. After adjusting odors for equal learnability, they tested escape behavior from binary mixtures after punishment training with one of its constituent elements and vice versa, how much flies avoid an odor element if it had been a component of a previously punished binary mixture. They found that learning scores are the same, when flies are trained with the component and tested with a component-containing mixture or when trained with the odor mixture and tested with only one component (Eschbach et al., 2011). The generalized avoidance is reduced compared to their learning baseline (pooled learning scores for one odor learning for all tested odor pairs). Thus the generalization is substantial, but partial. Further they could show that elements are equally similar to all mixtures containing it and that mixtures are equally similar to both their constituent elements. Including analyzes of the physicochemical properties they conclude that, the more distant the elements of a mixture are to each other, the more distant the flies regard the elements from the mixture. Again, such kinds of experiments would not be possible without the possibility to train and test single odors in a one-odor paradigm.

A thought experiment

One future project which comes to mind immediately by looking at Supplementary Figure 7 of Saumweber et al., 2011a could be to investigate decision like processes, comparing innate predictive *versus* innate relative preference of odors. Decision making, in itself an interesting process, is not well understood. Giving an example, a case study, if one would offer someone 10 euro than the one would be probably be glad and take it. If one would offer the same person 11 euro than this person would be about equally glad and have the same propensity to take it because the difference between 10 and 11 euro is ´marginal´ for most people. Instead, if one would offer the very same person the direct choice between 10 and 11 euro most people would take the 'marginally' better offer. This is an example of decision making where small differences in one-cue preferences can cause to an enormous difference in the relative preference. An according experiment possible using *Drosophila* larvae could be: One can give the larvae the choice between a naively new odor A and an empty odor container. Most larvae would prefer the odor side. Given the larvae the choice between another odor B and an empty container most larvae would prefer also that odor B. Similar to the procedure in Saumweber et al., 2011a, one can now balance the naïve responses to both odors by diluting it, such that larvae respond to both odors (A *versus* empty and B *versus* empty) to the same extent. In a

following experiment one can further test the relative preference between these two odors (A *versus* B in respective concentrations) to see whether there are differences in predicted and observed relative preferences to odors, similar to the thought experiment mentioned above for humans. Using further genetic intervention one has the possibility in the larvae to find the molecular basis underlying such a decision process.

From molecule to behavior: The role of SAP47 in larval behavior

On the molecular level I mainly focused on the role of the synaptic protein SAP47 in associative learning. In Saumweber et al., 2011b (chapter I.5) it is shown that the SAP47 protein is widely expressed in the neuropil regions of the larvae including the larval brain as well as larval neuromuscular junctions and cephalic organs. Concerning the intracellular localization of SAP47 it is associated with synaptic vesicles. It has no transmembrane domain so it is not an integral part of the synaptic vesicle membrane (Mastrogiacomo et al., 1994; Umbach et al., 1994; Arnold et al., 2004, loc. cit. Fig. 3). Natalja Funk generated the $Sap47^{156}$ deletion mutant by jump-out mutagenesis leading to a total absence of the SAP47 protein in larvae (for adult *Drosophila*: Funk et al., 2004). After extensive outcrossing larval mutants lacking this protein, either because their respective gene is deleted (shown for two deletion allels: $Sap47^{156}$ and $Sap47^{201}$) or because it has been knocked-down in their whole nervous system by means of RNAi, show a reduction in learning ability of about 50 % but retain all necessary sensory and motor functions for the learning task. Maybe most importantly, transgenic expression of the full length SAP47 protein driven by *elav*-Gal4 fully rescues associative function in the $Sap47^{156}$ mutant, providing compelling evidence for a function of SAP47 in behavioral associative plasticity. Together, and considering the outcrossing regimen for the $Sap47^{156}$ mutants, it seems reasonable to attribute the learning defect upon deletions in the *Sap47* gene to a lack of the SAP47 protein, rather than to spurious differences in genetic background. We note that the associative defect in all cases ($Sap47^{156}$, $Sap47^{201}$, in RNAi knockdown larvae, as well as in the genetic controls in both rescue experiments) is partial, arguing that there are SAP47-independent mechanisms to support associative function in our one odor as well as in a two odor version of the learning paradigm. Alternatively, there could be hitherto unknown *Sap47*– like genes in the fly genome. However, we can detect no SAP47 protein in the mutants either by antibody or by searching in the genome for SAP47-homologous sequence.

