Towards localizing the Synapsin-dependent olfactory memory trace in the brain of larval *Drosophila*

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Birgit Michels

aus Winterhausen

Würzburg, 2008

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender:

Erster Gutachter:

Zweiter Gutachter:

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Erklärung

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

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

Ich versichere, dass ich diese Dissertation in allen Teilen selbständig angefertigt habe und dazu keine anderen als die angegebenen Hilfsmittel benutzt habe.
Alle aus der Literatur entnommenen Textstellen sind als solche gekennzeichnet.

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

Würzburg, den

Birgit Michels Dr. Bertram Gerber

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 'General Introduction & Discussion' to give an overview of the thesis. Then, Chapter I deals, firstly, with the establishment of an odour-food associative learning paradigm in *Drosophila* larva. This robust assay laid the foundation to, secondly, study the neurogenetics underlying this kind of learning. Thirdly, I focussed on the cellular level, analyzing the cellular site of the Synapsin-dependent memory trace. These two aspects correspond to two publications concerning (1) the establishment and (2) the neurogenetic analysis of odour-food learning in larval *Drosophila*.
Chapter II studies predictive learning of pain-relief in adult fruit flies. It contains one manuscript prepared for publication and one publication *In Press*. These two, respectively, analyse (1) the role for Synapsin in an adult *Drosophila* learning paradigm using odours to be associated with electric shock punishment and (2) the role for the White protein in olfactory learning, in both adults and larvae.

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 the co-authors of each manuscript, whose contributions are explicated below.

Chapter I.1.

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B (2005) The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed Drosophila larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:265-279.

BM was responsible for establishing the olfactory learning paradigm in Wuerzburg. BM performed the larval behaviour experiments together with TH. KK and MBS performed the food uptake assay and a replication of the olfactory learning experiment. RM and FM did the

statistics reported in the Appendix. BM, TH and BG analyzed the data; BG wrote the paper with input from BM and TH.

Chapter I.2.

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B (2005) A role for Synapsin in associative learning: the Drosophila larva as a study case. Learn Mem 12:224-231.

BM performed the molecular and behavioural experiments (except for NMJ immunostainings done by IS). Experiments underlying Fig. 3 were part of my Diploma Thesis. BM and BG analysed the data and wrote the paper.

Chapter I.3.

Michels B, Tanimoto H, Engmann O, Saumweber T, Gerber B. Towards localizing a Synapsin-dependent olfactory memory trace in the brain of larval Drosophila.

BM performed all experiments (except for the mushroom body rescue using the *D52H*-Gal4 strain done by EO) and analysed the data. HT provided Gal4- fly strains. BM and TS prepared the figures. BM and BG wrote the manuscript.

Chapter II.1.

Niewalda, T, Yarali, A., Michels B, Gerber, B. Common involvement of Synapsin in predictive learning about the presence and absence of shock. (under revision)

BM generated and maintained critical fly stocks. TN performed the behavioural experiments. TN, AY and BM performed immunostainings. NT, BM and BG wrote the paper.

Chapter II.2.

Yarali A, Krischke M, Michels B, Saumweber T, Mueller MJ, Gerber B. (in press). Genetic Distortion of the Balance between Punishment and Relief Learning in Drosophila.

AY performed the behavioural experiments and analysed the data concerning adult flies. AY and MK performed the amine measurements and analysed the data. BM and TS performed the behavioural experiment concerning larval *Drosophila* and analyzed the data. AY and BG wrote the paper with input, concerning larval experiments, from BM and TS.

Würzburg, den

Birgit Michels Dr. Bertram Gerber

Table of Contents

Chapter II.

Predictive learning of pain-relief in adult fruit flies

General introduction & discussion

Animals need to adapt and modify their behaviour according to a changing environment. In particular, the ability to learn about rewarding or punishing events is crucial for survival, a process which requires regulated synaptic modification in the brain. A major topic in behavioural neuroscience therefore is to understand how associative plasticity on the synaptic level relates to associative changes in behaviour (Martin et al., 2000). *Drosophila* is an attractive model in this regard as the fruit fly has a relatively simple nervous system with relatively few neurons. Furthermore, transgenic techniques are available in *Drosophila* for behavioural research to block neurotransmission, induce cell death or to temporarily express any gene of interest in a defined set of cells, potentially allowing to bridge the gap between behavior and synaptic plasticity (Phelps and Brand, 1998; Kitamoto, 2001; Sokolowski, 2001; Heisenberg, 2003; Schroll et al., 2006). Additionally, ongoing innovations of techniques keep offering novel approaches, for example RNA interference previously published for *C. elegans*, which is now allowing to knock-down any gene of interest specifically in any genetically defined subset of cells.

The *Drosophila* larvae is a particularly suitable study case in this regard, as its nervous system has ten to a hundred times fewer cells than that of adult flies (e.g. Stocker [2001]; Python and Stocker [2002]), and appr. 10 million times fewer cells than that of humans. Still, despite this reduced cell number, the general layout of the olfactory system appears strikingly similar between larvae and adult (Gerber et al., 2008). Thus, a comprehensive account of olfactory learning, within reach for larval and adult *Drosophila*, may have bearings for our understanding of olfaction and learning in a more general sense.

Establishing an robust olfactory learning regime for Drosophila larvae

Despite this simplicity, *Drosophila* larvae can learn well, both associations between visual stimuli and gustatory reinforcement (Gerber et al., 2004a) and associations between odours and gustatory reinforcement (Dukas, 1999; Scherer et al., 2003).

Thus, the larva provides an attractive system for localizing memory traces at the cellular level. Indeed, Heisenberg et al. (1985) reported that late 3rd larvae with a mutation in the *mushroom body miniature* gene (*mbm*) have a strong defect in a larval version of odour-shock associative learning. However, given problems in replicating such odour-electroshock learning in wild-type larva (Forbes, 1993), we decided to develop a novel, and as it had turned out more robust olfactory learning paradigm, using food as reinforcer.

In Chapter I.1, we established an olfactory learning paradigm for *Drosophila* larvae that was the basis for all larval learning experiments performed in this Thesis. We first confirmed that *Drosophila* larvae are able to detect both the odours and the respective tastants (FRU, NaCl, Quinine) that are used during the behavioural task. We decided to use a reward learning paradigm because (1) *Drosophila* larvae are the main feeding stages in the life cycle of the fly and therefore taste and feeding play a major role and (2) we found that fructose possesses a strong and reliable positive reinforcing effect. These findings then were the basis to venture into a neurogenetics analysis of larval odour-food learning.

Neurogenetics of olfactory associative learning in Drosophila *larvae*

Since three decades learning and memory has been studied in *Drosophila* and various mutants have been found from mutagenesis screens that are impaired in memory formation or retrieval (reviewed in Skoulakis and Grammenoudi [2006]). The cAMP signaling cascade is the most extensively studied aspect that emerged from these screens: the learning mutant *rutabaga* affects a gene encoding a Ca^{2+}/ Calmodulin sensitive adenylyl cyclase (AC) which conceivably acts as molecular coincidence detector of conditioned and reinforcing stimulus processing (Dudai et al., 1988; Abrams et al., 1998). The AC is a doubly-regulated enzyme that is substantially activated only if both the conditioned stimulus signal drives the neuron in question activating the AC via Ca^{2+}/ Calmodulin and second the reinforcement signal gives input to the same neuron activating the AC via a G-protein coupled receptor (Abrams et al., 1991); interestingly, effective stimulation of the AC is only reached when activation via Ca^{2+}/ Calmodulin precedes activation via the G-protein which is in accordance with the temporal pairing requirements in classical conditioning (Abrams et al., 1998). The AC then activates a cAMP-dependent protein kinase (PKA) and PKA phosophorylates several target proteins– most of them being currently unknown– that ultimately mediate changes in neurotransmitter release. Consistent with this model, interfering with any component of the cAMP cascade results in learning impairments: *rutabaga* (AC) mutants show defects in a number of learning paradigms (Tully and Quinn, 1985; Zars et al., 2000); expressing a constitutively active form of a G-protein leads to learning impairments (Connolly et al., 1996) and flies that lack the catalytic or a regulatory subunit of PKA show decreased learning performance (Skoulakis et al., 1993; Goodwin et al., 1997). Consistently, also in mammals the cAMP/ protein kinase A (PKA) signaling cascade is critical for initial memory formation of learned fear (Byrne and Kandel, 1996; Abel et al., 1997; Siwicki and Ladewski, 2003). Furthermore besides the role

for PKA required for the acquisition of fear memory, it was also shown that PKA is a constraint for extinction of fear (Isiegas et al., 2006).

Besides components of the cAMP cascade several other learning mutants have been described (reviewed in Skoulakis and Grammenoudi [2006]). Recently a synaptic vesicle associated proteins has been found to be involved in learning and memory of adult *Drosophila*. Flies lacking Synapsin show reduced learning scores in odour-electroshock associative learning (Godenschwege et al., 2004). Synapsins are abundant phosphoproteins that are associated with synaptic vesicles (Hilfiker et al., 1999) for a controversial view see (Gaffield and Betz, 2007). They are highly conserved throughout the animal kingdom and share a common domain structure (Hilfiker et al., 1999; Hilfiker et al., 2005). In vertebrates three synapsin genes are found, whereas in invertebrates Synapsin is encoded by a single gene (Klagges et al., 1996; Hilfiker et al., 1999). Almost all Synapsins investigated so far share domains A, C and E, pointing to a conserved function mediated by these domains (Kao et al., 1999). In terms of molecular function, Synapsin is supposed to be involved in the regulation of synaptic transmission via tethering synaptic vesicles to the actin cytoskeleton in a phosphorylation-dependent manner and therefore regulating the number of vesicles available for release (Hosaka et al., 1999; Chi et al., 2001; Menegon et al., 2006; Gitler et al., 2008). Indeed, studies in mice showed that if Synapsin function is compromised, synaptic output per se remains functional, whereas the ability to maintain synaptic output at high, sustained spiking rates is compromised (Chi et al., 2003; Gitler et al., 2004). However, despite much work on the cellular, molecular, developmental, and physiological levels (Angers et al. [2002]; Ferreira and Rapoport [2002]; Chi et al. [2003]; Gitler et al. [2004]; Hilfiker et al. [2005] for reviews see Hilfiker et al. [1999] and Sudhof [2004]), the functional significance of Synapsin for behavior remains less well understood. In humans, Garcia et al. (2004) recently found that a mutation in the *synapsin I* gene causes severe neurological and behavioral phenotypes, including epilepsy and learning impairments. In the mouse, Silva et al. (1996) found learning impairments in *synapsin II*, but not *synapsin I* knockout mice; these results correlated with decreased post-tetanic potentiation in *synapsin II*, but not *synapsin I* mutants. In mice lacking all three *synapsin* genes, Gitler et al. (2004) documented that such triple mutants show delayed responses in a number of tested reflexes and diminished ability to hang from a suspended wire; they also noted that these animals show seizures upon disturbance by opening of the cage, reduced levels of piloerection, and difficulties maintaining balance when the cage is shaken. Importantly for the current context, Gitler et al. (2004) reported that in a

test for spatial memory in an eight-arm radial maze, these animals performed poorly; reportedly, this phenotype is not due to deficits in motivation or motor ability.
Given these previous findings, I asked in my Thesis whether Synapsin contributes to learning in *Drosophila* larva, and if so, where in the brain Synapsin may act to support such learning.

Memory trace localization

Also in humans much investigation on localizing memory traces is ongoing. Surgical, pharmacological and genetic lesion studies have revealed distinct anatomical sites involved with different forms of learning. Such 'maps' of a division of labour in the human brain are largely based on studies of patients with localized brain damage and on work in rodent model systems; for example, it was shown that the hippocampal formation participates in acquisition of declarative tasks but is not the site of their long-term storage (Milner et al., 1998; Scoville and Milner, 2000). However, a big disadvantage remains that in humans the patients´ 'natural' lesions are often ill-defined, individually unique, rare and also mostly irreversible, which has limited their use for dissecting the temporal processes of memories.
In flies, a distinct region of the brain, the mushroom body, likely houses an olfactory memory trace; this notion is based on the genetic repertoire available in *Drosophila* (for more detail see Wilson and Collier [1992]; Kitamoto [2001, 2002]; Martin et al. [2002]; McGuire et al. [2003]; Hay et al. [2004]). These methods also are the basis for analyses as to whether also in flies a division of labour exists between parts of the brain in terms of their roles in different kinds of learning task. Indeed, it turned out that whereas olfactory memory traces can be localized to the mushroom bodies, visual memory traces established in the flight simulator (Liu et al., 2006) are associated with the central complex, and place memory studied by heat box experiments (Zars et al., 2000) in the median bundle. Thus, as discussed in Heisenberg and Gerber (2008), the adult fly brain does seem to be functionally compartementalized in terms of a map of different memory traces in different brain regions. Is the same the case for larval *Drosophila*?
Larval *Drosophila* show visual (Gerber et al., 2004a) as well as olfactory learning using tastants as reinforcer (either sugars as reward, or high-salt or quinine as punishment, respectively) (Scherer et al., 2003; Hendel et al., 2005; Honjo and Furukubo-Tokunaga, 2005; Neuser et al., 2005; Niewalda et al., 2008; Schipanski et al., 2008). While at present nothing is known about the neurogenetics of visual learning (making it at present impossible to address the question of division of labour among regions of the larval brain), regarding odour-sugar learning, two studies speak towards a possible site of the memory trace: Firstly, Kaun et al.

(2007) investigated the *foraging* gene. This gene encodes a cGMP-dependent protein kinase (PKG) which is less expressed in sitters in contrast to rover flies (Osborne et al., 1997). Notably, the reduced learning scores in sitters can be increased to rover levels by boosting expression of PKG in the mushroom bodies. However, secondly, it was reported that the learning defect seen upon deletion of the *neurexin* gene ($Nrx\text{-}1^{\Delta 83}$; Zeng et al. [2007]) cannot be restored by Neurexin-1 expression in the mushroom bodies. Thus, with regard to memory trace localization in larval odour-sugar learning, we largely remain at sea.

In addition to these studies, Honjo and Furukubo-Tokunaga (2005) used a nonreciprocal training design, that makes the interpretation of the findings complicated as learning indices reflect not only the difference between reciprocally trained groups, but might be influenced by habituation, adaptation, changes in motivation, and changes in the concentration of the odors.

Given this relatively incomplete knowledge of the neurogenetics of larval learning, within this Thesis I venture into the first comprehensive account to localize the cellular site of odour-reward associative memory trace in larval *Drosophila*. I decided to use the presynaptic protein Synapsin for this effort. I found that acute pan-neural expression of Synapsin and constitutive local expression in only the mushroom body fully restore learning, whereas no such restoration of learning is found by Synapsin expression throughout large parts of the brain excluding the mushroom bodies, or by Synapsin expression in specifically the projection neurons. Thus, in order for learning to be restored, Synapsin expression in the mushroom bodies is evidently sufficient and likely necessary within the mushroom bodies of larval *Drosophila*.

To independently test for the local requirement of Synaspin, I produced transgenic flies that knock-down Synapsin by means of RNAi. Indeed, by a pan-neuronal knock-down of Synapsin protein the experimental larvae perform poorly in comparison to wild-type larvae in the learning task. This finding is consistent with the results of testing the deletion mutant syn^{97} in the larval learning regime. Thus, these flies can be used for further research to narrow down the necessity of Synapsin in the larval brain during larval olfactory learning.

Common involvement of Synapsin in predictive learning about the presence and absence of shock in adult flies

Given this role of Synapsin in larval odour-food learning, and in particular given that adult Synapsin null mutant *Drosophila* show an impairment in odour-shock associative learning (Godenschwege et al. [2004]; Michels et al. [2005]; see above), I took part in a study to ask

whether Synapsin plays a role for olfactory relief learning in the adult fly as well (Tanimoto et al., 2004; Yarali et al., 2006). We exploited the fact that an odour is avoided after odour _ shock training because it predicts shock, whereas after shock _ odour training the odour is approached because it signals no-shock. We show that a lack of Synapsin leads to reduced learning about both *shock*-predicting and *no-shock* predicting stimuli. These are the first results, in any experimental system, concerning how these antagonistic behavioural consequences of shock come about molecularly. In the next step we will test whether knocking down Synapsin by RNAi also causes a similar impairment of associative learning. To get insights where in the brain Synapsin expression is sufficient to rescue *shock* and *no-shock* learning, Synapsin will be expressed in different brain areas of interest. Taking into consideration that all transgenic flies have a mutation in the *white* gene because of the way these animals are generated (see below), this might lead to different amounts of White protein levels between different genotypes. We therefore asked whether a mutation in *white* gene causes leaning phenotypes in either *shock* or *no-shock* learning. It turned out that upon the loss of white-function, the balance between these two kinds of learning is distorted in favor of punishment learning: White mutants show stronger punishment learning and weaker relief learning, as compared to wild type flies. This prompted me to search for a putative effect of mutations in the *white* gene also in the larva. Such a phenotype, though, is not apparent. This is important for practical reasons: Transgenes typically carry a 'marker' mutation in the *white* gene. This 'marker' mutation causes white eye colour as compared to the wild-typeish red eye colour; fly transgenes therefore include a mini-*white* rescue gene (supporting red eye colour), such that loss of the construct would show by white eyes. In order to express any gene of interested in the flies one therefore has to cross two transgenic fly strains (each possessing one copy of the mini-white rescue gene). Such crossing this leads to progeny with two copies of *mini*-white rescue genes (one from each parental strain) whereas the corresponding controls do only posses one copy (from female or male parental strain, respectively). If one tests these animals for learning and the rescue flies perform well such a finding has to be interpreted carefully: On the one hand this result could be interpreted by the sufficiency of the protein of interest. On the other hand, it may rather be the higher levels of White protein in the rescued flies which endow these flies with increased learning ability. While such effects, obviously, have to be reckoned with in adult flies (see above), given the lack of effect of loss of *white* function in the larva, however, the experiments using the various transgenic constructs used for localizing the Synapsin-dependent memory trace can be interpreted without reference to *white* function.