Basic synaptic transmission and short-term plasticity at the neuromuscular junction were analyzed in cooperation with Annika Weyersmüller and Stefan Hallermann, to

investigate the physiological mechanism of SAP47 function. The pronounced synaptic depression during sustained bursts of neuronal activity is consistent with the hypothesis that SAP47 contributes to the recruitment of vesicles to the release site (Hallermann et al., 2010). Plasticity processes that underlie odorant-taste learning most likely happen within the central brain (Gerber and Stocker, 2007), but these cells are not identified in detail and are not amenable to comparable physiological analyses, yet. However, previous extrapolations between behavioral and synaptic plasticity at the neuromuscular synapses have been surprisingly successful (e.g. regarding the AC-cAMP-PKA cascade: Kidokoro et al., 2004; Ueda and Wu, 2009). Thus, the kind of distortion of vesicle recruitment necessary for short-term plasticity observed at the neuromuscular junction may be the cause for the impairment in associative function on the behavioral level (Abbott and Regehr, 2004; Rothman et al., 2009).

To summarize, this thesis provides the identification of a behavioral and physiological function of the phylogenetically conserved SAP47 protein.

Outlook and further projects

Isoforms

Interestingly in *Drosophila* eight different isoforms of SAP47 are annotated (http://flybase.bio.indiana.edu), three short and five longer ones. Expressing the shortest isoform of SAP47 (*sap47-RF*) in the whole nervous system of *Drosophila* larvae leads to a partial rescue of the defect in associative function of the mutant larvae, and expressing the full length isoform of SAP47 (*sap47-RA*) rescues the learning defect up to wildtype level. One may therefore speculate about different roles of different isoforms of SAP47. After extensive studies of possible epitops for polyclonal antisera production, I assigned Eurogentec (Seraing, Belgium) to generate different antisera from different species to investigate the expression pattern of these different isoforms. The corresponding epitopes are shown in Figure 4.

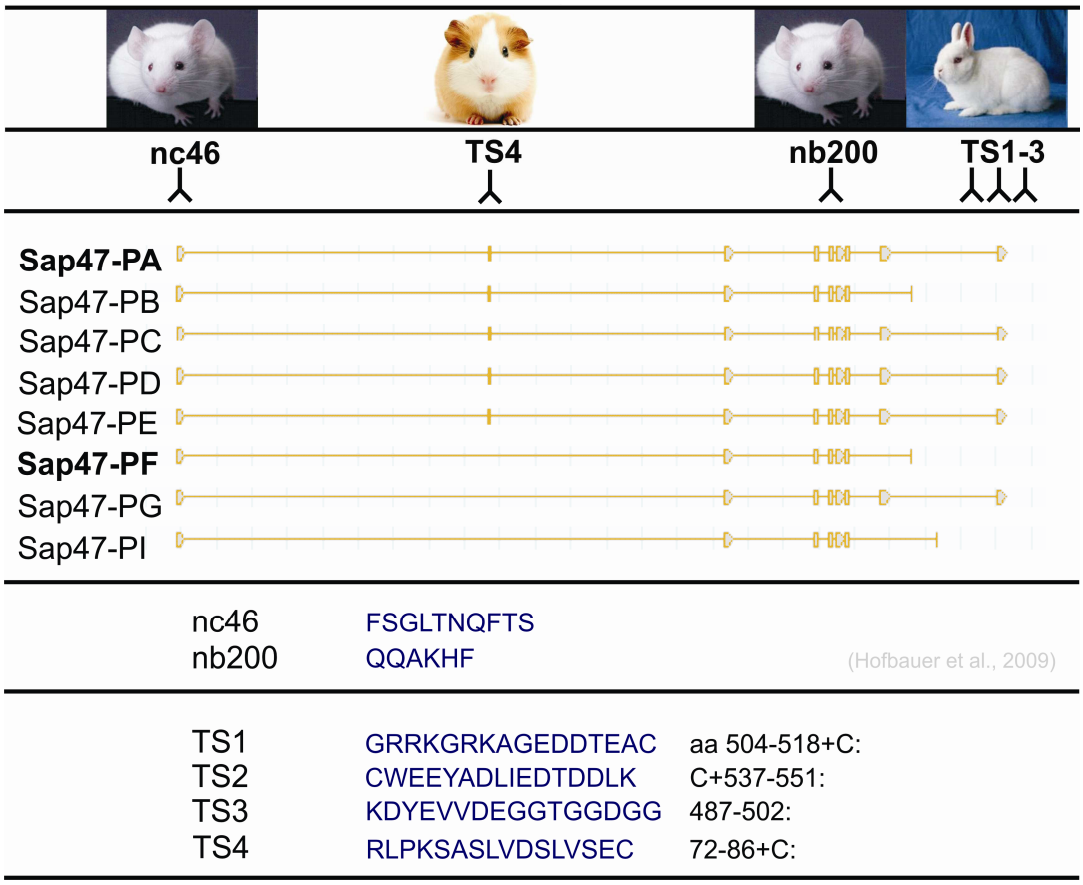


Figure 4:

Epitopes for monoclonal antibodies and polyclonal antisera.

Shown are the eight annotated transcripts of SAP47 and the epitopes for the two monoclonal mouse antibodies from the hybridoma library (Hofbauer et al., 2009), nc46 which epitope is in the first and the nb200 which epitope is in the fifth exon coded region. All of the short isoforms lack the eighths and ninth exon coded region providing epitopes for TS1, TS2 and TS3 for rabbit immmuniozation. Note that there are differences in the region coded by the second exon providing the epitope for TS4 from guinea pig. Using combinations of these antibodies and antisera enables to differentiate between isoforms.

So far we got these antisera and tried to get all of these antisera working in Western Blots as well as in immunocytochemistry. This is not trivial, because most all of them do not produce a clear, single band on Western Blots, as is usually the case for antisera. It is now necessary to enrich the specificity of sera cross-reacting with proteins other than SAP47. This can be achieved by allowing only these cross-reacting antibodies to bind to their epitopes during preincubation with *Sap47*^{156} mutant tissue. Then the supernatant can be used to probe the wild-type, which is an ongoing project (data not shown).

Given the knowledge of the coding sequences for the different isoforms, it would be further interesting to generate rescue strains for all eight isoforms putting every rescue

construct into the same insertion site by homologue recombination (Gao et al., 2008) to figure out which isoform, when, and in which brain region, is sufficient to restore memory formation.

Given that *Sap47*^{156} mutant larvae show a defect also in quinine avoidance (El-Keredy A and Schleyer M, Universität Würzburg; personal communication; please recall that sugar preference is unaffected: Saumweber et al., 2011b), these analyses of the function of SAP47 isoforms could now also be extended to figure out which isoform can rescue this quinine avoidance phenotype, and in which cells this would be possible.

Memory phase

Preliminary experiments performed by Dirk Planitzer show that *Sap47*^{156} mutant larvae cannot retrieve their memory established, using our standard paradigm, more than a few minutes in contrast to wildtype larvae, which can remember that kind of associations for about two hours (Planitzer, 2011). Similar experiments looking in Synapsin mutant larvae on the temporal dynamics about memory retrieval are performed at the moment by Andreas Hellmann, but data is not conclusive to date. Interestingly, Stefan Knapek compared short-term memory (3 min) and 5 hour memory in Synapsin mutant flies and could show that Synapsin null-mutants show a significantly decreased memory compared with wild-type flies tested immediately after training, but not when the flies are tested 5 hours after training (Knapek et al., 2010). This may suggest preferential (yet not exclusive) contributions of Synapsin and SAP47 for earlier and later phases of memory, respectively. Given that Synapsin is selectively required for anesthesia-sensitive but not amnesia-resistant memory (Knapek et al., 2010), it would therefore be interesting to see whether SAP47 may play a role in anaesthesia sensitive and/ or anaesthesia resistant memory.

Molecular cascade

Although the roles of Synapsin and SAP47 may be preferential for earlier *versus* later forms of memory, such assignment is certainly not exclusive: both *syn*^{97CS} and *Sap47*^{156} mutants show comparably partial phenotypes in immediate-term retention of larval odour-sugar memory Saumweber et al., 2011b; Michels et al., 2011). Also, both mutants may have similar phenotypes in terms of short-term synaptic plasticity at the neuromuscular junction (Saumweber et al., 2011b; Gerber B, Universität Leipzig; personal communication). Both observations therefore suggest that at least partially the same cascades may be affected in *syn*^{97CS} and *Sap47*^{156} mutants. Indeed, on behavioral level *Sap47*^{156}, *syn*^{97CS} double mutant

larvae (Albertova V, University of Würzburg) do not seem to show an additive defect in associative function (Bretzger J, Universität Würzburg; personal communication). Further, Western blots of head homogenates for SAP47 and Synapsin interaction were performed (Nuwal, 2010; also Funk N, Universität Würzburg; personal communication). SAP47 and Synapsin expression were compared in wild-type flies and looked at SAP47 expression in syn^{97} mutants and Synapsin expression in $Sap47^{156}$ mutants. It was found, and we also could confirm this result (see Fig. 5), that in $Sap47^{156}$ mutant flies there is at least one additional Synapsin band detectable not seen in wildtype using the monoclonal mouse antibody (3C11: anti-Synapsin). The band disappears after alkaline phosophatase treatment (Funk N, Universität Tübingen; personal communication). However, a direct interaction between these two proteins could not be detected, neither in a yeast-two hybrid screen (Funk N, Universität Tübingen; personal communication) nor in an *E. coli* cell based interaction assay (Wegener S, 2008).