OUTLOOK INTO MOLECULAR NETWORK

As I have shown that Synapsin is required for larval odour-food learning, and that the underlying memory trace likely is located in the mushroom bodies, the question of the molecular mechanism of Synapsin function emerged. Given that *Drosophila* Synapsin shows phosphorylation consensus sites for PKA, I asked whether Synapsin might be one of the target proteins of PKA and thus mediate its effects via the cAMP cascade. This is a question of somewhat broader interest: While the molecular mechanisms of coincidence detection by the type I adenylyl cyclase during training are well known and appear widely conserved across the animal kingdom (Dudai et al., 1988; Abrams et al., 1998; Renger et al., 2000), the question what the downstream processes are that allow more transmitter being released at the moment of testing remain a challenge. Indeed as argued above, it seems conceivable that the PKA consensus site(s) of Synapsin are required for reserve-pool vesicle recruitment: Fiumara et al. (2004) examined the substrate properties of *Aplysia* Synapsin. *Ap*Synapsin was found to be an excellent in vitro substrate for cAMP dependent protein kinase, which phosphorylated it at high stoichiometry on a single site (Ser-9) in the evolutionarily conserved A-domain. However, other kinases reported to phosphorylate mammalian Synapsins, phosphorylated *Ap*Synapsin to a much lesser extent. Therefore, I asked whether the phosphorylation consensus sites for cAMP-dependent protein kinase A (PKA) of *Drosophila* Synapsin are required to support learning. To this end, we have produced transgenic flies which express only a mutated version of the Synapsin protein with not phosphorylatable PKA sites. As these flies are in an otherwise synapsin-mutant background they role for phosphorylation of the Synapsin protein is feasible to be investigated in *Drosophila* olfactory learning.

Interestingly, it has been shown that the evolutionarily conserved PKA-1 phosphorylation consensus site undergoes ADAR-dependent RNA editing, thereby changing the consensus motif from R-R-F-S to R-G-F-S (Diegelmann et al., 2006b). This prompted me to ask for the role of this RNA editing, in particular with regard to learning and memory. Therefore, together with Jana Husse in her Diploma thesis, I aimed to generate transgenic flies that express an altered form of Synapsin protein with a non-edited PKA-1 under the control of an upstream activating sequence (UAS). We successfully generated these flies and provided evidence for PKA-1 being in the non-edited form or non-phosporylateable form, respectively, on both the genomic and the mRNA level. Furthermore, after crossing to a Gal4 driver strain, these UAS-*synapsin* flies express the altered Synapsin protein to a level comparable to Synapsin expression levels in wild-type flies. These transgenic flies can now be used for a number of experiments to elucidate the role of the RNA editing of *Drosophila synapsin*: on

the biochemical level phosphorylation assays can be performed and - most fascinating - on the behavioural level these flies will be tested for their learning performance.

REFERENCES

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88:615-626.

Abrams TW, Karl KA, Kandel ER (1991) Biochemical studies of stimulus convergence during classical conditioning in Aplysia: dual regulation of adenylate cyclase by Ca2+/calmodulin and transmitter. J Neurosci 11:2655-2665.

Abrams TW, Yovell Y, Onyike CU, Cohen JE, Jarrard HE (1998) Analysis of sequence-dependent interactions between transient calcium and transmitter stimuli in activating adenylyl cyclase in Aplysia: possible contribution to CS--US sequence requirement during conditioning. Learn Mem 4:496-509.

Angers A, Fioravante D, Chin J, Cleary LJ, Bean AJ, Byrne JH (2002) Serotonin stimulates phosphorylation of Aplysia synapsin and alters its subcellular distribution in sensory neurons. J Neurosci 22:5412-5422.

Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425-435.

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4:1187-1193.

Chi P, Greengard P, Ryan TA (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. Neuron 38:69-78.

Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ (1996) Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 274:2104-2107.

Diegelmann S, Nieratschker V, Werner U, Hoppe J, Zars T, Buchner E (2006b) The conserved protein kinase-A target motif in synapsin of Drosophila is effectively modified by pre-mRNA editing. BMC Neurosci 7:76.

Dudai Y, Corfas G, Hazvi S (1988) What is the possible contribution of Ca2+-stimulated adenylate cyclase to acquisition, consolidation and retention of an associative olfactory memory in Drosophila. J Comp Physiol [A] 162:101-109.

Dukas R (1999) Costs of memory: ideas and predictions. J Theor Biol 197:41-50.

Ferreira A, Rapoport M (2002) The synapsins: beyond the regulation of neurotransmitter release. Cell Mol Life Sci 59:589-595.

Fiumara F, Giovedi S, Menegon A, Milanese C, Merlo D, Montarolo PG, Valtorta F, Benfenati F, Ghirardi M (2004) Phosphorylation by cAMP-dependent protein kinase is essential for synapsin-induced enhancement of neurotransmitter release in invertebrate neurons. J Cell Sci 117:5145-5154.

Forbes B (1993) Larval learning amd memory in Drosophila melanogaster [Diploma Thesis]. University of Würzburg, Department of Genetics and Neurobiology.

Gaffield MA, Betz WJ (2007) Synaptic vesicle mobility in mouse motor nerve terminals with and without synapsin. J Neurosci 27:13691-13700.

Garcia CC, Blair HJ, Seager M, Coulthard A, Tennant S, Buddles M, Curtis A, Goodship JA (2004) Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy. J Med Genet 41:183-186.

Gerber B, Stocker RF, Tanimura T, Thum AS (2008) Smelling, Tasting, Learning: Drosophila as a Study Case. Springer-Verlag Berlin Heidelberg.

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, Heisenberg M (2004a) Visual learning in individually assayed Drosophila larvae. J Exp Biol 207:179-188.

Gitler D, Cheng Q, Greengard P, Augustine GJ (2008) Synapsin IIa controls the reserve pool of glutamatergic synaptic vesicles. J Neurosci 28:10835-10843.

Gitler D, Takagishi Y, Feng J, Ren Y, Rodriguiz RM, Wetsel WC, Greengard P, Augustine GJ (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. J Neurosci 24:11368-11380.

Godenschwege TA, Reisch D, Diegelmann S, Eberle K, Funk N, Heisenberg M, Hoppe V, Hoppe J, Klagges BR, Martin JR, Nikitina EA, Putz G, Reifegerste R, Reisch N, Rister J, Schaupp M, Scholz H, Schwarzel M, Werner U, Zars TD, Buchner S, Buchner E (2004) Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. Eur J Neurosci 20:611-622.

Goodwin SF, Del Vecchio M, Velinzon K, Hogel C, Russell SR, Tully T, Kaiser K (1997) Defective learning in mutants of the Drosophila gene for a regulatory subunit of cAMP-dependent protein kinase. J Neurosci 17:8817-8827.

Hay BA, Huh JR, Guo M (2004) The genetics of cell death: approaches, insights and opportunities in Drosophila. Nat Rev Genet 5:911-922.

Heisenberg M (2003) Mushroom body memoir: from maps to models. Nat Rev Neurosci 4:266-275.

Heisenberg M, Gerber B (2008) Behavioral Analysis of Learning and Memory in Drosophila. InR Menzel (Ed), Learning Theory and Behavior Vol [1] of Learning and Memory: A Comprehensive Reference, 4 vols (JByrne Editor), pp [549-560] Oxford: Elsevier.

Heisenberg M, Borst A, Wagner S, Byers D (1985) Drosophila mushroom body mutants are deficient in olfactory learning. J Neurogenet 2:1-30.

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B (2005) The carrot, not the stick: appetitive rather than

aversive gustatory stimuli support associative olfactory learning in individually assayed Drosophila larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:265-279.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354:269-279.

Hilfiker S, Benfenati F, Doussau F, Nairn AC, Czernik AJ, Augustine GJ, Greengard P (2005) Structural domains involved in the regulation of transmitter release by synapsins. J Neurosci 25:2658-2669.

Honjo K, Furukubo-Tokunaga K (2005) Induction of cAMP response element-binding protein-dependent medium-term memory by appetitive gustatory reinforcement in Drosophila larvae. J Neurosci 25:7905-7913.

Hosaka M, Hammer RE, Sudhof TC (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. Neuron 24:377-387.

Isiegas C, Park A, Kandel ER, Abel T, Lattal KM (2006) Transgenic inhibition of neuronal protein kinase A activity facilitates fear extinction. J Neurosci 26:12700-12707.

Kao HT, Porton B, Hilfiker S, Stefani G, Pieribone VA, DeSalle R, Greengard P (1999) Molecular evolution of the synapsin gene family. J Exp Zool 285:360-377.

Kaun KR, Hendel T, Gerber B, Sokolowski MB (2007) Natural variation in Drosophila larval reward learning and memory due to a cGMP-dependent protein kinase. Learn Mem 14:342-349.

Kitamoto T (2001) Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol 47:81-92.

Kitamoto T (2002) Targeted expression of temperature-sensitive dynamin to study neural mechanisms of complex behavior in Drosophila. J Neurogenet 16:205-228.

Klagges BR, Heimbeck G, Godenschwege TA, Hofbauer A, Pflugfelder GO, Reifegerste R, Reisch D, Schaupp M, Buchner S, Buchner E (1996) Invertebrate synapsins: a single gene codes for several isoforms in Drosophila. J Neurosci 16:3154-3165.

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L (2006) Distinct memory traces for two visual features in the Drosophila brain. Nature 439:551-556.

Martin JR, Keller A, Sweeney ST (2002) Targeted expression of tetanus toxin: a new tool to study the neurobiology of behavior. Adv Genet 47:1-47.

Martin KC, Barad M, Kandel ER (2000) Local protein synthesis and its role in synapse-specific plasticity. Curr Opin Neurobiol 10:587-592.

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003) Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302:1765-1768.

Menegon A, Bonanomi D, Albertinazzi C, Lotti F, Ferrari G, Kao HT, Benfenati F, Baldelli P, Valtorta F (2006) Protein kinase A-mediated synapsin I phosphorylation is a central modulator of Ca2+-dependent synaptic activity. J Neurosci 26:11670-11681.

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B (2005) A role for Synapsin in associative learning: the Drosophila larva as a study case. Learn Mem 12:224-231.

Milner B, Squire LR, Kandel ER (1998) Cognitive neuroscience and the study of memory. Neuron 20:445-468.

Neuser K, Husse J, Stock P, Gerber B (2005) Appetitive olfactory learning in Drosophila larvae: effects of repetition, reward strength, age, gender, assay type and memory span. Animal behaviour 69:891-898.

Niewalda T, Singhal N, Fiala A, Saumweber T, Wegener S, Gerber B (2008) Salt Processing in Larval Drosophila: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way. Chem Senses.

Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Coulthard A, Pereira HS, Greenspan RJ, Sokolowski MB (1997) Natural behavior polymorphism due to a cGMP-dependent protein kinase of Drosophila. Science 277:834-836.

Phelps CB, Brand AH (1998) Ectopic gene expression in Drosophila using GAL4 system. Methods 14:367-379.

Python F, Stocker RF (2002) Adult-like complexity of the larval antennal lobe of D. melanogaster despite markedly low numbers of odorant receptor neurons. J Comp Neurol 445:374-387.

Renger JJ, Ueda A, Atwood HL, Govind CK, Wu CF (2000) Role of cAMP cascade in synaptic stability and plasticity: ultrastructural and physiological analyses of individual synaptic boutons in Drosophila memory mutants. J Neurosci 20:3980-3992.

Scherer S, Stocker RF, Gerber B (2003) Olfactory learning in individually assayed Drosophila larvae. Learn Mem 10:217-225.

Schipanski A, Yarali A, Niewalda T, Gerber B (2008) Behavioral analyses of sugar processing in choice, feeding, and learning in larval Drosophila. Chem Senses 33:563-573.

Schroll C, Riemensperger T, Bucher D, Ehmer J, Voller T, Erbguth K, Gerber B, Hendel T, Nagel G, Buchner E, Fiala A (2006) Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. Curr Biol 16:1741-1747.

Scoville WB, Milner B (2000) Loss of recent memory after bilateral hippocampal lesions. 1957. J Neuropsychiatry Clin Neurosci 12:103-113.

Silva AJ, Rosahl TW, Chapman PF, Marowitz Z, Friedman E, Frankland PW, Cestari V, Cioffi D, Sudhof TC, Bourtchuladze R (1996) Impaired learning in mice with abnormal short-lived plasticity. Curr Biol 6:1509-1518.

Siwicki KK, Ladewski L (2003) Associative learning and memory in Drosophila: beyond olfactory conditioning. Behav Processes 64:225-238.

Skoulakis EM, Grammenoudi S (2006) Dunces and da Vincis: the genetics of learning and memory in Drosophila. Cell Mol Life Sci 63:975-988.

Skoulakis EM, Kalderon D, Davis RL (1993) Preferential expression in mushroom bodies of the catalytic subunit of protein kinase A and its role in learning and memory. Neuron 11:197-208.

Sokolowski MB (2001) Drosophila: genetics meets behaviour. Nat Rev Genet 2:879-890.

Stocker RF (2001) Drosophila as a focus in olfactory research: mapping of olfactory sensilla by fine structure, odor specificity, odorant receptor expression, and central connectivity. Microsc Res Tech 55:284-296.

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.

Tanimoto H, Heisenberg M, Gerber B (2004) Experimental psychology: event timing turns punishment to reward. Nature 430:983.

Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol [A] 157:263-277.

Wilson BA, Collier RJ (1992) Diphtheria toxin and Pseudomonas aeruginosa exotoxin A: active-site structure and enzymic mechanism. Curr Top Microbiol Immunol 175:27-41.

Yarali A, Hendel T, Gerber B (2006) Olfactory learning and behaviour are 'insulated' against visual processing in larval Drosophila. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 192:1133-1145.

Zars T, Wolf R, Davis R, Heisenberg M (2000) Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: in search of the engram. Learn Mem 7:18-31.

Zeng X, Sun M, Liu L, Chen F, Wei L, Xie W (2007) Neurexin-1 is required for synapse formation and larvae associative learning in Drosophila. FEBS Lett 581:2509-2516.

Chapter I.

I.1. Establishing an robust olfactory learning regime for *Drosophila* larvae

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B

J Comp Physiol A (2005) 191: 265–279
DOI 10.1007/s00359-004-0574-8

ORIGINAL PAPER

**Thomas Hendel · Birgit Michels · Kirsa Neuser
Angela Schipanski · Karla Kaun · Marla B. Sokolowski
Frank Marohn · René Michel · Martin Heisenberg
Bertram Gerber**

The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larvae

Received: 7 January 2004 / Revised: 1 October 2004 / Accepted: 2 October 2004 / Published online: 19 January 2005

Abstract The ability to learn is universal among animals; we investigate associative learning between odors and "tastants" in larval *Drosophila melanogaster*. As biologically important gustatory stimuli, like sugars, salts, or bitter substances have many behavioral functions, we investigate not only their reinforcing function, but also their response-modulating and response-releasing function. Concerning the response-releasing function, larvae are attracted by fructose and repelled by sodium chloride and quinine; also, fructose increases, but salt and quinine suppress feeding. However, none of these stimuli has a nonassociative, modulatory effect on olfactory choice behavior. Finally, only fructose but neither salt nor quinine has a reinforcing effect in associative olfactory learning. This implies that the response-releasing, response-modulating and reinforcing functions of these tastants are dissociated on the behavioral level. These results open the door to analyze how this dissociation is brought about on the cellular and molecular level; this should be facilitated by the cellular simplicity and genetic accessibility of the *Drosophila* larva.

T. Hendel · B. Michels · K. Neuser · A. Schipanski
M. Heisenberg · B. Gerber (✉)
Department of Genetics and Neurobiology,
University of Würzburg, Biozentrum,
Am Hubland, 97074 Würzburg, Germany
E-mail: bertram.gerber@biozentrum.uni-wuerzburg.de
Tel.: +49-931-8884483
Fax: +49-931-8884452

K. Kaun · M. B. Sokolowski
Department of Biology, University of Toronto,
3359 Mississauga Rd, North, Mississauga,
ON L5L 1C6, Canada

F. Marohn · R. Michel
Institute of Applied Mathematics and Statistics,
University of Würzburg, Am Hubland,
97074 Würzburg, Germany

Keywords *Drosophila* larva · Learning · Olfaction · Taste · Reinforcement

Abbreviations AM: Amylacetate · EMP: Empty · FRU: Fructose · LI: Learning index · NaCl: Sodium chloride · OCT: 1-octanol · PREF: Preference · QUI: Quinine hemisulfate · SOL: Solvent · +: Positive reinforcement · −: Negative reinforcement

Introduction

Associative plasticity is a fundamental feature of behavior. We chose to characterize associative learning between odors and "tastants" in larval *Drosophila melanogaster*. These animals offer a fortunate combination of learning ability (Aceves-Pina and Quinn 1979; Heisenberg et al. 1985; Tully et al. 1994; Dukas 1998; Scherer et al. 2003; Gerber et al. 2004) and cellular simplicity. As the chemosensory system of the larva has recently become the focus of intense investigations on the molecular and cellular level (Cobb 1999; Heimbeck et al. 1999; Scott et al. 2001; Python and Stocker 2002; Gendre et al. 2003; Liu et al. 2003), and as the larval neuromuscular junction is a much-used preparation for the study of synaptic plasticity (Koh et al. 2000), our research on the behavioral level might be a helpful contribution towards a multi-level understanding of associative plasticity.

Beyond their potential to support olfactory learning, it is clear that gustatory stimuli play many different roles in the biology of animals. To analyze these behavioral functions, it seems useful to choose an organism for which gustation and feeding play a particularly prominent role. Larvae of the fruit fly *Drosophila* meet this demand as they are the main feeding stages in the life cycle of the fly. We chose to

investigate the behavioral functions of fructose (FRU), sodium chloride (NaCl) and quinine hemisulfate (QUI) in this animal. We did so with respect to three of their potential functions: (1) response-releasing, (2) response-modulating, and (3) reinforcing function (we use the term "reinforcer" synonymous with unconditioned stimulus).

To study the reinforcing function of gustatory stimuli in the *Drosophila* larva, we used the olfactory learning paradigm of Scherer et al. (2003). For that paradigm, larvae were exposed to one odorant, for example amylacetate, in the presence of FRU (+), and to a second odorant, for example 1-octanol, in the presence of NaCl (−) (AM+/OCT−). A second group of animals was trained reciprocally (AM−/OCT+). In a subsequent test, individual animals were given a choice between AM and OCT. Associative learning was shown by higher preferences for AM after AM+/OCT− training than after the reciprocal AM−/OCT+ training. In these experiments, the authors always used two potential reinforcers in a differential conditioning procedure, one presumably appetitive (FRU), the other presumably aversive (either NaCl or QUI). It was found that both combinations of stimuli (i.e. FRU-NaCl, and FRU-QUI) support learning. It remained unclear, however, whether FRU, NaCl or QUI alone would effectively support learning and would thus qualify as reinforcers. This issue is addressed in the current study, which uses either FRU, or NaCl or QUI alone as reinforcers. As only one reinforcer is used and the alternative odor is presented simply without any such overt reinforcer, we call this procedure " absolute" conditioning.