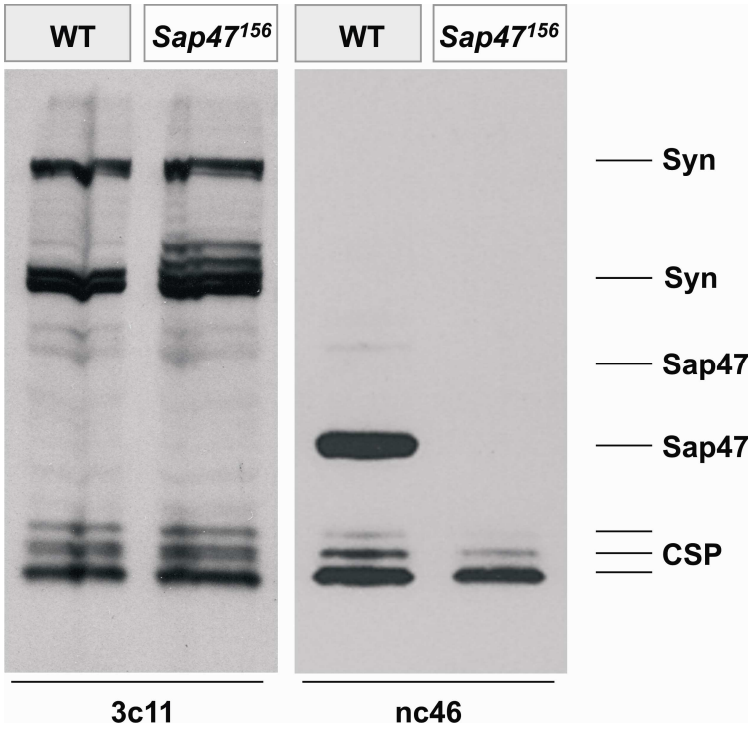


Figure 5:

Western Blot

Shown are Western Blots from 3 adult brains of WT and $Sap47^{156}$ mutant flies.

Left: 3c11 was used as antibody detecting Synapsin at 143 and 74 kDa. An additional Synapsin band is detectable in $Sap47^{156}$ mutants in comparison to wildtype flies, which may be due to a bandshift caused by phosphorylated Synapsin.

Right: nc46 antibody was used to detect SAP47 in wildtype, whereas no SAP47 expression is detectable in $Sap47^{156}$ mutants. Both blots were probed with the ab49 antibody labeling the Cysteine String Protein (CSP) at 32 kDa as a loading control

Taken these findings together there may be an interaction between SAP47 and Synapsin in a phospholylation dependent manner, although this interaction may not be direct. Rather, SAP47 may either activate e.g. any phosphatase or inactivate e.g. any kinase which then further regulates the phosphorylation status of Synapsin (see Fig. 6). This in turn is necessary to regulate the vesicle recruitment from the synaptic vesicles from the reserve pool to the ready releasable pool mediating plasticity effects.

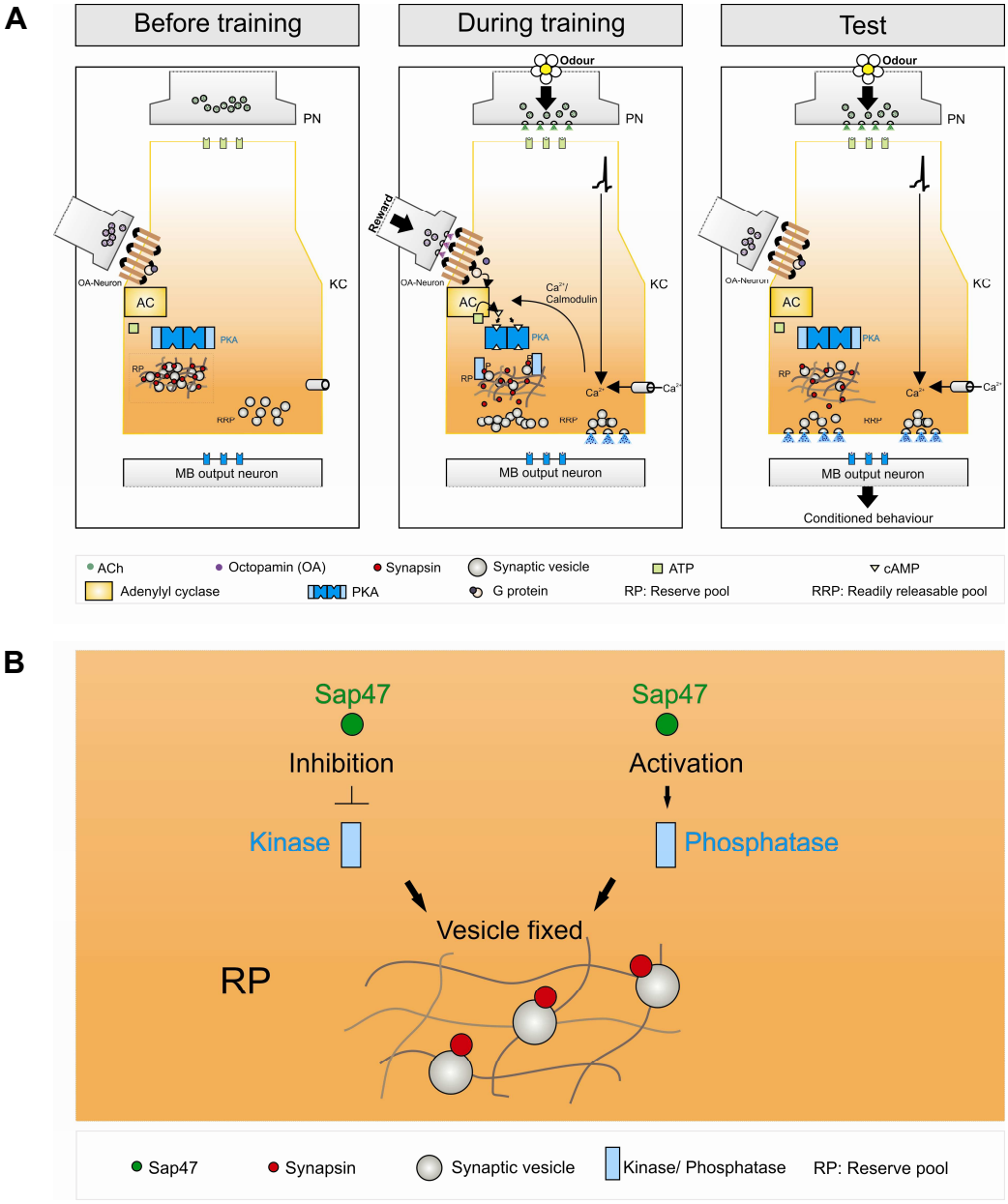


Figure 6:

Cartoon of molecular mechanism in the presynapse

A: Shown is a working hypothesis of the molecular role of Synapsin and SAP47 in associative learning. Our results suggest that type I adenylyl cyclase (AC) acts as coincidence detector (Michels et al., 2011). The odor leads to presynaptic calcium influx, and hence to an activation of calmodulin, whereas the reward leads to an activation of most 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 (Michels et al., 2011).
B: Two possible hypotheses of the molecular role of SAP47: Important for vesicle recruitment is the regulated balance of phosphorylation status of Synapsin. First hints suggest that SAP47 puts a break on this phosphorylation, either by inhibiting a kinase, or by activating a phosphatase. This may contribute to set a proper threshold for learning-induced phosphorylation of Synapsin by e.g. the AC-cAMP-PKA cascade (see text).
This is based on the observation that in *Sap47*^{156} null mutants a shifted Synapsin band on Western Blots of head homogenates is detectable (Fig. 5), which is gone after alkaline phosphatase treatment (Funk N and Nuwal T, Universität Würzburg; personal communication).

Salt processing in larval *Drosophila*: Choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way.

Niewalda et al., 2008

Together with Thomas Niewalda I used salt (sodium chloride) as reinforcer to investigate how salt is affecting larval behavior (Niewalda et al., 2008). We could show that low salt concentrations are attractive, whereas increasing salt concentrations shift the preferences from attraction to repulsion; similarly, the effects of salt on feeding behavior turn from increasing to decreasing feeding. We further found that, depending on salt concentrations the effect of salt as reinforcer also switches, from acting as reward to acting as punishment.
Interestingly, learned behavior after these kinds of training is not automated. Rather, after odor-LOWsalt training the odor informs a *search for reward*, but after odor-HIGHsalt it informs escape from HIGHsalt: conditioned appetitive behavior is disabled if the sought-for reward is actually present at test, whereas conditioned aversive behavior remains suppressed as long as the testing situation does not require escape. Thus, it is the expected outcome which determines whether memory is behaviorally expressed- or not.

Based on these findings it would be interesting to ask whether the respective memory trace includes, in addition to the valence ("How bad?") also quantitative ("How much?") as well as qualitative aspects ("What kind?") of the reinforcer. Notably, Eschbach et al., (*Experimental Biology, In Press*) lately introduced a mechanical stimulation ("Buzz") as a negative reinforcer. These "Buzz-memories" are retrieved in the presence, rather than the absence of the Buzz, and interestingly also in the presence of HIGH salt or quinine. In contrast, larvae which are trained with HIGH salt or quinine and are tested in the presence of

the Buzz do not show conditioned behavior. This might mean either that the Buzz is just less "bad", or that the outcome expectation which the Buzz memory trace is supporting is less specific than the one confered by HIGHsalt or quinine memory traces.

A Behavior-based circuit-model of how outcome expectations organize learned behavior in larval *Drosophila.*

Schleyer et al., In Press

Together with Michael Schleyer we developed a behavior-based circuit model of how outcome expectations are organized in the larval brain.