To study a potential nonassociative, response-modulating effect of FRU, NaCl, or QUI on olfactory choice behavior, we tested whether olfactory choice is altered when tested in the presence of either of these stimuli. It was previously shown (Scherer 2002) that presentation of NaCl immediately before testing did not have any effect on olfactory choice. That is, no evidence for a nonassociative, sensitization-like effect was found; therefore, in this study we chose a yet more rigid test and assayed olfactory choice in the presence of either FRU, NaCl, or QUI to see whether these substances modulate olfactory choice.

To study the response-releasing function of these three gustatory substances, we tested the gustatory choice of the larvae between two substrates. One substrate was plain agarose (PURE), whereas the other, in addition, contained either FRU, NaCl, or QUI. We also tested whether any of these substances induces or suppresses feeding behavior.

We found that FRU supports appetitive and NaCl and QUI aversive responses. However, none of these stimuli has a response-modulating, nonassociative effect on olfactory choice. Finally, only FRU but not NaCl and not QUI has an apparent reinforcing effect.

Methods

General

In all cases, experimenters were blind with respect to the experimental conditions (reinforcer presence and identity), which were decoded only after the experiment. Also, the experimental groups to be statistically compared were run in strict temporal parallelity to avoid false positive differences between groups which could result from variations over time as they are typical for behavior in invertebrates. All statistical analyses were performed with Statistica 6.0 for PC and/or StatView 4.51 for the Macintosh (significance level: $P < 0.05$).

We used third instar feeding stage larvae aged 5 days (±12 h) after egg lay. Flies of the Canton-S wild-type strain were used and kept in mass culture, maintained at 25°C, 60–70% relative humidity and a 14/10 h light/dark cycle (12/12 h for Figs. 3, 5). Experiments were performed in red light under a fume hood at 20–24°C room temperature.

Petri dishes (Sarstedt, D) of 90-mm inner diameter were used throughout, unless stated otherwise. These were filled with 1% freshly boiled aqueous agarose solution (electrophoresis grade; Roth, D), which was then allowed to solidify, covered with their lids, and left untreated at room temperature until the following day. As potentially reinforcing "tastant" stimuli, we used fructose, (FRU, purity: 99%, Sigma–Aldrich, D), sodium chloride (NaCl, purity 99.5%, Fluka, D) or quinine hemisulfate (QUI, purity 92%, Sigma–Aldrich, D) added to the agarose solution after boiling. We used 2 mol FRU or 4 mol NaCl, respectively, dissolved in 1 l of water; for QUI, we used 2 g dissolved in 1 l to obtain a 0.2% w/w solution. These values are at the upper limit for NaCl and QUI, avoiding crystallization in the petri dishes.

Immediately before experiments, we replaced the regular lids of the petri dishes with lids perforated in the center by 15 1-mm holes to improve aeration. For experiments, a spoonful of food medium containing larvae was taken from the food bottle and transferred to a glass vial. From there, larvae were picked on demand, briefly washed, and transferred into the middle of the petri dish for the start of experiments.

In our choice of olfactory stimuli, we followed Scherer et al. (2003) and used 1-octanol (OCT, purity: 99.5%; Sigma-Aldrich, D) and amyl acetate (AM, purity: 99%, diluted 1:50 in paraffin oil; Sigma-Aldrich, D); at these concentrations, we expected naïve animals to show about equal distribution between the two odors in a choice assay (Scherer et al. 2003; Fig. 1). Odorants were applied by adding 10 µl of odor substance into Teflon containers (inner diameter 5 mm) which could be closed by a perforated lid (seven holes, 0.5-mm diameter). These containers were then placed onto the agarose surface in the petri dishes.

Detectability of odors

To test for odorant detectability, we performed olfactory choice tests (for a sketch see insets of Fig. 1). Individual larvae were put into the middle of petri dishes containing pure agarose and two odorant containers on opposite sides, approximately 7 mm from the edges. The content of the containers differed on both sides to achieve a choice situation. Specifically, we compared choices between the sides equipped with an empty (EMP) container versus a container filled with AM dissolved in paraffin, a container with that solvent (SOL) versus OCT, with SOL versus EMP or with AM versus OCT. Larvae were allowed to move freely on the test plate and their position (defined by the mouth hooks) was scored every 20 s for 5 min as "AM", "OCT", or "neutral" (a 7-mm-wide zone in the middle of the assay plate). On these data, we calculated an odor preference for each animal. We determined the number of times a given animal was observed on the AM side during the test minus the number of times that animal was observed on the OCT side, divided by the total number of observations (if experiments involved EMP or SOL, calculations were done analogously):

$$\mathrm{PREF} = \frac{\text{number of observations}_{\mathrm{AM}} - \text{number of observations}_{\mathrm{OCT}}}{\text{number of observations}_{\mathrm{Total}}} \quad (1)$$

Thus, positive values indicate a preference of that animal for AM, and negative values a preference for OCT. These data were tested against random by one-sample sign tests and compared between groups with Mann-Whitney *U*-tests.

Response-releasing function of "tastants": testing avoidance and approach

To test the response-releasing functions of FRU, NaCl and QUI, we performed gustatory choice assays. Larvae could choose between two substrates, one consisting of pure agarose (PURE), and one of agarose with a "tastant" added (for a sketch see insets of Fig. 2). Petri dishes of 52-mm inner diameter were equipped with a vertical barrier in the middle. These barriers were made from overhead transparencies and fixed to the plates with small stripes of tape. Parafilm was used to tighten the barrier. Then, the respective agarose solutions were gently poured into either side of the split petri dish to yield the desired combination of substrates on either side. Shortly before the substances solidified, the barriers were torn out. This procedure leads to a smooth yet sharp border between sides. After 20 min of cooling, the plates were covered with their standard lids and left at room temperature over night to be used for experiments on the next day. As this preparation of plates is rather laborious, we chose to use an en masse assay to determine gustatory choice. Groups of ten animals were put into the middle of the plate. Then animals could move about the plate for 15 min until we determined the number of animals located on either the "tastant" side or the PURE side. Animals that dug into the agarose or crawled up the lids of the plates were not considered in data analysis. A preference index was calculated as

$$\mathrm{PREF} = \frac{\text{number of animals}_{\mathrm{TASTANT}} - \text{number of animals}_{\mathrm{PURE}}}{\text{number of animals}_{\mathrm{Total}}} \quad (2)$$

Thus, positive values indicate attraction to the "tastant" while negative values indicate repulsion; to statistically analyze these data between tastants, Kruskal–Wallis and Mann–Whitney *U*-tests were used; to test the preference values against random levels, one-sample sign tests were used. We chose to allow 15 min for choice, as in mass assays larvae typically remain in a clump for a while before dispersing (Gordesky–Gold et al. 1996).

Response-releasing function of "tastants": testing for an influence on feeding

To test for an influence of the "tastants" on feeding, carmine red (BDH Chemical Ltd., distributed via VWR International, Mississauga, Ontario, Canada) was added to the agarose, which upon feeding leads to staining of larval guts. We dissolved 200 mg carmine red powder in 200 µl distilled water and added to 100 ml hot 1% agarose solution, leading to a 0.2% final concentration of carmine red in the agarose; then, "tastants", either 2 M FRU, 4 M NaCl, or 0.2% QUI, were stirred into the hot agarose solution. Pure, dyed agarose plates were used as reference. Groups of ten larvae were placed onto such dyed agar plates. After 15 min of feeding, larvae were removed from the petri dishes, gently rinsed and placed in 70–90°C distilled water for approximately 20 s to achieve full body extension. They were then placed ventral side up on a small petri dish and digital images were taken. For each animal, the percent area of food intake was measured

by counting the number of red-dyed pixels in the gut (see Fig. 3) and dividing this by the number of pixels of the entire body. This measure is used to estimate the amount of dyed agarose swallowed. Statistical analyses of these data were done between "tastant" conditions with Kruskal–Wallis and Mann–Whitney *U*-tests. To compare feeding levels to zero, we used one-sample sign tests. We analyzed the same data also after classifying larvae as either "eaters" or "noneaters" and compared their frequencies by χ^2 tests.

Response-modulating function

To test for an influence of "tastants" on olfactory preferences, we performed olfactory choice tests as described above, but in the presence of the "tastants" (FRU, NaCl, QUI) (for a sketch see insets of Fig. 4); olfactory preferences were measured on pure agarose for comparison. Olfactory preferences were calculated as in Eq. 1; any difference in these preference data between PURE versus FRU, NaCl, or QUI as determined in a Kruskal–Wallis test would point to a nonassociative modulation of odor responses by "tastants". A one-sample sign test was used to test the pooled data from these groups against zero.

Reinforcing function as tested in "absolute" conditioning

In "absolute" conditioning, we compare individual animals which underwent either of two reciprocal training regimes (for a sketch see Fig. 5a): one received AM with e.g. appetitive reinforcement by FRU (AM+) and OCT without reinforcement (AM+/OCT); the second was trained reciprocally (AM/OCT+). Then, animals from both treatment conditions are individually tested in a choice situation for their preference between AM versus OCT. Associative learning is indicated by differences of individuals from reciprocal treatment conditions during test. This conclusion is compelling as during training individuals from the AM+/OCT group and the AM/OCT+ group have identical exposure to odorants and reinforcement. What differs between treatment conditions is solely the contingency between them. Importantly, in all cases the reciprocally trained animals were run alternatedly, which allows stringent pairing of data for the calculation of a learning index (LI; see below).

A group of eight larvae was transferred to a training plate. These plates contained either pure agarose or pure agarose plus one of the reinforcers (FRU, NaCl, or QUI). We started with pure agarose as substrate for half of the cases, and for the other half of the cases, we started with a reinforcer-containing plate (see also legend of Fig. 5a).

Immediately before a trial, two containers loaded with the same odorant were placed on opposite sides of the plate, 7 mm from the edges. We started with AM for half the animals and with OCT for the other half. Then, lids were closed and the larvae were allowed to move about the plate for 1 min. Thereafter, animals were transferred to a completely empty petri dish for a 1-min inter-trial interval. The larvae were then transferred to a plate with the alternative odorant and the respective other substrate for 1 min, followed by another 1-min inter-trial-interval. This cycle was repeated three (Fig. 5) or ten (Figs. 6, 7, 8) times. Fresh assay plates were used for each conditioning cycle.

After this training, each larva was individually tested for its odor choice; thus, animals were trained in small groups of eight, but tested as individuals. For testing, each larva was placed on a fresh, pure-agarose assay plate with a container of AM on one side and a container of OCT on the other side to create the desired choice situation; sides were changed for every other animal. Individual larvae were placed in the center of the petri dish, the lid was closed and the position of the larvae was scored for 5 min every 20 s as "AM", "OCT", or "neutral". After this was completed, animals from the reciprocal training group were run. In all cases, we discarded larvae that moved onto the lid or onto the odorant containers (< 5% of animals). We present test performance in three consecutive steps:

First (see Fig. 5b), for a time-resolved description of the animals' performance, we present the preference of the population of larvae by calculating for each time point the number of animals located on the AM side minus the number of animals located on the OCT side, divided by the total number of animals:

$$\mathrm{PREF} = \frac{\text{number of animals}_{\mathrm{AM}} - \text{number of animals}_{\mathrm{OCT}}}{\text{number of animals}_{\mathrm{Total}}} \tag{3}$$

Thus, a value of 1 indicates that all larvae were recorded on the AM side at that time point, whereas a value of −1 indicates that all were on the OCT side.

Second (see Fig. 5c), we calculate the odor preference for each animal as described in Eq. 1. In order to test for an associative effect of training, we took the paired PREF values from the alternatedly run, reciprocally trained animals and compared them with a Wilcoxon signed ranks test.

Third, to quantify learning, we calculated a learning index from these pairs ranging from −1 to 1 as:

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM+/OCT}} - \mathrm{PREF}^{\mathrm{AM/OCT+}}}{2} \quad \text{for FRU;} \tag{4a}$$

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM/OCT-}} - \mathrm{PREF}^{\mathrm{AM-/OCT}}}{2} \quad \text{for NACL or QUI.} \tag{4b}$$

Accordingly, in both Eq. 4a and Eq. 4b, positive LI values indicate associative learning. An exhaustive

analysis using the bootstrap technique reveals that these LIs are a reasonable basis for statistical analysis (see Appendix). The bootstrap analyses were warranted to test whether results might be distorted by a random component when assigning animals into pairs; that is, such pairing errors might lead to a changed distribution of LI values and hence to changed variances and/or medians; for small sample sizes, which is not the case in this study, the possibility exists that this can affect the outcome of statistical tests. However, the bootstrap analyses suggest that such pairing errors are negligible (see Appendix). Therefore, we use the LIs as basis for our statistical analyses. We use nonparametric statistics throughout: for comparisons of LIs against zero, we use the one-sample sign test; for multiple-group comparisons of the LIs we use the Kruskal–Wallis test; for two-group comparisons of LIs we use the Mann–Whitney *U*-test.

For the first learning experiment to be reported in the Results, we present all of the three steps outlined above (see Fig. 5b–d); for the subsequent learning experiments, we present the data only in the "condensed" format of the LIs and/or the PREF values (Figs. 6, 7, 8).

Reinforcing function as tested in differential conditioning

In the "differential" conditioning version of this paradigm, animals received two reinforcers: both appetitive reinforcement by FRU and aversive reinforcement by either NaCl or QUI; thus, in this case we compared the test performance of animals trained AM+/OCT− to ones trained AM−/OCT+; LI values were calculated as

$$\mathrm{LI} = \frac{\mathrm{PREF}^{\mathrm{AM+/OCT-}} - \mathrm{PREF}^{\mathrm{AM-/OCT+}}}{2} \qquad (4c)$$

Results

Detectability of odors

Larvae show clear attraction to both odors used: that is, they choose the side of the assay plate equipped with the AM container over that side with an empty container, and they prefer the OCT side over the side with a solvent-filled container (Fig. 1; $P<0.05$ in either case; $N=72, 79$). The response to AM is a genuine response to AM and not to the paraffin oil used as solvent, as the paraffin oil does not elicit significant responses (Fig. 1; $P>0.05$; $N=48$). If signs for the OCT group are reversed, we find that the degree of preference for AM and OCT is equal (no Fig.; $U=2632.5$; $P>0.05$; Ns as above). Interestingly, however, if larvae are given a choice between AM and OCT, they show a preference for OCT (Fig. 1; $P<0.05$; $N=77$). Thus, although both odors are equally attractive when tested in isolation, in a binary choice situation OCT is preferred over AM, at least in naïve larvae (for animals which had undergone associative training, see below). In the simplest account, this implies that a relative preference is a more sensitive measure than an absolute preference. In any event, because during associative training the odors are used in isolation, we chose to use AM and OCT at the present concentrations as olfactory stimuli in the subsequent learning experiments.

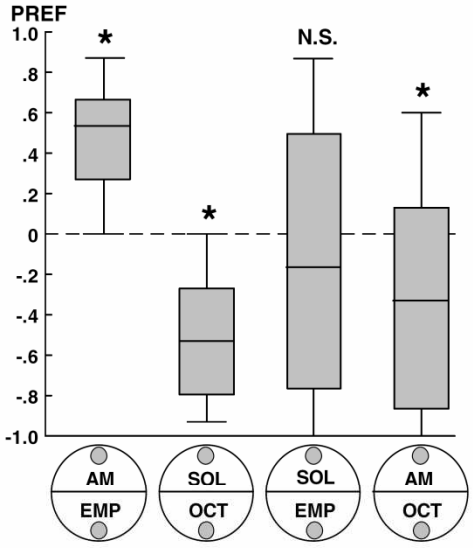


Fig. 1 Detectability of odors: AM and OCT are well detectable. Insets below the figure depict the procedure for testing the detectability of odors. From *left to right*, groups were tested for their choice between: an empty container (EMP) and a container filled with AM dissolved in paraffin; a container with that paraffin solvent (SOL) versus OCT; with SOL versus EMP; or with AM versus OCT. Animals were observed for 5 min, and every 20 s, the position of the animals was noted as being AM, neutral, or OCT. For each animal, the odor preference is calculated by summing up the number of times it was observed on the AM side minus the number of times it was observed on the OCT side; the result was then divided by the total number of times the animal was observed. Thus, positive values indicate AM preference and negative values OCT preference. *: $P<0.05$; NS: $P>0.05$. The *box plots* represent the median as the *middle line* and 10 and 90, and 25 and 75% quantiles as *whiskers and box boundaries*, respectively. Ns are from *left to right* 72, 79, 48, 77

Response-releasing function of "tastants": avoidance and approach

Larvae show attraction to fructose (FRU) and are repelled by sodium chloride (NaCl) and quinine (QUI) (Fig. 2; $P<0.05$ in all cases; $N=19, 21, 20$). Avoidance of NaCl is, statistically speaking, about as strong as that of QUI (Fig. 2; $P>0.05$, $U=150.5$; Ns as above). If one reverses signs for the aversion responses, one can ask whether the extent to which FRU, NaCl, and QUI

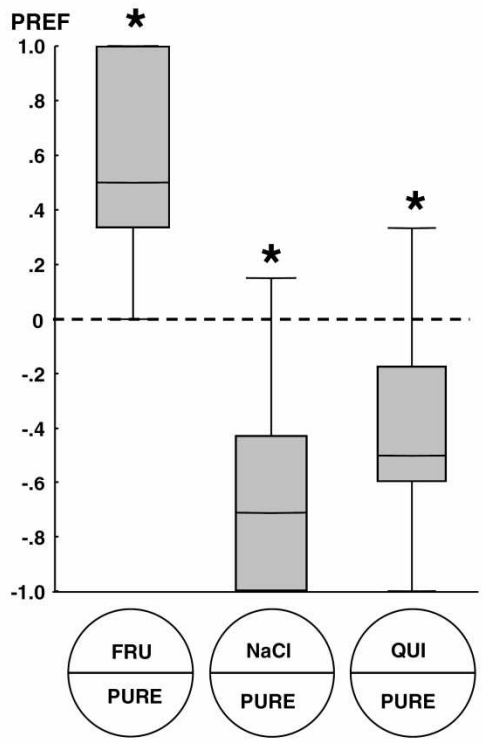


Fig. 2 Response-releasing function: appetitive response to FRU, aversive responses to NaCl and QUI. Insets below the figure depict the procedures for the gustatory response tests. In all cases, preferences between plain agarose (PURE) versus "tastant" were measured; groups differed with respect to "tastant" used: either FRU, NaCl, or QUI. Groups of ten animals each were placed in the middle of the test plate and after 15 min the number of animals located on the PURE, or "tastant" side was determined. To calculate gustatory preferences, the number of animals located on the "tastant" side was subtracted from the number of animals located on the PURE side; that value was then divided by the total number of animals. Thus, positive values indicate attraction and negative values repulsion of a "tastant". $*P<0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 19, 21, 20

possess response-releasing functions is different. No such difference is found (no Fig.; $P>0.05$, $H=2.308$, $df=2$; Ns as above). Thus, all three "tastants" are detected well and, at the concentrations used, about equally potent in releasing avoidance and approach responses.

Response-releasing function of "tastants": influence on "appetite"

As another way of testing appetitive and aversive responses, we asked whether FRU, NaCl, and QUI would have an effect on "appetite", i.e. on feeding behavior. We find that larvae swallow agarose even without any "tastant" added (Fig. 3a; $P<0.05$, $N=60$). Larvae also eat when FRU is added (Fig. 3a; $P<0.05$, $N=60$), but not if either NaCl or QUI are present in the substrate: in none of these animals did we observe any sign of feeding (Fig. 3a; $P>0.05$, $N=60$ in both cases). Thus, feeding clearly depends on the kind of "tastant" present (Fig. 3a: $P<0.05$, $H=149.0$, $df=3$, Ns as above); specifically, FRU increases (Fig. 3a; $P<0.05$, $U=1{,}172$; Ns as above), whereas NaCl and QUI decrease feeding (Fig. 3a; $P<0.05$, $U=600$ in both cases; Ns as above) as compared to the pure condition. These conclusions remain unaltered if larvae are scored as "eaters" and "noneaters" and the frequency of these cases is compared (FRU: 57/3; PURE: 44/16; NaCl: 0/60; QUI: 0/60) (no Fig.: $P<0.05$, $\chi^2=165.6$, $df=3$; for FRU versus pure: $\chi^2=4.36$, $df=1$; for pure versus NaCl: $\chi^2=69.47$, $df=1$).

Taken together, in accord with the literature (Heimbeck et al. 1999; Scherer et al. 2003), all stimuli used in this study are detected well by the larvae under our experimental conditions and at the concentrations used. Concerning "tastants", our results in particular show that FRU, NaCl, and QUI possess substantial response-releasing properties for appetitive and aversive responses, respectively.

Response-modulating function of "tastants"?

Given that FRU, NaCl and QUI are triggering strong appetitive and aversive responses, we asked whether these same stimuli would modulate olfactory responses in a nonassociative way: would the choice between AM and OCT be different on a neutral versus a sweet, salty or bitter substrate? For example, larvae might ignore olfactory stimuli altogether if crawling on an unpleasant substrate. However, we find that larvae perform similarly on all four substrates (Fig. 4; $P>0.05$, $H=2.876$, $df=3$; $N=68$, 66, 64, 66). If the data from all these experimental groups are pooled, we find a preference for OCT (no Fig.; $P<0.05$; $N=264$). This OCT preference is indistinguishable from the data for the AM versus OCT condition in Fig. 1 (no Fig.; $P>0.05$; $U=7{,}018.0$; Ns as above) arguing that patterns of preference have reasonable stability over repetitions. Importantly, the equal olfactory choice performance on PURE, FRU, NaCl and QUI substrates suggests that FRU, NaCl and QUI, although potent response-releasing stimuli, do not lead to nonassociative modulations of olfactory choice behavior.

Reinforcing function: Do FRU, NaCl or QUI support associative learning?

We next asked whether these "tastants" are able to act as reinforcers in associative learning. We first report in some detail on a pilot experiment performed in the Toronto laboratory using only FRU in an "absolute" conditioning experiment (Fig. 5a–d). In a next step, we report three "absolute" conditioning experiments using

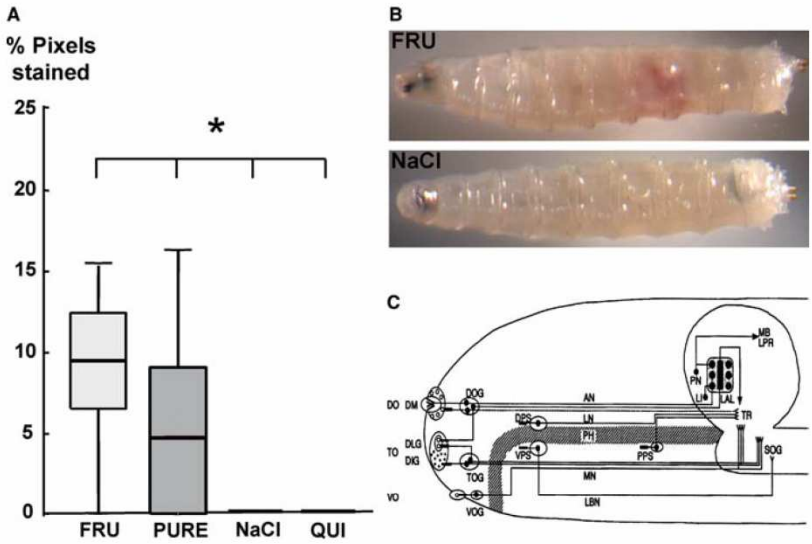


Fig. 3 Response-releasing function: appetitive effect of FRU, aversive effect of NaCl and QUI in a feeding assay. **a** To estimate the amount of food intake, the percentage of stained pixels is shown for animals that fed on different carmine-red stained substrates: From *left to right* the results are shown for stained agarose with FRU added, without any "tastant", with NaCl and with QUI. Animals eat on pure agarose; this is increased by the presence of fructose and suppressed by the presence of sodium chloride or quinine. The conclusions remain unaltered if alternatively the frequencies of "eaters" and "noneaters" are evaluated (see text). **b** Examples of animals after opportunity to feed on stained agarose with added FRU (*upper picture*) or NaCl (*lower picture*). **c** Schematic (from Python and Stocker 2002) showing the external mixed olfactory/gustatory sense organ (dorsal organ, *DO*), and the external gustatory sense organs (terminal and ventral organ, *TO*, *VO*). The internal gustatory sensillae are situated along the pharynx (*DPS, VPS, PPS* dorsal, ventral, and posterior pharyngeal sensilla). For all these sensory structures, the central projections to the antennal lobe (*AL*), tritocerebrum (*TR*) and suboesophageal ganglion (*SOG*) are shown. From the AL, projection neurons (*PN*) relay onto the lateral protocerebrum (*LPR*) and provide collaterals into the mushroom bodies (*MB*). *AN, LN, MN, LBN* antennal, labral, maxillary, labial nerves. *DLG, DIG* dorsolateral and distal group of TO. *DOG, TOG, VOG* ganglia of DO, TO, VO. The figure does not cover other potentially chemosensitive structures on cephalic, thoracic and abdominal segments. *: $P<0.05$. For an explanation of the *box plots*, see legend of Fig. 1. Ns are from *left to right* 60, 60, 60, 60

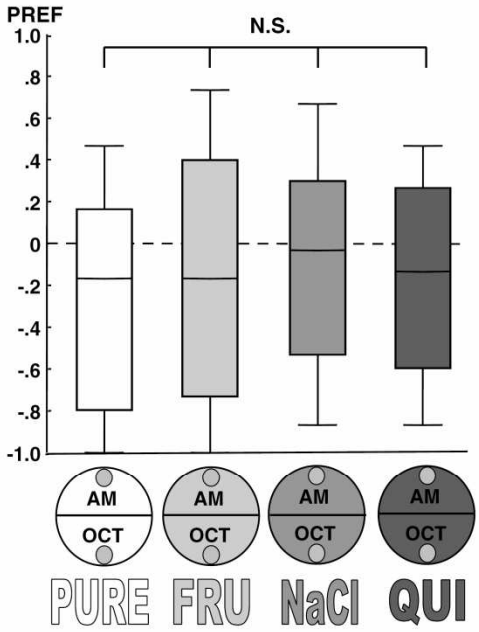


Fig. 4 Response-modulating function: Olfactory choice is unaffected by FRU, NaCl, or QUI. Insets depict the experimental procedure. Groups differ in that the odor choice between AM and OCT was measured on different substrates: plain agarose (PURE), FRU, NaCl, or QUI. Odor preferences, calculated as explained in the legend of Fig. 2, do not differ between these groups. NS: $P>0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 68, 66, 64, 66

either only FRU as reinforcement, or only NaCl or only QUI; it will turn out that FRU, but neither NaCl nor QUI can induce learning (Fig. 6).

We compared the performance of two reciprocally trained groups (Fig. 5a): one received the reinforcer with AM and received OCT without reinforcement (AM+/OCT); the other group was trained with OCT being accompanied by the "tastant" (AM/OCT+). As shown in Fig. 5b, c animals which had received AM+/OCT training showed a higher AM preference than animals which had received AM/OCT+ training (Fig. 5b, c; $P<0.05$, $Z=2.71$; Ns = 46). We quantified this difference by a learning index (LI); this LI is positive in about 75% of the cases (Fig. 5d). The median LI is 0.10, which represents that LIs are significantly above chance level (Fig. 5d; $P<0.05$; $N=46$). This result must lead to the conclusion that individually assayed *Drosophila* larvae show associative learning between olfactory stimuli and FRU reinforcement. It should be emphasized that this

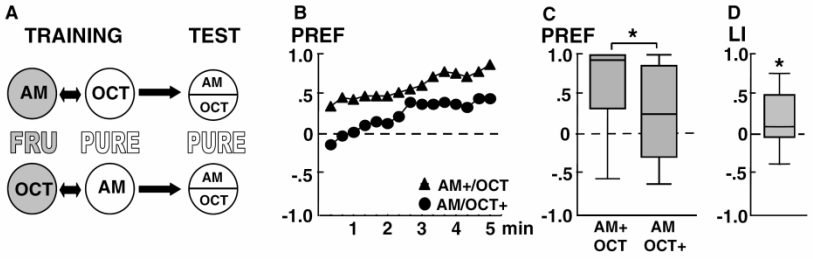


Fig. 5 Reinforcing function: FRU supports "absolute" conditioning. **a** Diagram of the procedure for the conditioning experiment; please note that for half of the cases, the sequence of training trials within each reciprocal group was as indicted (i.e. AM+/OCT and OCT+/AM), whereas for the other half of the cases (not shown), the sequence of training trials was reversed (i.e. OCT/AM+ and AM/OCT+). **b** Time-resolved description of the odor preferences during test calculated as the number of animals located on the AM side at a given time point minus the number of animals located on the OCT side, divided by the total number of animals. Thus, positive values indicate that a majority of larvae were recorded on the AM side at that time point, whereas negative values indicate that a majority was located on the OCT side. **c** Peference values were calculated for each animal as explained in the legend of Fig. 2. Positive values indicate AM preference and negative values OCT preference. The AM preference was higher after AM+/OCT training than after AM/OCT+ training, indicating associative learning. **d** A learning index (LI) was calculated for pairs of animals which underwent either of the reciprocal training regimes, e.g. either AM+/OCT or AM/OCT+, by subtracting the PREF values of both animals and dividing the result by two. The LIs are significantly larger than zero, indicating associative learning. $*P<0.05$. For an explanation of the box plots, see legend of Fig. 1 $N=46$

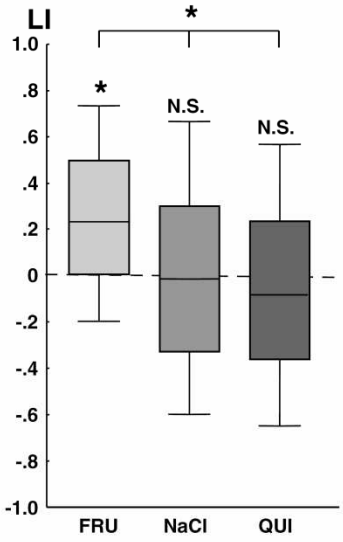


Fig. 6 Reinforcing function: FRU but neither NaCl nor QUI supports learning. Direct comparison of the effectivity of "absolute" conditioning between the different "tastants". The LI values are significantly different from zero for FRU, but not for NaCl or QUI. In a direct comparison, the LI values differ significantly between the three reinforcers. $*P<0.05$; *NS* $P>0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 115, 122, 120

conclusion is drawn from the comparisons between pairs of animals which had undergone reciprocal training regimes (AM+/OCT versus AM/OCT+). As only the relation of odors and reinforcement differs between these training regimes, only associative learning can account for differences during the test. This conclusion is unaffected by the overall preference for AM over OCT (see below); this preference merely leads to an offset of the preference values for both reciprocal groups (Fig. 5b, c) but cannot cause differences in preference values between them as measured by the learning index (Figs. 5d, 6, 8). Therefore, the conclusion that larval *Drosophila* form associations between olfactory stimuli and FRU reinforcement is compelling.

The next experiment, using ten instead of three conditioning cycles, compared the effectiveness of FRU, NaCl, and QUI in "absolute" conditioning. Concerning FRU, the results from the previous experiment nicely reproduced: preferences for AM are higher after AM+/OCT training than after AM/OCT+ training (no Fig.; $P<0.05$, $Z=6.02$; Ns=115). This difference can be quantified by an LI of 0.23, which was significantly above chance level (Fig. 6; $P<0.05$; $N=115$). With NaCl and QUI as reinforcers, the training regime has no influence on the behavior during the test: after training with AM−/OCT and AM/OCT−, odor preferences were indistinguishable (no Fig.; for NaCl: $P>0.05$, $Z=0.31$; Ns=122; for QUI: $P>0.05$, $Z=1.76$; Ns=120). The corresponding learning indices were in both cases indistinguishable from zero (Fig. 6; $P>0.05$ in both cases, Ns=122 and 120, respectively). Thus, despite the fact that both NaCl and QUI are potent in eliciting avoidance responses (Figs. 2, 3), they are apparently not potent as reinforcers in associative learning. This is in marked contrast to the effectiveness of FRU in this respect (Figs. 5, 6, 8). Importantly, a direct comparison between the experiments which used either FRU or NaCl or QUI showed a significant difference in the effectiveness across these three stimuli (Fig. 6; $P<0.05$, $H=32.28$; $df=2$; Ns as above). This directly demonstrates that reinforcer identity is a determinant for olfactory associative learning in the *Drosophila* larva.

In a further analysis of our data, we asked whether FRU reinforcement could bi-directionally modulate odor preferences. We reasoned that, as animals do not learn associatively when using NaCl and QUI, pooling the preference data from the two reciprocal NaCl groups

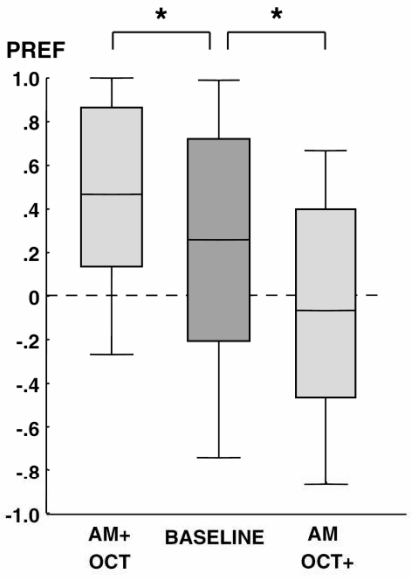


Fig. 7 Reinforcing function: Bi-directional effects of FRU in the reciprocally trained groups. Post-hoc analysis of the experiment shown in Fig. 5, comparing the PREF values of animals after FRU training to baseline; the baseline was provided by pooling the data of the two reciprocal NaCl groups as well as the two reciprocal QUI groups. After AM+/OCT training, animals had a higher AM preference than baseline, and after AM/OCT+ training, they had a lower preference than baseline. $*P < 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 115, 484, 115

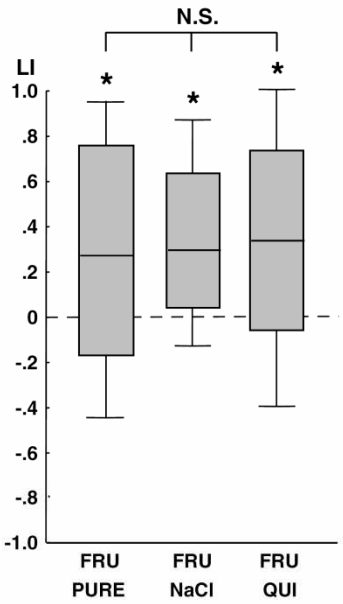


Fig. 8 Reinforcing function: Neither NaCl nor QUI potentiates FRU reinforcement. Comparison of "absolute" and differential conditioning. Groups differed in that either only FRU was used for "absolute" conditioning (*left*) or differential conditioning was performed using FRU for positive as well as either NaCl (*middle*) or QUI (*right*) for negative reinforcement. Under all three conditions, the same amount of learning as measured by the LI was observed; in all three cases, the LIs are above chance level. $*P < 0.05$; *NS* $P > 0.05$. For an explanation of the box plots, see legend of Fig. 1. Ns are from *left to right* 59, 58, 47

as well as the two reciprocal QUI groups would result in a baseline, post-training measure of odor preference against which the performance of the FRU-trained groups (AM+/OCT and AM/OCT+) could be compared. As shown in Fig. 7, animals from the AM+/OCT group have a higher AM preference than baseline (Fig. 7; $P < 0.05$, $U = 22{,}210$; $N = 484, 115$), whereas the ones from the AM/OCT+ group are below baseline (Fig. 7; $P < 0.05$, $U = 21{,}185$; $N = 484, 115$). In the simplest account, this opposite effect of FRU in the reciprocally trained groups suggests that larvae can associate FRU with both AM and OCT.

Interestingly, the baseline performance shows a preference for AM over OCT (Fig. 7; $P < 0.05$; N as above). This is in contrast to the naïve preference for OCT over AM (Figs. 1, 4). This shift in overall preference in animals that show no associative learning (for OCT before training, for AM after training) might be due to either: (1) the passage of time, affecting the animals sensory system and/or the physical or chemical properties of the odors; (2) odor exposure, leading to sensory adaptation and/or habituation; (3) handling of the larvae and the stress this might entail; (4) previous exposure to "tastants". At present, we cannot distinguish between these possibilities. Interesting and obvious as this effect is, it should be stressed that it cannot dismiss the associative effects as measured by the learning indices. This is because the reciprocally trained groups are equal with respect to all the above-mentioned parameters.

Reinforcing function: do NaCl or QUI potentiate associative learning?