The olfactory circuits of the larva (Fig. 7) are fairly well understood on the cellular level (e.g.. Stocker, 2006). The breakthrough was the identification of a *Drosophila* family of odorant receptor genes (Clyne et al., 1999; Vosshall et al., 1999). Of this *Or*-gene family, adult *Drosophila* express ~ 60 (Clyne et al., 1999; Vosshall et al., 1999; Robertson et al., 2003) and larvae 25 members (Fishilevich et al., 2005; Couto et al., 2005; Kreher et al., 2005). 11 of these 25 are larval-specific, while the remaining 14 are expressed in both developmental stages (Kreher et al., 2008). One of the 25 genes (*Or83b,* synonymous to *Orco)* encodes a chaperone-like protein. *Orco* is required as a coreceptor for *Or*-receptor function and is expressed in all larval olfactory sensory neurons (Fishilevich et al., 2005). Beside *Orco,* olfactory sensory neurons typically express one other *Or* gene, which by virtue of the ligand profile of the encoded receptor protein determines the receptive range of the sensory neuron (Kreher et al., 2005; Fishilevich et al., 2005). As exception only two sensory neurons coexpress besides *Orco Or33b*/ *Or47a* and *Or94a*/ *Or94b*, respectively (Fishilevich et al., 2005; Kreher et al., 2008). The recently discovered ionotropic receptor gene family members are expressed in developmentally distinct (i.e. *Orco*-negative) sensory lineages, and in analogy to the situation in adults may mediate chemosensory information as well (Benton et al., 2009), but the expression patterns and functions of the larval-expressed *Ir* genes remain opaque).

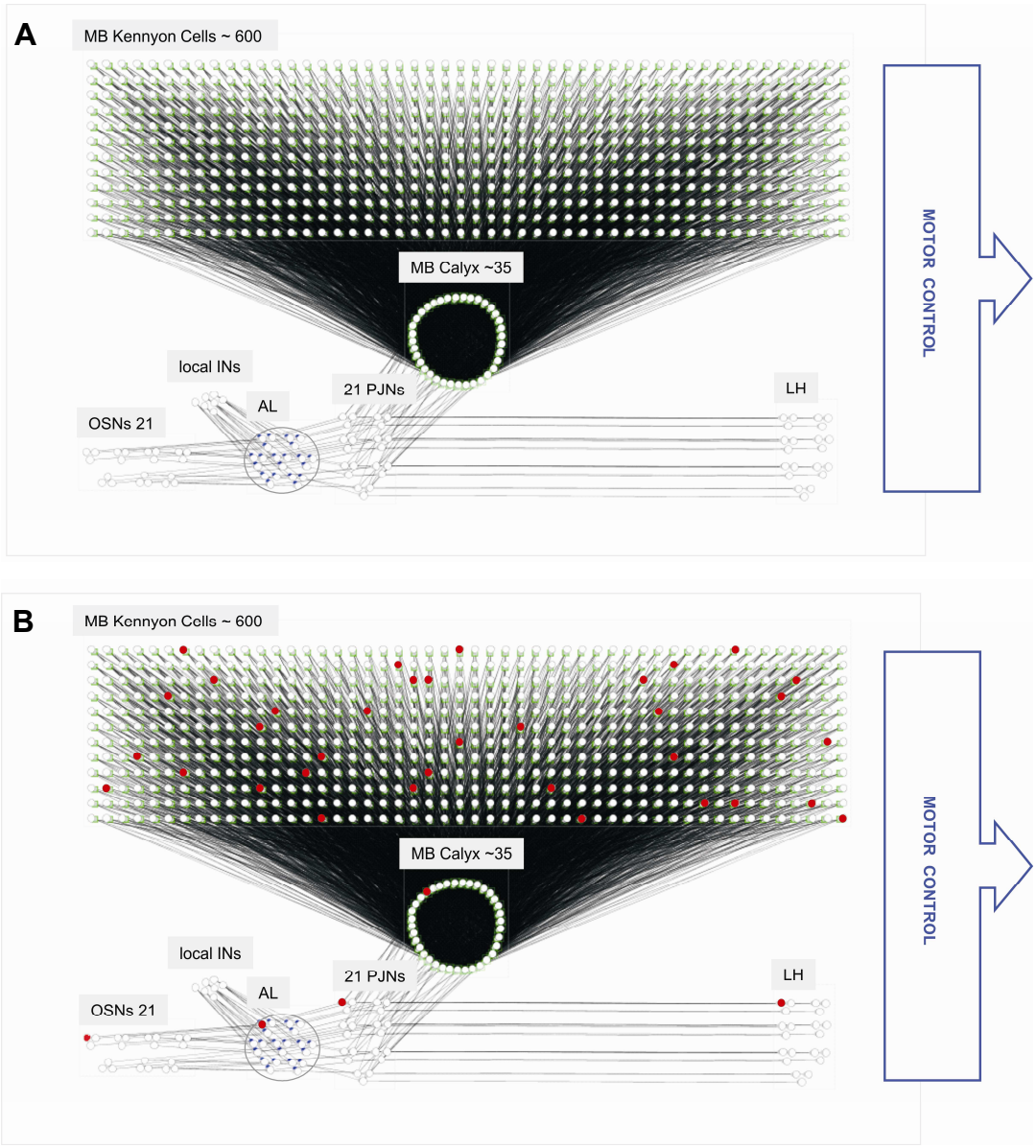


Figure 7:

Cartoon of the larval olfactory pathway

A: Shown is the olfactory pathway of *Drosophila* larva and the number of involved cells. Odor detection is accomplished by 21 olfactory sensory neurons expressing olfactory receptors located in the dorsal organ. They send their axons to 21 larval antennal lobe glomeruli. Some local interneurons interconnect the glomeruli modulating odor tuning. Olfactory information is further processed via uni-glomerular projection neurons to higher brain centres, the lateral horn and the mushroom bodies. Projection neurons choose mostly a single from about 35 calyx glomeruli as targets and many of these projection neurons stereotypically link a specific antennal lobe glomerulus with a specific calyx glomerulus. Larval Kenyon cells either innervate a single calyx glomerulus (Ramaekers et al., 2005) or establish arbors in multiple, about 6 out of 35 glomeruli, implying that information from ~6 calyx glomeruli can converge to a single Kenyon cell (Masuda-Nakagawa et al., 2005 and 2009).
B: Visualized is an activation pattern along the olfactory pathway if only a single olfactory sensory neuron were activated.
AL antennal lobe; IN interneuron; LH lateral horn; MB mushroom body; OSN olfactory sensory neuron; PJN projection neuron.

In any event, as in adult *Drosophila* as well as in mammals, in the larva each olfactory sensory neuron projects to a single glomerulus in the antennal lobe. But compared to the 1,300 olfactory sensory neurons of the adult, there are no more than 21 of these in the larvae. Each of these is unique and projects to one of 21 glomeruli in the antennal lobe (Stocker, 2006). However, olfactory coding does not simply rely on additive activation of 21 parallel pathways, but also involves interactions via interneurons in the antennal lobe (and possibly also further downstream). Single-clone analysis at the level of projection neurons suggests that each projection neuron essentially connects a single antennal glomerulus to a single calyx glomerulus (Masuda-Nakagawa et al., 2009). Asahina et al., 2009 also reported that stimulation of a single olfactory sensory neuron strongly activates projection neuron terminals in only 1 or exceptionally 2 calyx glomeruli, and suggested that this finding was consistent with a 1 : 1 : 1 connectivity between olfactory sensory neurons, antennal lobe glomeruli, and calyx glomeruli. The larval mushroom body calxy comprises approximately 35 - 40 glomeruli. Postsynaptic to the projection neurons in the mushroom bodies are the Kenyon cells. Each of the approximately 600 Kenyon cells receives input from an apparently random selection of 1 - 6 glomeruli (Masuda-Nakagawa et al., 2005, 2009). This suggests that they may receive several different combinations of heterogeneous odor inputs, which allows them to discriminate a large variety of odor identities and intensities (Masuda-Nakagawa et al., 2009). The mushroom bodies receive further input from modulatory, aminergic reinforcement neurons - dopaminergic and octopaminergic, such that within the mushroom bodies the association of odor-evoked activity with reward or punishment signals can take place (regarding adult Drosophila: Busch et al., 2009, 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) (please note that these cells are not included in the the circuitry diagram of Fig. 7, yet). The Kenyon cells in turn synapse onto remarkably few (based on findings in adult flies [Ito et al. 1998; Tanaka et al., 2008; Séjourné et al., 2011; Tanimoto H, MPI für Neurobiologie, München; Gerber B, Universität Leipzig and Thum A, Université de Fribourg; personal communication]) output neurons that entertain connections towards pre-motor centres. It indicates that the mushroom body organize learnt olfactory behavior (see discussions in Gerber et al., 2004a and 2009; Heisenberg and Gerber, 2008). However, the exact connectivity between the mushroom bodies, as well as the lateral horn, and motor circuits is largely unknown. The paper by Schleyer et al., (*In Press*) proposes, on the basis of a series of behavioral experiments, a scaffold for these circuits. In particular, a circuitry is proposed that accommodates the organization of learned behavior with regard to

its expected outcome. Obviously, the *Drosophila* toolkit, in particular the reversible block of synaptic output via *shibire*^{ts}, should now be use to test these proposals.