Although NaCl and QUI did not induce associative learning on their own in "absolute" conditioning (Fig. 6), they still might have a potentiating effect if used in combination with FRU in differential conditioning. Therefore, three learning experiments were compared. In one, larvae were trained in "absolute" conditioning with FRU reinforcement alone (this replicates the FRU experiment shown in Fig. 6). In the other two, animals received differential conditioning using two reinforcers: either the combination FRU–NaCl or FRU–QUI. All three procedures resulted in indistinguishable learning indices (Fig. 8; $P > 0.05$, $H = 0.38$, $df = 2$; $N = 59, 58, 47$). Within all three experiments, the learning indices were above chance level (Fig. 8; $P < 0.05$ in all cases; Ns as above). Thus, neither NaCl nor QUI had an apparent

potentiating effect on olfactory learning when used in differential conditioning together with FRU. If one compares the learning indices between repetitions of the FRU-learning experiments in Fig. 6 versus Fig. 8, no difference is found (no Fig.; $P > 0.05$; $U = 3{,}305{,}5$; Ns as above). This suggests that, with boundary conditions unchanged, the learning indices have reasonable stability over repetitions.

Discussion

Among the behavioral functions of gustatory stimuli, we investigated their potential response-releasing, response-modulating, and reinforcing function. We found that these functions are dissociated: FRU, NaCl and QUI can all release appetitive or aversive responses, respectively (Figs. 2, 3). Still, none of these stimuli has detectable nonassociative, response-modulating effects on olfactory choice (Fig. 4). Finally, only FRU but neither NaCl nor QUI has an apparent reinforcing effect for olfactory learning (Fig. 6). Taken together, this implies that these functions are dissociated between the three tastants on the behavioral level. Clearly, such a dissociation can come about only by a dissociation of the underlying neuronal circuitry. To this end, Menzel et al. (1999) found that octopamine is sufficient to rescue the reinforcing, but not the response-releasing function of sucrose in honeybees depleted of biogenic amines by reserpine. Dopamine, on the other hand, was able to rescue the response-releasing but not the reinforcing function. This is in line with the finding of Hammer (1993) who showed that driving the identified putatively octopaminergic neuron VUM_{mx1} is sufficient to substitute for the reinforcing, but not the response-releasing function of reward. In mammals, dopamine plays a role in mediating the reinforcing function of reward (Waelti et al. 2001), but not its response-releasing function (Cannon and Palmiter 2003). Also, the analyses of classical conditioning of eyeblink conditioning in the rabbit have shown that in the brainstem processing of the corneal air puff is separated out into a direct connection to the motor nuclei to release the reflex, and an extensive reinforcing cerebellar loop targeting the site of synaptic plasticity to support learned responses (Christian and Thompson 2003). Together, these data suggest that, as a general rule, processing of potentially reinforcing, unconditioned stimuli might diverge already at early processing stages into separate circuitries to on the one hand directly trigger reflexes, and on the other hand a reinforcement signal to induce learning.

Effectiveness of FRU as reinforcer

With respect to reinforcement function, we report three "absolute" conditioning experiments and find that FRU but not NaCl or QUI can act as a reinforcer for associative olfactory learning (Fig. 6). Furthermore, neither NaCl nor QUI have a potentiating effect on learning when used together with FRU in differential conditioning (Fig. 8). The reinforcing effect of FRU was repeatedly found in the Würzburg laboratory (Figs. 6, 8) and was, even with only three conditioning cycles, replicated in the Toronto laboratory as well (Fig. 5). These results provide the first evidence of appetitive olfactory learning in *Drosophila* larvae to date. Concerning NaCl and QUI, however, an absence of proof for learning is not a proof of its absence, which, on principle grounds, is difficult to obtain.

The same differential reinforcement effectiveness for FRU was found in a companion paradigm in which larvae were trained to associate visual stimuli with the same three gustatory reinforcers as in the current study (Gerber et al. 2004). As FRU, NaCl and QUI are well detectable to larvae (Figs. 2, 3), we suggest that larvae are, in general, more susceptible to appetitive than to aversive reinforcement. The relatively low reinforcement effectiveness of aversive stimuli might be one reason why the studies of Aceves-Pina and Quinn (1979), Heisenberg et al. (1985), and Tully et al. (1994), which used electric shock for aversive reinforcement, were compromised in their reproducibility (Forbes 1993; F. Python, University of Fribourg, Switzerland, personal communication).

A possible ultimate reason for the effectiveness of FRU reinforcement

On an ultimate level, the high effectiveness of appetitive stimuli as reinforcers in the larva might reflect their evolutionary design as a feeding stage. In other words, the larvae might be "clamped" into a feeding motivation, similar to the situation in the honeybee worker (Hammer and Menzel 1995; Menzel et al. 1999), and therefore appetitive, nutritious stimuli might be particularly rewarding to them. Interestingly, in adults the situation is reversed, in that aversive reinforcers are more effective than appetitive ones (Schwaerzel et al. 2003; see below). This reversed pattern of results might reflect the life style of adults as reproductive stages for which feeding and growth play less of a role than for larvae. A similar developmental switch, by the way, is also seen in odor and light preferences, as larvae are usually attracted by odors and repelled by light (Sawin-McCormack et al. 1995; Cobb 1999; Hassan et al. 2000), whereas adults are usually repelled by odors, at least at intermediate and high concentrations, and attracted by light (Ballinger and Benzer 1988; Ayyub et al. 1990).

Possible proximate reasons for the effectiveness of FRU reinforcement

On a proximate level, reinforcement signals triggered by aversive gustatory stimuli might not converge with processing of olfactory and visual input, and hence no associations can possibly form. Concerning QUI, how-

ever, we were informed that if very long training trial durations are used in an en masse version of our assay (30 min, instead of 1 min as in this study), QUI is able to support aversive olfactory learning in the larva (F. Mery, University of Fribourg, Switzerland, personal communication). This is in line with the fact that also in adult flies quinine has been successfully applied in olfactory learning experiments (Medioni and Vaysse 1975; DeJianne et al. 1985; Bouhouche et al. 1995; Mery and Kawecki 2002). This implies that in both larvae and adults the neuronal circuitry to support aversive olfactory learning by QUI does, in principle, exist. With respect to the current study, it underlines the cautious conclusion (see above) that QUI is less effective than FRU as a reinforcer, but might not be totally ineffective. Concerning NaCl, prolonged training trials might eventually also reveal aversive learning. It should then be interesting to test different concentrations of NaCl, as low concentrations might be appetitive but high concentrations might be aversive reinforcers (Miyakawa 1982).

Another proximate reason for the reduced effectivity of NaCl and QUI reinforcement could be that the reinforcing effects of the gustatory stimuli depend on food intake. Larvae carry three paired groups of external gustatory sensilla on their head segment, in the so called dorsal-, terminal- and ventral organs; in addition, they also possess three paired groups of internal gustatory sensilla in the pharynx (Python and Stocker 2002; Gendre et al. 2003). These latter sensilla might be used to sample swallowed food, whereas external sensilla might be used to sample the gustatory environment. Thus, it is conceivable that the external gustatory sensillae mediate attraction and avoidance responses (Heimbeck et al. 1999) as well as the decision to swallow food or not, whereas the pharyngeal sensilla mediate the reinforcing effect of food. We found that larvae swallow crumbs of agarose under our assay conditions and are more likely to do so if FRU, but less likely if high concentration NaCl or QUI, are added to the agarose (Fig. 3). Thus, any reinforcing effect as mediated by the pharyngeal sensillae would be negligible as only minute amounts of NaCl or QUI are swallowed, whereas the large amount of swallowed FRU might be sufficient to induce a reinforcing effect. Interestingly, despite extensive reorganization of the larval nervous system during metamorphosis, the majority of the pharyngeal sensilla persist into the adult stage (Gendre et al. 2003). As most other larval sensory neurons die during metamorphosis (but see Tissot and Stocker 2000; Helfrich-Foerster et al. 2002), this might indicate that pharyngeal gustatory sensilla play a particularly important role in the life of the fly.

Outlook

Interestingly, in adult olfactory learning, appetitive sucrose conditioning requires octopaminergic, but not dopaminergic neurons, while aversive learning with electric shock reinforcement in turn requires dopaminergic, but not octopaminergic neurons (Schwaerzel et al. 2003). This dissociation of possible reinforcement systems is, albeit with reversed sign, similar to the situation in monkeys where activations of midbrain dopamine neurons were found to be stronger and more frequent by appetitive compared to aversive stimuli (Mirenowicz and Schultz 1996). Furthermore, stimuli that predict the occurrence of reward activate these neurons in the monkey, whereas stimuli that predict the absence of reward lead to their inhibition (Tobler et al. 2003). It is of interest to determine whether similar dissociations might apply in *Drosophila* larvae, which due to their cellular simplicity, transparent cuticle and genetic as well as optophysiological accessibility (Liu et al. 2003) might be a fruitful system for such an analysis.

Acknowledgements Support for this study came from the Deutsche Forschungsgemeinschaft (SFB 554, to M. H.), the Canada Research Chair Program (M.B.S.) and the Company of Biologists (travel grant, to T.H.). The Volkswagen Foundation is acknowledged for having provided the start-up funds for this research program (I/76 240 to B.G.). Thanks are extended to the members of the Würzburg and Toronto labs, especially A. Fiala, C.A.L. Riedl, and H. Tanimoto for discussion. Our experiments comply with German law, as well as the NIH "Principles of animal care", 86-23, 1985.

Appendix

Is the distribution of LI values influenced by pairing? A bootstrap analysis

The LI values are calculated as the difference in odor preference between reciprocally trained animals. A statistical analysis of these LI values makes only sense if their distribution does not depend on the pairing of the preference values on which they are based. This is because different values of sample variance and sample median may be obtained depending on which particular animals are assigned into pairs (the sample mean, however, is independent of pairing). Consequently, one may argue that the outcome of statistical tests of these LIs may be due to a particular choice of pairing. In the following, we investigate whether this is indeed the case. It turns out that the distribution of the LIs is not influenced by pairing; specifically, the bootstrap method is used to test whether the distributions of sample variance and sample median are influenced by pairing. Several hundred samples of randomly taken pairs are thereby considered.

The basic idea behind bootstrapping (inventor: Efron 1979) is that it treats the empirical distribution of the data (probability mass $1/n$ on $x_1,\ldots, x_n$) as if it were the underlying true (but unknown) distribution. Thus, the sampling distribution of a certain statistic of interest, in our case variance and median, can be estimated by

generating a large number of new samples (called bootstrap samples) by drawing with replacement from the actual sample. From these many samples, bootstrap can provide us with confidence intervals for the variance (var) and the median (med) of the "original pairing". The same can then be done for LI values obtained by various (random) pairings of the preference values. Note that bootstrapping makes no assumption about the underlying distribution.

Various methods are known for using bootstrap to determine confidence intervals. In the present paper, we use the percentile method (Efron and Tibshirani 1993, Chap. 13), which is briefly described in the following. We prefer this over the standard method to approximate confidence intervals for an unknown parameter via standard errors, because standard errors are crude measures of statistical accuracy, and transformations that may improve the normal approximation (if they exist) would have to be known. Denote by $x_1,..., x_n$ a LI sample, based on the "original" or a randomly generated pairing, of size n. A bootstrap sample $x_1^*,..., x_n^*$ is a random sample of size n drawn with replacement from

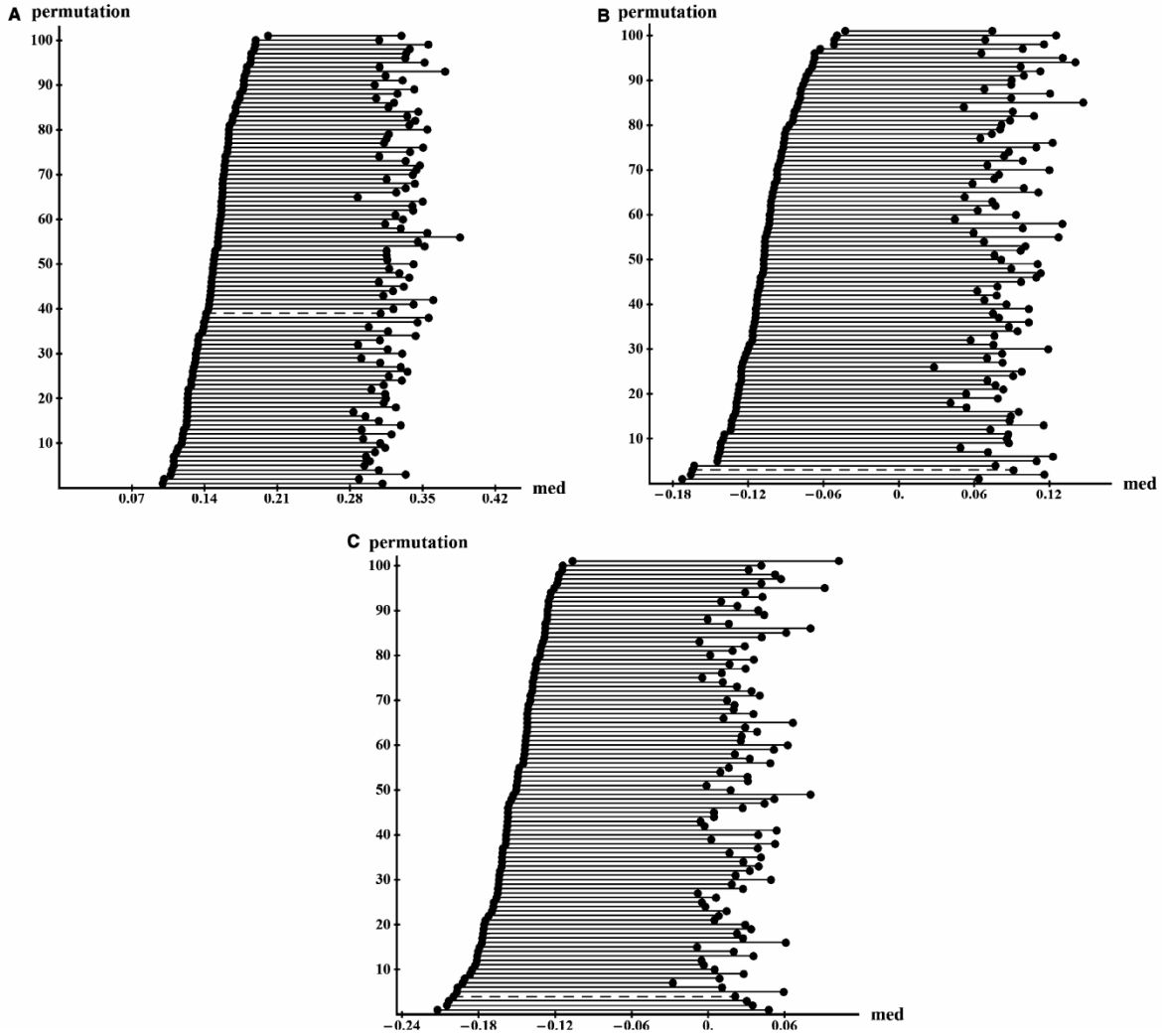


Fig. 9 The median of the LIs is not influenced by pairing. For each of the 100 randomly permutated pairings of PREF values the corresponding LI values were calculated and the confidence interval for the median derived by the bootstrap method using that particular pairing is presented; from *bottom* to *top*, the permutations are ordered by increasing lower limits of the confidence interval. The *dashed confidence interval* labels the median for that sample which is based on the original pairing; the original pairing uses the animals trained alternately for the calculation of LIs and is the basis of data presentation and statistical analysis in the main text (Fig. 6). In all three experimental conditions (FRU, NaCl, QUI in *a*, *b* and *c*, respectively), the confidence interval for the median of the original pairing overlaps with the confidence intervals for the median of the permutated combinations. This indicates that pairing does not substantially alter the median. The same analysis was performed on the data presented in Figs. 5 and 8, leading to the same result (not shown)

the actual sample $x_1,..., x_n$. For the bootstrap sample, we evaluate the statistics of interest; here, the variance of the bootstrap sample:

$$\mathrm{var}\left(x_1^*,\ldots,x_n^*\right)=\frac{1}{n}\sum_{j=1}^{n}\left(x_j^*-\frac{1}{n}\sum_{i=1}^{n}x_i\right)^2$$

(note that $\sum_{i=1}^{n} x_i/n$ is the mean of the bootstrap sample), and median of the bootstrap sample $med(x_1^*,..., x_n^*)$.

The bootstrap algorithm begins by generating a large number, for example, $N=20{,}000$, of independent bootstrap samples:

$$\text{Resample \# 1}, \text{Resample \# 2}, \ldots, \text{Resample \# } N.$$

For each bootstrap sample, we calculate the variance:

$$\mathrm{var}(\text{Resample \# 1}), \ldots, \mathrm{var}(\text{Resample \# } N) \quad (5)$$

and the median:

$$\mathrm{med}(\text{Resample \# 1}), \ldots, \mathrm{med}(\text{Resample \# } N). \quad (6)$$

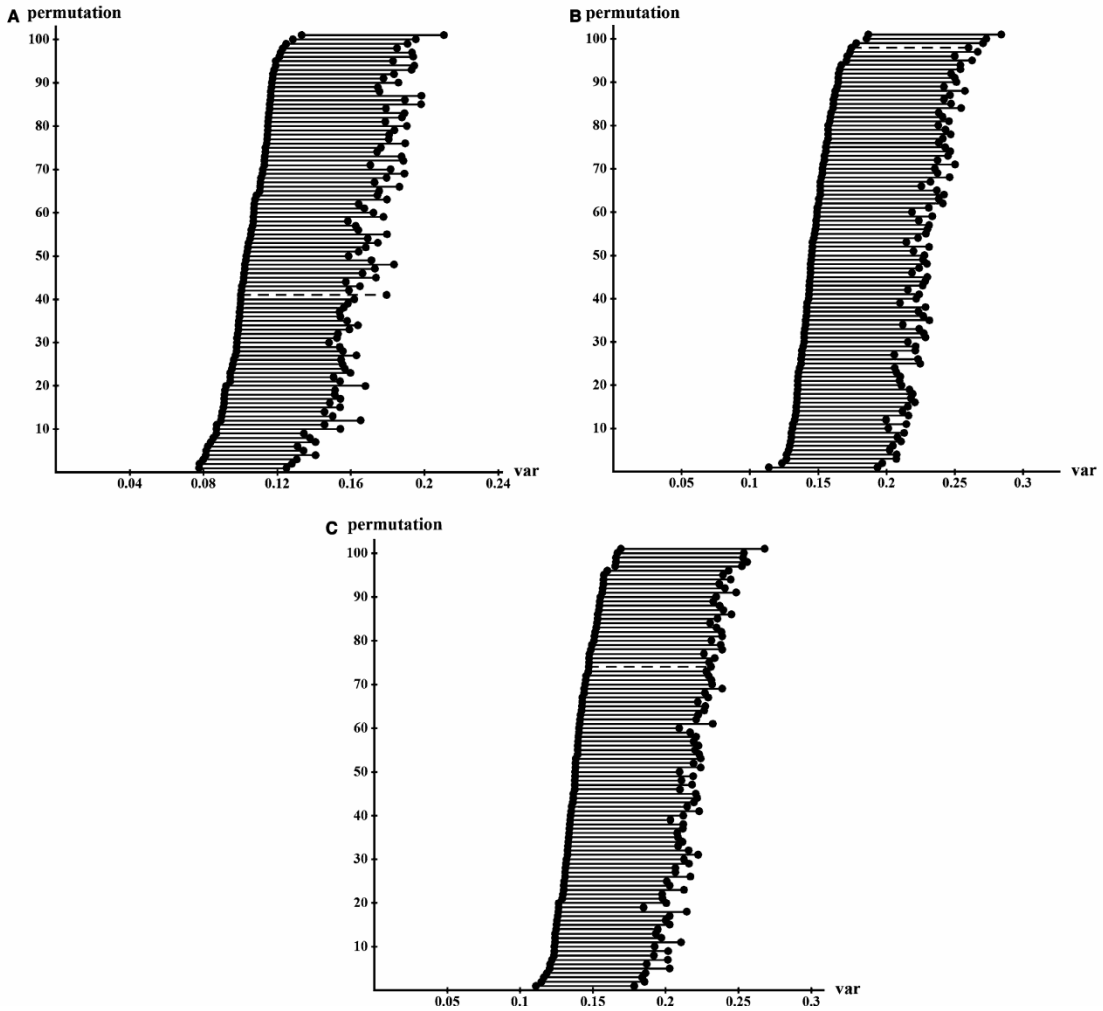


Fig. 10 The variance of the LIs is not influenced by pairing. For each of the 100 randomly permutated pairings of PREF values the corresponding LI values were calculated and the confidence interval for the variance derived by the bootstrap method using that particular pairing is presented; from *bottom* to *top*, the permutations are ordered by increasing lower limits of the confidence interval. The dashed confidence interval labels the variance for that sample which is based on the original pairing; the original pairing uses the animals trained alternately for the calculation of LIs and is the basis of data presentation and statistical analysis in the main text (Fig. 6). In all three experimental conditions (FRU, NaCl, QUI in *a*, *b* and *c*, respectively), the confidence interval for the variance of the original pairing overlaps with the confidence intervals for the variance of the permutated combinations. This indicates that pairing does not substantially alter the variance. The same analysis was performed on the data presented in Figs. 5 and 8, leading to the same result (not shown)

We then consider the empirical distribution pertaining to the bootstrap samples Eqs. 5 and 6, respectively. The confidence interval with coverage probability 1-α is the interval between the $100\times\alpha/2$ and $100\times(1-\alpha/2)$ percentile of the empirical distribution of the statistic of interest. In our simulations we chose $\alpha = 0.05$. Note that in the same way we obtained bootstrap confidence intervals for randomly permutated LI data.

For the experimental data shown in Fig. 6, Figures 9 and 10 show that for all 100 randomly chosen pairings and the original pairing (dashed line), the confidence intervals overlap, showing that variance and median are not influenced by pairing. The same is true for the experimental data in Figs. 5 and 8. This indicates that the "original" LI values are a reasonable basis for further statistical analysis.

It is known that for the median an unreasonably high number of bootstrap samples would be necessary to yield reliable bootstrap estimates. In such cases, the bootstrap approach can be improved by involving a smoothed version of the sample quantile function; for a survey of smoothed bootstrap we refer to Falk and Reiss (1992). In our simulations the normal kernel was used in order to obtain such a smoothed version of the sample quantile function. The simulations were carried out with the software package *MATHEMATICA*, Version 5.0.

References

Aceves-Pina EO, Quinn WG (1979) Learning in normal and mutant *Drosophila* larvae. Science 206:93–96

Ayyub C, Paranjape J, Rodrigues V, Siddiqi O (1990) Genetics of olfactory behavior in *Drosophila melanogaster*. J Neurogenet 6:243–262

Ballinger DG, Benzer S (1988) Photophobe (Ppb), a *Drosophila* mutant with a reversed sign of phototaxis; the mutation shows an allele-specific interaction with sevenless. Proc Natl Acad Sci USA 85:3960–3964

Bouhouche A, Elkhessaimi A, Vaysse G, Choulli MK (1995) The role of quinine chlorhydrate in the conditioned inhibition of the tarsal reflex in *Drosophila melanogaster* (in French). Can J Exp Psychol 49:520–529

Cannon CM, Palmiter RD (2003) Reward without dopamine. J Neurosci 23:10827–10831

Christian K, Thompson RF (2003) Neural substrates of eyeblink conditioning: acquisition and retention. Learn Mem 10:427–455

Cobb M (1999) What and how do maggots smell? Biol Rev 74:425–459

DeJianne D, McGuire TR, Pruzan-Hotchkiss A (1985) Conditioned suppression of proboscis extension in *Drosophila melanogaster*. J Comp Psychol 99:74–80

Dukas R (1998) Ecological relevance of associative learning in fruit fly larvae. Behav Ecol Sociobiol 19:195–200

Efron B (1979) Bootstrap methods: another look at the jackknife. Ann Stat 7:1–26

Efron B, Tibshirani R (1993) An introduction to the bootstrap. Chapman and Hall, New York

Falk M, Reiss R-D (1992) Bootstrapping the sample quantile: a survey. In: Jöckel KH, Sendler W, Rothe G (eds) Bootstrapping and related techniques. Lecture Notes in Economics and Mathematical Systems. Springer, Berlin Heidelberg New York, pp 173–180

Forbes B (1993) Larval learning and memory in *Drosophila melanogaster*. Diploma Thesis, Julius Maximilian University, Würzburg, Germany

Gendre N, Luer K, Friche S, Grillenzoni N, Ramaekers A, Technau GM, Stocker RF (2003) Integration of complex larval chemosensory organs into the adult nervous system of *Drosophila*. Development 131:83–92

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, Heisenberg M (2004) Visual learning in individually assayed *Drosophila* larvae. J Exp Biol 207:179–188

Gordesky-Gold B, Warrick JM, Kutzler DP, Neal KC, Coughlin M, Tompkins L (1996) Interstrain variability of larval photokinesis in *Drosophila melanogaster*. Behav Genet 26:49–54

Hammer M (1993) An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. Nature 366:59–63

Hammer M, Menzel R (1995) Learning and memory in the honeybee. J Neurosci 15:1617–1630

Hassan J, Busto M, Iyengar B, Campos AR (2000) Behavioral characterization and genetic analysis of the *Drosophila melanogaster* larval response to light as revealed by a novel individual assay. Behav Genet 30:59–69

Heimbeck G, Bugnon V, Gendre N, Häberlin C, Stocker RF (1999) Smell and taste perception in *D. melanogaster* larva: toxin expression studies in chemosensory neurons. J Neurosci 19:6599–6609

Heisenberg M, Borst A, Wagner S, Duncan B (1985) *Drosophila* mushroom body mutants are deficient in olfactory learning. J Neurogenet 2:1–30

Helfrich-Foerster C, Edwards T, Yasuyama K, Wisotzki B, Schneuwly S, Stanewsky R, Meinertzhagen IA, Hofbauer A (2002) The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. J Neurosci 22:9255–9266

Koh YH, Gramates LS, Budnik V (2000) *Drosophila* larval neuromuscular junction: molecular components and mechanisms underlying synaptic plasticity. Microsc Res Tech 49:14–25

Liu L, Yermolaieva O, Johnson WA, Abboud FM, Welsh MJ (2003) Identification and function of thermosensory neurons in *Drosophila* larvae. Nat Neurosci 6:267–273

Medioni J, Vaysse G (1975) Conditional suppression of a reflex in *Drosophila melanogaster*: acquisition and extinction (in French). C R Seances Soc Biol Fil 169:1386–1391

Menzel R, Heyne A, Kinzel, C, Gerber B, Fiala A (1999) Pharmacological dissociation between the reinforcing, sensitizing, and response-releasing functions of reward in honeybee classical conditioning. Behav Neurosci 113:744–754

Mery F, Kawecki T (2002) Experimental evolution of learning ability in fruit flies. Proc Natl Acad Sci USA 99:14274–14279

Mirenowicz J, Schultz W (1996) Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature 379:449–451

Miyakawa Y (1982) Behavioral evidence for the existence of sugar, salt and amino acid recptor cells and some of their properties in *Drosophila* larvae. J Insect Physiol 28:405–410

Python F, Stocker R (2002) Adult-like complexity of the larval antennal lobe of *Drosophila melanogaster* despite markedly low numbers of odorant receptor neurons. J Comp Neurol 445:374–387

Sawin-McCormack EP, Sokolowski MB, Campos AR (1995) Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. J Neurogen 10:119–135

Scherer S (2002) Associative olfactory learning in individually assayed *Drosophila* larvae. Diploma Thesis, University of Fribourg, Fribourg, Switzerland

Scherer S, Stocker RF, Gerber B (2003) Olfactory learning in individually assayed *Drosophila* larvae. Learn Mem 10:217–225

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. J Neurosci 23:10495–10502

Scott K, Brady R Jr, Cravchik A, Morozov P, Rzhetsky A, Zuker C, Axel R (2001) A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. Cell 104:661–673

Tissot M, Stocker RF (2000) Metamorphosis in *Drosophila* and other insects: the fate of neurons throughout the stages. Prog Neurobiol 62:89–111

Tobler PN, Dickinson A, Schultz W (2003) Coding of predicted reward omission by dopamine neurons in a conditioned inhibition paradigm. J Neurosci 23:10402–10410

Tully T, Cambiazo V, Kruse L (1994) Memory through metamorphosis in normal and mutant *Drosophila*. J Neurosci 14:68–74

Waelti P, Dickinson A, Schultz W (2001) Dopamine responses comply with basic assumptions of formal learning theory. Nature 412:43–48

Chapter I.

I.2. Neurogenetics of olfactory associative learning in *Drosophila* larvae

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B

Research

A role for Synapsin in associative learning: The *Drosophila* larva as a study case

Birgit Michels, Sören Diegelmann, Hiromu Tanimoto, Isabell Schwenkert, Erich Buchner, and Bertram Gerber[1]

University of Würzburg, Biocenter, Am Hubland, Department of Genetics and Neurobiology, D 970 74 Würzburg, Germany

Synapsins are evolutionarily conserved, highly abundant vesicular phosphoproteins in presynaptic terminals. They are thought to regulate the recruitment of synaptic vesicles from the reserve pool to the readily-releasable pool, in particular when vesicle release is to be maintained at high spiking rates. As regulation of transmitter release is a prerequisite for synaptic plasticity, we use the fruit fly *Drosophila* to ask whether Synapsin has a role in behavioral plasticity as well; in fruit flies, Synapsin is encoded by a single gene (*syn*). We tackled this question for associative olfactory learning in larval *Drosophila* by using the deletion mutant syn^{97CS}, which had been backcrossed to the Canton-S wild-type strain (CS) for 13 generations. We provide a molecular account of the genomic status of syn^{97CS} by PCR and show the absence of gene product on Western blots and nerve-muscle preparations. We found that olfactory associative learning in syn^{97CS} larvae is reduced to ~50% of wild-type CS levels; however, responsiveness to the to-be-associated stimuli and motor performance in untrained animals are normal. In addition, we introduce two novel behavioral control procedures to test stimulus responsiveness and motor performance after "sham training." Wild-type CS and syn^{97CS} perform indistinguishably also in these tests. Thus, larval *Drosophila* can be used as a case study for a role of Synapsin in associative learning.

Synapsins are phylogenetically conserved and highly abundant presynaptic phosphoproteins associated with the cytoplasmic side of synaptic vesicles. The working model of Synapsin function in synaptic vesicle housekeeping (review by Hilfiker et al. 1999; for a critical review see Sudhof 2004) proposes that the balance between the readily-releasable and the reserve pool of synaptic vesicles, the latter being tethered to the cytoskeleton, is regulated by the phosphorylation status of Synapsins; thus, phosphorylation of Synapsins regulates the number of vesicles available for release. If Synapsin function is compromised, synaptic output per se remains functional, whereas the ability to maintain synaptic output at high, sustained spiking rates is compromised (Chi et al. 2003; Gitler et al. 2004). Given a role in regulating synaptic output, which is a prerequisite for synaptic plasticity, we ask whether Synapsin might have a role in behavioral plasticity as well. This seems timely, because despite much work on the cellular, molecular, developmental, and physiological levels (Angers et al. 2002; Chin et al. 2002; Ferreira and Rapoport 2002; Chi et al. 2003; Gitler et al. 2004; Hilfiker et al. 2005; for reviews see Hilfiker et al. 1999 and Sudhof 2004), the functional significance of Synapsin for behavior remains less well understood. In humans, Garcia et al. (2004) recently found that a mutation in the *synapsin I* gene causes severe neurological and behavioral phenotypes, including epilepsy and learning impairments. In the mouse, Silva et al. (1996) found learning impairments in *synapsin II*, but not *synapsin I* knockout mice; these results correlated with decreased post-tetanic potentiation in *synapsin II*, but not *synapsin I* mutants. In mice lacking all three *synapsin* genes, Gitler et al. (2004) documented that such triple mutants show delayed responses in a number of tested reflexes and diminished ability to hang from a suspended wire; they also noted that these animals show seizures upon disturbance by opening of the cage, reduced levels of piloerection, and difficulties maintaining balance when the cage is shaken. Importantly for the current context, Gitler et al. (2004) reported that in a test for spatial memory in an eight-arm radial maze, these animals performed poorly; reportedly, this phenotype is not due to deficits in motivation or motor ability.

In the genome of the fruit fly *Drosophila melanogaster*, only one *synapsin* gene (*syn*) is found (Klagges et al. 1996), which makes interpretation of phenotypes relatively straightforward. syn^{97} was recently described as carrying a 1.4-kb deletion spanning parts of the regulatory sequence of the *syn* gene and half of its first exon (Fig. 1A). As a consequence, adult syn^{97} mutants lack detectable Synapsin (Godenschwege et al. 2004) and hence—regarding adult flies—qualify as null mutants. Whether this is also true for larvae is at present unknown. In any event, the availability of a null mutant provides an opportunity to test whether behavioral plasticity might depend on Synapsin function. We tackled this question with regard to olfactory associative learning in larval *Drosophila* (Scherer et al. 2003; Hendel et al. 2005; Neuser et al. 2005). Such an endeavor seems timely, as the larva is a widely used model system to study synaptic physiology (Koh et al. 2000).

We exerted much effort in avoiding confounding effects of "marker" genes and genetic background. We outcrossed syn^{97} to the wild-type control strain CS for 13 generations such that the resulting syn^{97CS} and wild-type CS essentially share the same genetic background. Such care is warranted given the effects of genetic background (De Belle and Heisenberg 1996) and of "marker" genes (Zhang and Odenwald 1995), which are often used to monitor the presence of transgenic constructs. We are thus confident that phenotypes in syn^{97CS} are indeed attributable to the syn^{97} mutation and allow conclusions about Synapsin function.

After confirming the genomic status of syn^{97CS} by PCR, we provide a characterization of syn^{97CS} in the larva at the protein

[1]**Corresponding author.**
E-mail bertram.gerber@biozentrum.uni-wuerzburg.de; fax +49 931 888 4452.
Article and publication are at http://www.learnmem.org/cgi/doi/10.1101/lm.92805.

12:224–231 ©2005 by Cold Spring Harbor Laboratory Press ISSN 1072-0502/05; www.learnmem.org

level. We show that in syn^{97CS} immunoreactivity for Synapsin is absent on Western blots and from synaptic boutons at the neuromuscular junction. We then investigated whether syn^{97CS} are defective in olfactory associative learning, and found that learning ability is reduced to ~50% of wild-type CS levels. By introducing two additional, novel "sham training" control procedures, we made a special effort to test whether this learning defect may be secondary to any sensory or motor defects, which we found is not the case.

Results

Larval syn^{97} is null mutant on the protein level

In a single-larva approach, syn^{97CS} showed a PCR product only for that combination of primers which lie up- and downstream of the deletion (primers 1 and 3; Fig. 1A,B), but not for those primers which lie upstream and within the deletion (primers 1 and 2; Fig. 1A,B). In wild-type CS, however, the 1/2 combination gives a product, but the 1/3 combination does not (Fig. 1B). This confirms the genomic status of syn^{97CS} as carrying the reported 1.4-kb deletion of the *syn* gene (Godenschwege et al. 2004).

At the protein level, syn^{97CS} clearly is a null mutant. syn^{97CS} lack Synapsin immunoreactivity on the Western blot: Bands for the expected isoforms of Synapsin at 143 and 74 kDa (Klagges et al. 1996; Godenschwege et al. 2004) were detected in homogenates from larval brains of wild-type CS, but not from syn^{97CS} (Fig. 1C). In Figure 1C, the blot was successively probed with the SYNORF1 antibody and, after stripping, with an antibody labeling the Cysteine String Protein (CSP) (band at 32 kDa) as a loading control.

To verify the absence of Synapsin immunoreactivity in situ, we investigated the synaptic terminals innervating the larval body wall musculature. We focused on the much-investigated muscle pair 6/7 and double-labeled the preparation with the SYNORF1 antibody and, in order to visualize the motorneurons, with an anti-HRP antiserum labeling neuronal cell membranes. Synapsin immunoreactivity was clearly seen in wild-type CS but not in syn^{97CS} (top panels in Fig. 1D). Synapsin immunoreactivity colocalized with HRP immunoreactivity (overlay for wild-type CS in Fig. 1D). Specifically, a magnification of the boxed areas in Figure 1D shows that the membrane at the circumference of the synaptic boutons is stained by the anti-HRP antiserum, whereas Synapsin staining is seen cen-trally in these boutons; at these central sites, Synapsin colocalizes with Synaptotagmin immunoreactivity (Godenschwege et al. 2004), confirming its synaptic localization. Thus, the syn^{97CS} strain obviously carries the genomic deletion of the *syn* gene as reported by Godenschwege et al. (2004) (Fig. 1A,B) and, also at the larval stage, qualifies as a null mutant for Synapsin at the protein level (Fig. 1C,D). In a next step, we therefore asked whether these mutants would be altered in their learning ability.

Larval syn^{97} are impaired in learning

We tested wild-type CS and syn^{97CS} larvae for their ability to associate odors with a fructose reward in an en masse assay (Neuser et al. 2005); we found that both wild-type CS (Fig. 2; one-sample sign test: $P < 0.05$; $n = 27$), and syn^{97CS} (Fig. 2; one-sample sign test: $P < 0.05$; $n = 27$) learn this association; however, wild-type CS learn significantly better than syn^{97CS} (Fig. 2; $P < 0.05$, $U = 233$, sample sizes as above), which show only 50% of the median wild-type CS learning score.