Site and mode of Synapsin action in associative learning

Michels et al., 2011

Synapsin is required for larval odor-food learning (Michels et al., 2005). Here we investigated the molecular mechanism of Synapsin function and the localization of the Synapsin dependent memory trace (Michels et al., 2011). *Drosophila* Synapsin contains phosphorylation consensus sites for PKA, so it might be one of the target proteins of PKA and thus mediate its effects via the cAMP cascade. As it is well described that the molecular mechanisms of coincidence detection appears by the type I adenylyl cyclase during training (Dudai et al., 1988; Abrams et al., 1998; Renger et al., 2000), but is an open question which process 'translates' activation of the AC-cAMP-PKA cascade to stronger transmitter release at the moment of test. Because *Aplysia* Synapsin was found to be an excellent in vitro substrate for cAMP dependent protein kinase (Fiumara et al., 2004), it came into consideration that one or both of the PKA consensus sites of Synapsin are required for reserve-pool vesicle recruitment. Therefore a mutated Synapsin protein was expressed, which cannot be phosphorylated at the two predicted PKA sites due to a replacement of the serine by alanine. We could show that transgenically expressing this mutated Synapsin cannot rescue the defect of the s*yn*^{97CS} mutant in associative function, thus assigning Synapsin as a behaviorally relevant effector downstream of the AC-cAMP-PKA cascade. To figure out if both or which of these two sites are necessary and if there are further phosphorylation sites is under investigation at the moment, including the role of the ADAR-dependent RNA editing at one of these sites (Diegelmann et al., 2006b).

On cellular level, a Synapsin-dependent memory could be assigned to only a handful cells using *D52h*-Gal4 (6-12 cells). 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 the *D52h*-Gal4 pattern, to then perform learning experiments combined with physiology during memory acquisition and/ or memory retrieval.

Genetic distortion of the balance between punishment and relief learning in *Drosophila.*

Yarali et al., 2009b

An experience with electric shock can support two opposing kinds of behavioral effects (Solomon and Corbit, 1974; Wagner, 1981): Stimuli that precede shock during training are subsequently avoided as predictors for punishment, whereas stimuli that follow shock during training are later on approached, as they predict relief (for adult fruit fly: Tanimoto et al., 2004, also found in rat and man; Gerber B, Universität Leipzig; personal communication). Ayse Yarali demonstrated for adult *Drosophila* that upon the loss of *white*-function (Morgan et al., 1915), the balance between these two kinds of learning is distorted. $white^{1118}$ mutants show increased punishment learning and decreased relief learning, as compared to wild type flies, suggesting that $white^{1118}$ mutants establish, overall, more ‘‘negative’’ memories for the shock experience. As discussed in detail within Yarali et al., (2009b), this phenotype of the $white^{1118}$ mutant is unrelated to the levels of biogenic amines.. Interestingly, learning about reward is apparently unaffected in adult flies. In this project I tested whether $white^{1118}$ mutant larvae are affected in associative function using our standard appetitive learning paradigm. I made a comparison of larval odor-reward learning between wild-type and the $white^{1118}$ mutant. This is important in regard of genetic background effects of transgenic flies being usually in the $white^{1118}$ mutant background: A *mini-white* gene as marker in the transgenic constructs for generating transgenic fly strains helps to keep track of the construct. It turned out that the $white^{1118}$ mutation has no effect on larval odor-reward learning (Yarali et al., 2009b; Saumweber et al., 2011b; Michels et al., 2011), so this paradigm can be used for behavioral analysis in transgenic animals without considering *white* function. For transgenic studies of adult flies, however, *white* function should be considered.

Closing remark

During my thesis I tried to understand fundamental mechanisms of learning and memory in more detail. I looked at different aspects of olfactory associative larval learning – on the behavioral, cellular and molecular level. I provide a detailed parametric analysis of reward learning and could show that innate attraction and learnability can be dissociated and that Drosophila larva also can learn about the absence of a reward. Further on behavioral level I investigated, how salt affects larval behavior. On cellular level we tried to generate a model based on behavior of the larvae, to figure out how outcome expectations can be generated and

are controlled by the larval brain. On molecular level I was involved in investigating three proteins. Together with Birgit Michels, I investigated the cellular site and molecular mode of Synapsin function. For White we could show that in mutant larvae reward-learning is unaffected. Further, I could show that SAP47 plays a role in associative function as well as synaptic plasticity.

From these findings many questions arise, as usual and wanted in research. From my perspective, the 'biggest' of these questions are firstly, how decision like processes come about and secondly, whether a given memory trace includes in addition to the valence also quantitative as well as qualitative aspects of the reinforcer. Thirdly it would be important to figure out the exact connectivity between the mushroom bodies, as well as the lateral horn, and motor circuits. Our proposed model that accommodates the organization of learned behavior, has now to be proven using the *Drosophila* toolkit. Fourthly, which isoforms of SAP47 is required to rescue the learning defect in $Sap47^{156}$ mutants and where in the larval brain and how SAP47 on molecular level can regulate the balance of phosphorylation status of Synapsin, which is important for vesicle recruitment and further to find also other interaction partners of SAP47 to complete the whole pathway involved in olfactory associative learning and memory.

In many regards *Drosophila* larvae are suitable to tackle these questions, and thus to understand exactly how it comes that also a *Drosophila* larva is what it is, because of what it has learnt and what it remembers.

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2. A Yarali, M Krischke, B Michels, **T Saumweber**, MJ Mueller and B Gerber.
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