We confirmed this effect in the individual-animal version of this learning paradigm (Scherer et al. 2003; Hendel et al. 2005; Neuser et al. 2005). We found that wild-type CS learn well also in this paradigm (Fig. 3A; one-sample sign test: $P < 0.05$; $n = 39$), whereas syn^{97CS} do not show significant learning (Fig. 3A; one-

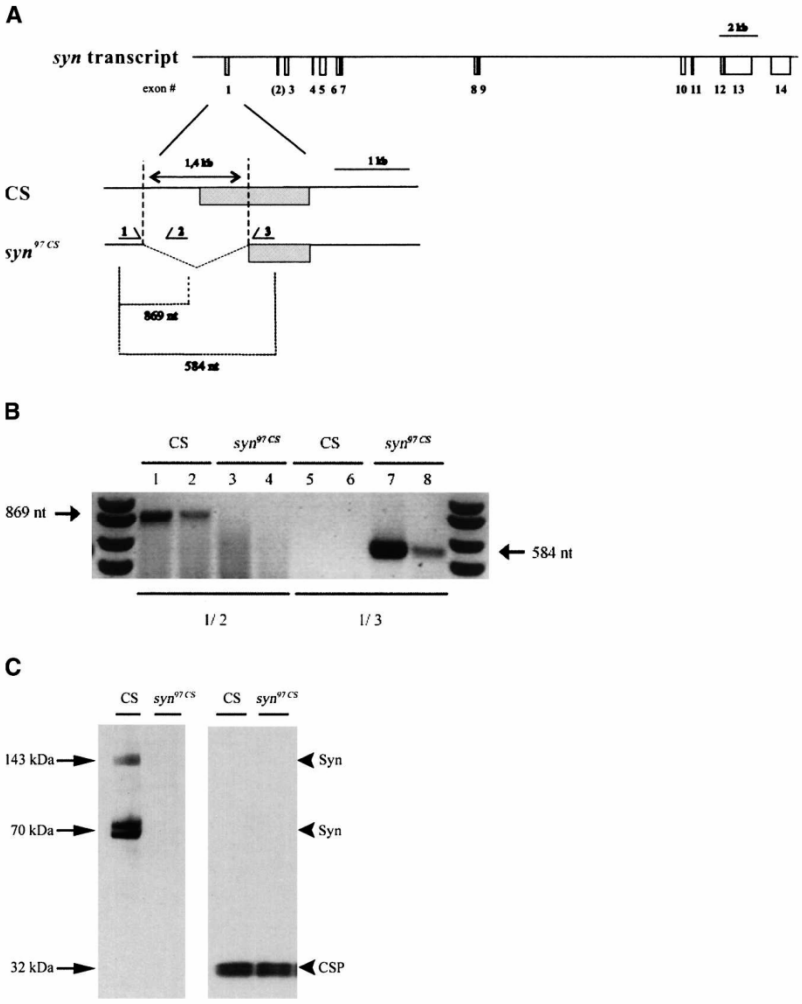


Figure 1. (Continued on next page)

sample sign test: $P > 0.05$; $n = 45$). In a direct comparison, wild-type CS learn significantly better than syn^{97CS} (Fig. 3A; $P < 0.05$, U = 621.5; sample sizes as above), which show a >50% reduction in learning ability.

Behavioral controls: No defect of syn^{97} in canonical, naive animal tests

Low learning scores may, apart from "genuine" defects in learning, result from more general defects in the ability to taste or smell or to behaviorally respond to tastants and odors. The canonical approach to these problems is to compare experimentally naive, untrained animals in terms of their responses to the to-be-associated stimuli. Both wild-type CS and syn^{97CS} larvae show a significant preference for fructose over pure agarose (Fig. 3B; wild-type CS: one-sample sign test: $P < 0.05$; $n = 32$; syn^{97CS}: one-sample sign test: $P < 0.05$; $n = 32$). Importantly, there is no difference between the genotypes with respect to fructose preference (Fig. 3B; $P > 0.05$; U = 509.0; sample sizes as above).

Concerning odors, typically one chooses concentrations for the learning experiments such that naive, wild-type animals distribute about equally between them (Scherer et al. 2003). Therefore, if one would compare naive odor choice between wild-type and mutant, one would "ideally" expect both to be indifferent between the two odors. This indifference, however, may come about for different reasons in the two genotypes: the wild-type may be truly indifferent between the two odors, whereas the mutant may be anosmic. This problem of interpretation is typically avoided by testing olfactory detection ability in an odor versus no-odor setup. In the present case, both wild-type CS and syn^{97CS} larvae show significant attraction to both of the odors used (wild-type CS: for amylacetate (AM) in Fig. 3C, one-sample sign test, $P < 0.05$, $n = 72$; for 1-octanol (OCT) in Fig. 3D, one-sample sign test, $P < 0.05$, $n = 64$) (syn^{97CS}: for AM in Fig. 3C, one-sample sign test, $P < 0.05$, $n = 72$; for OCT in Fig. 3D, one-sample sign test, $P < 0.05$, $n = 60$). Importantly, there is no difference between the genotypes with respect to preference for either odor (for AM: Fig. 3C, $P > 0.05$, U = 2400.0; for OCT: Fig. 3D, $P > 0.05$, U = 1675.5; sample sizes as above). Thus, syn^{97CS} likely are impaired specifically in associating odors with a fructose reward.

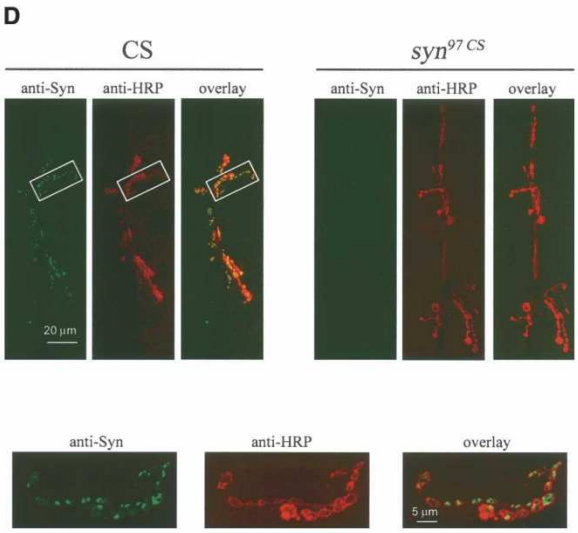


Figure 1. (*A*) Genomic organization of the *Drosophila synapsin* locus. syn^{97CS} carries a 1.4-kb deletion spanning parts of the regulatory sequence and half of the first exon of the *syn* gene. The arrows indicate the binding sites for the PCR primers upstream (primer 1), within (primer 2), and downstream (primer 3) of the deletion. (*B*) syn^{97CS} is a deletion mutant. In a single-larva PCR approach, primer combination 1/2 yields a 869-nt product in wild-type CS (two independent samples in lanes *1,2*) but not in syn^{97CS} (two independent samples in lanes *3,4*), whereas primer combination 1/3 yields a 584-nt product in syn^{97CS} (two independent samples in lanes *7,8*) but not in wild-type CS (two independent samples in lanes *5,6*). (*C,D*) syn^{97CS} lack Synapsin. (*C*) Western blot from brains of larval *Drosophila*. The blot shows separate staining for Synapsin (*left* panel) and, after stripping the blot from the SYNORF1 antibody, for CSP as loading control (*right* panel). The *left* lanes were loaded from wild-type CS, the *right* lane from syn^{97CS}.The SYNORF1 antibody labels bands at 74 and 143 kDa, where Synapsin is expected (Klagges et al. 1996; Godenschwege et al. 2004). These bands represent fused triple and double bands, respectively, and are absent in syn^{97CS}. (*D*) Synapsin localizes to synaptic terminals. Immunofluorescence images of synaptic terminals innervating the larval body wall muscle pair 6/7 using double labeling with the SYNORF1 antibody and, for visualization of the motorneuron terminals, an anti-HRP antiserum. The anti-HRP antiserum stains neuronal cell membranes and thus visualizes motorneuron terminals (*middle* panels for wild-type CS and syn^{97CS} in *D*). Synapsin immunoreactivity is seen exclusively in boutons of wild-type CS (*leftmost* panel for wild-type CS in *D*), where it colocalizes with anti-HRP (*right* panel for wild-type CS in *D*). In syn^{97CS} larvae, no Synapsin immunoreactivity can be found (*left* panel for syn^{97CS} in *D*). The *insets* in the lower part of the figure show magnifications of the area boxed in the *upper* panel; *left* and *middle insets* show Synapsin and HRP labeling, respectively; the *right inset* shows the overlay. Obviously, the membrane of the synaptic boutons is stained by the anti-HRP antiserum; the *center* of these terminals shows Synapsin immunoreactivity.

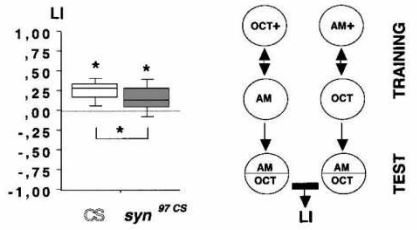


Figure 2. syn^{97CS} larvae are impaired in learning: en masse assay. In an en masse assay for olfactory associative learning, syn^{97CS} show ~50% of the learning index (LI) of wild-type CS. The *inset* figure illustrates the behavioral procedure; please note that in half of the cases we started training with OCT+ or AM+ as indicated; for the other half of the cases, we started training with AM or OCT. *: $P < 0.05$. Box plots represent the median as the *middle* line, 25% and 75% quantiles as box boundaries, as well as 10% and 90% quantiles as whiskers, respectively.

Two novel behavioral controls: No defect of syn^{97} after "sham training"

Clearly, learning can be measured only after training. Therefore, rather than testing experimentally naive animals, one may argue that the olfactory and motor abilities which the animals need during testing must be investigated (as no gustatory abilities are required during testing, this objection does not apply concerning taste). This is because such training by necessity encompasses handling, exposure to reinforcers, and exposure to odors, all of which may alter odor responsiveness on their own behalf (see Discussion). In particular, handling and/or stimulus exposure may render mutants unresponsive to odors, an effect that may feign a "learning" phenotype in such mutants. We therefore tested whether syn^{97CS} are still able to detect and respond to the odors after either of two "sham-training" treatments. These do not involve associative training but the very same handling as during training plus (1) exposure to the odors (but not the reinforcer); (2) exposure to the reinforcer (but not the odors). We found that responses to either odor are equal between wild-type

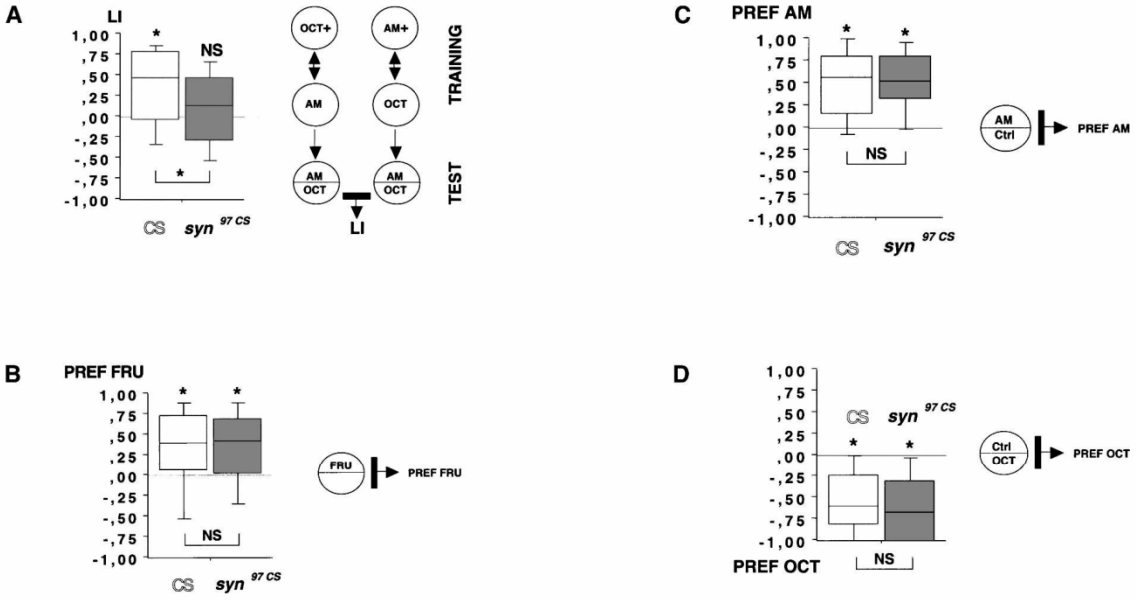


Figure 3. (*A*) Confirming the learning deficit in syn^{97CS} larvae in an individual-animal version of the learning assay. Learning in syn^{97CS} is reduced to <50% of wild-type CS levels. (*B*,*C*,*D*) Behavioral controls in naive larvae. Responses to the positive reinforcer (*B*: FRU) and detection of the used odors (*C*,*D*: AM and OCT, respectively) are not different between genotypes; thus, the learning impairment in syn^{97CS} is not due to deficits in detecting the to-be-learned stimuli. All experiments used individually assayed larvae. *Insets* in each figure illustrate the behavioral procedure. *: $P < 0.05$. For explanation of the box plots, see Figure 2 legend.

CS and syn^{97CS} after both sham training with odor exposure (for AM responses: Fig. 4A, $P > 0.05$ U = 786, n = 40, 40; for OCT responses: Fig. 4B, $P > 0.05$, U = 1053.5, n = 48, 48) and after "sham training" with reward exposure (for AM responses: Fig. 4C, $P > 0.05$ U = 1014.5, n = 48, 48; for OCT responses: Fig. 4D, $P > 0.05$, U = 1103.5, n = 48, 48). These results argue that the learning deficit in syn^{97CS} is not secondary to an altered susceptibility to effects of handling, odor exposure, or reward exposure. As the motor abilities that are required for odor detection after sham training are the same as those required to express memory after training, these results also argue that no critical motor abilities are impaired in syn^{97CS}.

After pooling the data in Figure 4 across genotypes, it is obvious that OCT responses are lower after odor-exposure sham training compared to reward-exposure sham training (pooled data from Fig. 4B vs. pooled data from Fig. 4D; $P < 0.05$, U = 2005, n = 96, 96); the same effect is, albeit less obviously, seen for AM as well (pooled data from Fig. 4A vs. pooled data from 4C; $P < 0.05$, U = 2817.5, n = 80, 96). Statistical comparisons to naive odor responses (Fig. 3C,D) are not possible, as the data in Figures 3 and 4 were gathered some months apart; therefore in a formal sense it must remain an open question whether this effect represents an increase from naive odor responses due to reward-exposure sham training, or a decrease from naive odor responses due to odor-exposure sham training. Contemplating Figure 3D versus Figure 4B, though, the latter possibility seems the better guess. In any event, whatever the reason(s) for this effect (habituation, adaptation, changes in motivation, changes in the concentration of the odors), three points are important to note: first, there is no reason to question the interpretation of the learning index as a pure measure of associative learning. This is because the learning index reflects the difference between reciprocally trained groups, and the change in odor responses by necessity will happen in both these reciprocally trained groups. In other words, an effect that occurs in *both* groups cannot cause differences *between* them. Second, obviously sham training does have effects on odor responses; thus, it is necessary to control for possible between-genotype differences in these effects because they could feign "learning phenotypes." Third and most important for the present study, wild-type CS and syn^{97CS} are equal in terms of both naive odor responses (Fig. 3C,D), and in terms of odor responses after sham training (Fig. 4A–D). This means that any changes in odor responses that come along with training affect both genotypes in the same way. Thus, the difference between the genotypes in their learning ability (Figs. 2, 3A) cannot be secondary to differences in terms of changed odor responses.

Discussion

We report that syn^{97CS} is a protein-null mutant at the larval stage (Fig. 1C,D), and that associative learning in syn^{97CS} larvae is reduced to ~50% of wild-type CS levels (Figs. 2, 3A). Concerning the behavioral specificity of this learning defect, we tested experimentally naive, untrained animals in terms of their responses to the to-be-associated stimuli and found no difference between wild-type CS and syn^{97CS} (Fig. 3B,C,D). This shows that at the beginning of training, genotypes are equal with respect to their olfactory ability and thus have the same ability to establish odor memories. These kinds of behavior-specificity controls have been state of the art until to date. We took an extra effort and compared olfactory behavior in wild-type CS and in syn^{97CS} after "sham training," i.e. after (1) handling and exposure to the odors; (2) handling and exposure to the reinforcer. These procedures seem critical to evaluate whether in syn^{97CS} handling or stimulus exposure may deteriorate olfactory or motor abilities, as they are required to express memory during test. That is, handling may deteriorate motivation, lead to fatigue, and/or change the value of odors; repeated odor exposure may reduce olfactory responses by sensory adaptation (Cobb and Domain 2000) or habituation (concerning adult flies: Cho et al. 2004), and sugar

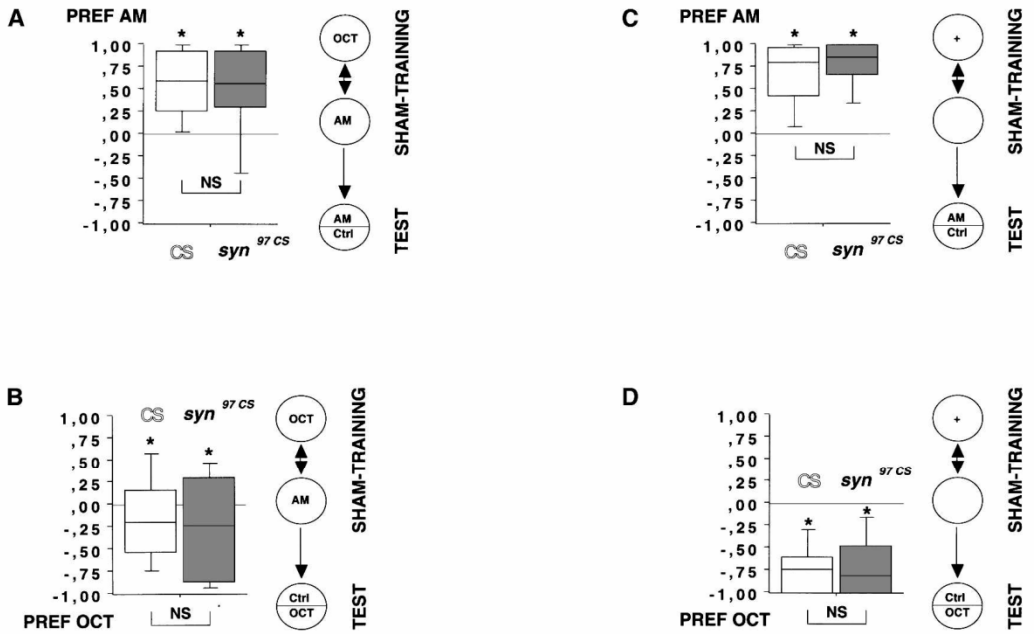


Figure 4. (*A–D*) No genotype differences after sham training. The two sham training procedures involve the same training procedure as shown in the Fig. 3A *inset*, except that either the reinforcer (*A,B*) or the odors (*C,D*) were omitted. After sham training, animals were tested for their ability to detect AM (*A,C*) and OCT (*B,D*), respectively. In neither of the sham training experiments did we uncover any difference between wild-type CS and syn^{97CS}. All experiments used individually assayed larvae. *Insets* in each figure illustrate the behavioral procedure. *: $P < 0.05$. For explanation of the box plots, see Figure 2 legend.

exposure may entail motivational changes which distort olfactory behavior (for an analogous effect of electric shock in adult flies, Preat 1998). However, wild-type CS and syn^{97CS} did not differ in odor responses after either sham training regime (Fig. 4A–D); thus, the low learning scores in syn^{97CS} reflect a genuine learning defect.

Gross brain anatomy (data not shown), as well as basic synaptic function (measured by excitatory junction potentials at the neuromuscular junction), the number of synaptic boutons on muscles 6/7 and 12/13, and the number of synaptic vesicles around the active zone of type Ib synapses on these muscles are unaltered in syn^{97} (Godenschwege et al. 2004). Together, these data suggest a specific contribution of Synapsin for behavioral associative plasticity in *Drosophila* larva.

In adult *Drosophila*, a phenotype of syn^{97CS} in odor-shock learning is more moderate than in larvae, i.e., adult syn^{97CS} retain ~80% of wild-type CS learning levels (Godenschwege et al. 2004). Together with our data, this supports the notion that relatively low levels of learning can be achieved without Synapsin; beyond that level, however, Synapsin is needed. In any event, the common, yet unequally strong, associative learning phenotypes of syn^{97CS} across different learning paradigms and across the stages of metamorphosis suggest a rather general contribution of Synapsin to associative plasticity.

With respect to synaptic plasticity, there is at present no way to directly and in vivo observe synaptic plasticity in central brain neurons in *Drosophila*—one is limited to observing synaptic plasticity at the larval neuromuscular junction (Koh et al. 2000). Inferences from plasticity phenomena at the larval motorneuron-to-muscle synapse to central brain synapses, however, are of debatable value. Still, the robust larval learning phenotype of syn^{97CS} reported here lays the foundation for three lines of doable further research: (1) When and in which parts of the brain would transgenic expression of the wild-type protein be sufficient to restore learning in the mutant background, and where is Synapsin function necessary in the normal brain? (2) Which functional domains of Synapsin are playing a role (Hilfiker et al. 2005), in particular with respect to the putative phosphorylation sites of the protein? (3) Which role does the editing of the *syn* mRNA (Diegelmann et al. 2003) play in this respect? The latter two questions may be relevant for Synapsin function in general, and in particular may contribute to our understanding of Synapsin-dependent forms of epilepsy and learning impairments in mice (Silva et al. 1996; Gitler et al. 2004) and humans (Garcia et al. 2004). Along these lines, research on *Drosophila*, including the larva, should be helpful.

Materials and Methods

We used third-instar feeding stage larvae aged 5 d after egg laying. Flies were kept in mass culture and maintained at 25°C, 60%–70% relative humidity, and a 14/10-h 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 compared wild-type CS larvae to the deletion mutant syn^{97CS}. This strain was generated by 13 outcrossing steps from syn^{97}, which had been obtained from a jump-out mutagenesis of Syn^{P1+P2} (Godenschwege et al. 2004). This jump-out line is characterized by a 1.4-kb deletion spanning parts of the regulatory sequence and half of the first exon of the *syn* gene (Fig. 1A). The outcrossing regime ensured that the residual phenotypic markers for the presence of the P-element in syn^{97} were removed and that the genetic background of syn^{97} and wild-type CS is essentially the same. Outcrossing steps always involved several single couples of heterozygous syn^{97}/CS crossed with CS/CS flies. This resulted in a first filial generation where the genotype of each

individual is unknown, but which consists again of syn^{97}/CS and CS/CS flies. These were mated in single pairs to wild-type CS, and the genotype of the questionable part among the parents was determined via single-fly PCR (Gloor et al. 1993). Finally, single crossings among the heterozygous progeny resulted in the newly established strain syn^{97CS}. Heterozygous flies could be identified as they show two PCR products, in contrast to the homozygous wild-type CS and syn^{97CS}.

Single-animal PCR

PCRs were carried out according to Gloor et al. (1993), using material from individual larvae. The primer binding sites were upstream (primer 1: 5′-AGAAAATTTGGCTTGCATGG-3′), within (primer 2: 5′-CGGGGTCTCAGTTTTGTTG-3′), or downstream (primer 3: 5′-CCTCTACTTTTGGCTGCCTG-3′) of the deletion (Fig. 1A). The primer pair 1/2 gives an 869-nucleotide product in only wild-type CS, whereas primer pair 1/3 results in a 584-nucleotide product in only syn^{97CS} flies (because in wild-type CS the template is too long for amplification).

Western blot

For each lane in the Western blots, 10 larval brains were homogenized in 10 µL 2 × SDS gel loading buffer; whole-larva homogenates do not yield a signal in Western blots because of insufficient protein concentration and/or degradation by proteases. The sample was heated to 70°C for 5 min and centrifuged for 2 min before electrophoresis. Proteins were separated by 8.5% SDS-PAGE in a Multigel chamber (150 V, 3 h; Biometra) and transferred to nitrocellulose membranes (Khyse-Andersson 1984). Immunoreactions were successively performed with two monoclonal antibodies: SYNORF1 for Synapsin detection (Klagges et al. 1996) (dilution 1:100), and ab49 (Zinsmaier et al. 1990, 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) according to the manufacturer's specifications. We stripped and reprobed the blot: The membrane was first stained for Synapsin, then incubated for 30 min in stripping buffer to remove the SYNORF1 antibody (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris HCL pH 6.8; 58°C), and only then probed for CSP as loading control. To reduce the background staining of the membrane, in both staining steps the antibodies were dissolved in blocking buffer (5% milk powder in 1 × TBST).

Immunohistochemistry

For double immunofluorescence analyses, larval body wall muscles were dissected in Ca^{2+}-free saline (Stewart et al. 1996) and fixed in ice-cold 4% paraformaldehyde for 30 min. The preparations were washed in PBS/0.1% Triton (PBST) followed by a 1-h incubation with blocking solution, and then incubated overnight at 4°C with the monoclonal anti-Synapsin mouse antibody SYNORF1 (diluted 1:10). The primary antibody was detected after 1 h incubation with Alexa 488 goat antimouse Ig (diluted 1:250) (green); during that step, the preparation was coincubated with a Texas Red-coupled rabbit anti-HRP antibody (diluted 1:200) (Jackson labs) (red). All incubation steps were followed by multiple PBST washes; the detector incubation step was performed under light protection. Finally, preparations of the larvae were examined under a confocal microscope, aiming at muscle pair 6/7 of the body wall and its innervation by motor neurons.

Learning experiments

Methods for learning experiments follow previous work (Scherer et al. 2003; Hendel et al. 2005; Neuser et al. 2005) (see insets of Figs. 2A and 3A for sketches of the learning paradigm). In brief, we trained groups of 30 larvae and compared olfactory choice performance after either of two reciprocal training regimes: For one regime, animals received amylacetate (AM) with a positive reinforcer and 1-octanol (OCT) without such reinforcer (AM+/OCT); for the second regime, animals were trained reciprocally (AM/OCT+). Then, animals were tested for their choice between AM versus OCT. Associative learning is indicated by systematic differences in test performance between the reciprocal treatment conditions. This conclusion is compelling, as during training animals from both training regimes had identical exposure to both odorants and the reward; what differs between them is solely the contingency between these stimuli. The reciprocally trained groups were run alternately, which allows stringent pairing of data for the calculation of a learning index (LI; see below).

Petri dishes (85-mm inner diameter; Sarstedt) were filled with 1% agarose (electrophoresis grade; Roth), allowed to solidify, covered with their lids, and then left untreated until the following day. As positive reinforcer we used 2 mol fructose (FRU, purity: 99%) added to 1 L of agarose 10 min after boiling.

Experiments were performed in red light under a fume hood at 21°–24°C. Before experiments, we replaced the regular lids of the petri dishes 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, briefly washed in tap water, and as a group transferred to the assay plates for the start of training. Each training trial lasted 1 min. Immediately before a trial, two containers loaded with the same odorant (for details see below) were placed on the assay plate on opposite sides of the plate, 7 mm from the edges. Within each reciprocal training condition, for half of the cases we started with AM, for the other with OCT. Thus, for half of the cases we started with an agarose plate that had FRU added to the substrate, and for the other we started with a plate without FRU. Then, the lid was closed and the larvae were allowed to move for 1 min. The larvae were then transferred to a plate with the alternative odorant and the respective other substrate for 1 min. This cycle was repeated three times. Fresh assay plates were used for each trial.

After this training, animals were tested for their odor choice. The larvae were placed in the middle of a fresh, pure agarose assay plate with a container of AM on one side and one of OCT on the other side to create a choice situation. After 3 min, the number of animals on the "AM" or "OCT" side was counted. After this test was completed, the next group of animals was run and trained reciprocally. For both groups, we then calculated an odor preference ranging from −1 to 1. We determined the number of animals observed on the AM side ($\#_{AM}$) minus the number of animals observed on the OCT side ($\#_{OCT}$), divided by the total number of larvae ($\#_{TOTAL}$):

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

To determine whether these preferences are different depending on training regime, we took the paired data from the alternately run, reciprocally trained groups and calculated a learning index ranging from −1 to 1 as:

$$LI = (PREF_{AM+/OCT-} - PREF_{AM/OCT+})/2 \quad (2)$$

After the data for one such LI value in one genotype had been collected, the corresponding data for an LI value of the other genotype were gathered, i.e., data from both genotypes were obtained alternately. In a conservative approach, we used nonparametric analyses throughout; comparisons of LIs against zero, i.e., random level, were made with one-sample sign tests, and comparisons of LIs between two genotypes were done with Mann-Whitney U-tests.

Regarding olfactory stimuli, we followed previous work (Scherer et al. 2003; Hendel et al. 2005; Neuser et al. 2005) and used OCT (purity: 99.5%) and AM (purity: 99%, diluted 1:50 in paraffin oil). Odorant was applied by adding 10 µL of odor substance into Teflon containers (5-mm inner diameter) which could be closed by a perforated lid (seven holes, 0.5-mm diameter).

We wanted to back up our results in the paradigm of Scherer et al. (2003), which used individually assayed animals. This assay differs from the above en masse assay introduced by Neuser et al.

(2005) in that (1) a group of eight, rather than 30, was trained; (2) a 1-min break was introduced between training trials; (3) 10, rather than three training trials were given. Most importantly, (4) the test was performed on individual animals. Following Scherer et al. (2003), the position of the individual larva during the test was noted every 20 sec for 5 min as "AM," "OCT," or "neutral" (a 7-mm-wide zone in the middle of the assay plate). To calculate the odor preferences for each animal we determined the number of times a given animal was observed on the AM side during the test minus the number of times that animal was observed on the OCT side, divided by the total number of observations. For calculating the LI, we took the pairs of individuals from either of the two training conditions and calculated analogous to equation 2. These data were then statistically compared as detailed above.

Controls for detection of FRU and the odors

In corresponding control assays, we determined the ability of individually assayed animals to detect FRU and the odors. To test the ability to detect FRU, we prepared split petri dishes according to Heimbeck et al. (1999), with one side pure agarose and the other with FRU added to the agarose (for a sketch, see Fig. 3B inset). To test the ability of larvae to detect the odorants used, we took experimentally naive animals and gave them the choice between either paraffin-diluted AM versus paraffin, or between undiluted OCT versus an empty container (for sketches, see Fig. 3C,D insets). For both FRU detection and odor detection, animals were assayed individually; data acquisition, calculation of the PREF values, and data analysis follow the procedure for the odor choice test in individual animals detailed in the preceding paragraph.

Two novel "sham training" controls

Additionally, we introduced two novel sham training controls (see Discussion for a more detailed description of the motivation for these experiments). This seemed warranted to test whether genotype differences in learning may be secondary to differences in the susceptibility to odor or reward exposure. Therefore, we determined the ability of individually assayed animals from both genotypes to detect the odors after either of two sham training treatments. The first tests for genotype-differences with respect to the effects of odor exposure: it consists of the same treatment as in the individual animal learning assay, except that the reinforcer was omitted (for a sketch, see Fig. 4A,B insets). The second tests for differences in terms of reward exposure: it also consists of the same treatment as in the learning assay, but in turn omits the odors (for a sketch, see Fig. 4C,D insets). The tests for odor detection after either kind of sham training involved choices either between paraffin-diluted AM versus paraffin, or between undiluted OCT versus an empty container.

All statistical analyses were performed with StatView on a MacIntosh (significance: $P < 0.05$).

Acknowledgments

Start-up funds for this research program were provided by the VolkswagenFoundation (I/76 240, to B.G.). Current support comes from the Deutsche Forschungsgemeinschaft (SFB 554-A2 and Graduiertenkolleg GRK 200 Arthropod Behavior to E.B.; SFB 554-B6 to M. Heisenberg in support of B.G.), the International Human Frontiers Science Program Organization (long-term fellowship to H.T.), and the Studienstiftung des Deutschen Volkes (to I.S.). Thanks to K. Gerber for assistance with and B. Poeck for advice concerning the PCR experiments; to M. Porsch for advice to improve Western blotting; to colleagues from the Würzburg lab, in particular D. Dudazek, K. Neuser, X.-b. Mao, and A. Yarali for many helpful contributions. Finally, special thanks go to the referee who pointed to adaptation/habituation as a source of the *synapsin* phenotype and who thus inspired the sham training controls.

Dedicated to the memory of U. Werner.

References

Angers, A., Fioravante, D., Chin, J., Clearly, L.J., Bean, A.J., and Byrne, J.H. 2002. Serotonin stimulates phosphorylation of *Aplysia* synapsin and alters its subcellular distribution in sensory neurons. *J. Neurosci.* **22:** 5412–5422.

Arnold, C., Reisch, N., Leibold, C., Becker, S., Prüfert, K., Sautter, K., Palm, D., Jatzke, S., Buchner, S., and Buchner, E. 2004. Structure-function analysis of the cysteine string protein in *Drosophila*: Cysteine string, linker and C terminus. *J. Exp. Biol.* **207:** 1323–1334.

Chi, P., Greengard, P., and Ryan, T.A. 2003. Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. *Neuron* **38:** 69–78.

Chin, J., Angers, A., Clearly, L.J., Eskin, A., and Byrne, J.H. 2002. Transforming growth factor β 1 alters synapsin distribution and modulates synaptic depression in *Aplysia*. *J. Neurosci.* **22:** RC220.

Cho, W., Heberlein, U., and Wolf, F.W. 2004. Habituation of an odorant-induced startle response in *Drosophila*. *Genes Brain Behav.* **3:** 127–137.

Cobb, M. and Domain, I. 2000. Olfactory coding in a simple nervous system: Adaptation in *Drosophila* larvae. *Proc. Royal Soc. London (B)* **267:** 2119–2125.

De Belle, J.S. and Heisenberg, M. 1996. Expression of *Drosophila* mushroom body mutations in alternative genetic backgrounds: A case study of the mushroom body miniature gene (*mbm*). *Proc. Natl. Acad. Sci.* **93:** 9875–9880.

Diegelmann, S., Werner, U., Godenschwege, T.A., Reisch, D., Putz, G., Schwaerzel, M., and Buchner, E. 2003. Molecular and phenotypical characterization of the *Drosophila synapsin* mutant. In *The Neurosciences from research to therapy. Proceedings of the 29th Göttingen Neurobiology Conference.* (eds. N. Elsner and H. Zimmermann). Abstract 759. Thieme, Stuttgart, New York.

Ferreira, A. and Rapoport, M. 2002. The synapsins: Beyond the regulation of neurotransmitter release. *Cell. Mol. Life Sci.* **59:** 589–595.

Garcia, C.C., Blair, H.J., Seager, M., Coulthard, A., Tennant, S., Buddles, M., Curtis, A., and Goodship, J.A. 2004. Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy. *J. Med. Genet.* **41:** 183–186.

Gitler, D., Takagishi, Y., Feng, J., Ren, Y., Rodriguiz, R.M., Wetsel, W.C., Greengard, P. and Augustine, G.J. 2004. Different presynaptic roles of synapsins at excitatory and inhibitory synapses. *J. Neurosci.* **24:** 11368–11380.

Gloor, G.B., Preston, C.R., Johnson-Schlitz, D.M., Nassif, N.A., Phillis, R.W., Benz, W.K., Robertson, H.M., and Engels, W.R. 1993. Type I repressors of P element mobility. *Genetics* **135:** 81–95.

Godenschwege, T.A., Reisch, D., Diegelmann, S., Eberle, K., Funk, N., Heisenberg, M., Hoppe, V., Hoppe, J., Klagges, B.R.E., Martin, J.R., et al. 2004. Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *Eur. J. Neurosci.* **20:** 611–622.

Hendel, T., Michels, B., Neuser, K., Schipanski, A., Kaun, K., Sokolowski, M.B., Marohn, F., Michel, R., Heisenberg, M., and Gerber, B. 2005. The carrot, not the stick: Appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larvae. *J. Comp. Physiol. (A)* **191:** 265–279.

Heimbeck, G., Bugnon, V., Gendre, N., Häberlin, C., and Stocker, R.F. 1999. Smell and taste perception in *Drosophila melanogaster* larva: Toxin expression studies in chemosensory neurons. *J. Neurosci.* **19:** 6599–6609.

Hilfiker, S., Benfenati, F., Doussau, F., Nairn, A.C., Czernik, A.J., Augustine, G.J., and Greengard, P. 2005. Structural domains involved in the regulation of transmitter release by synapsins. *J. Neurosci.* **25:** 2658–2669.

Hilfiker, S., Pieribone, V.A., Czernik, A.J., Kao, H.-T., Augustine, G.J., and Greengard, P. 1999. Synapsins as regulators of neurotransmitter release. *Phil. Trans. Royal Soc. (B)* **354:** 269–279.

Khyse-Anderson, J. 1984. Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Meth.* **10:** 203–209.

Klagges, B., Heimbeck, G., Godenschwege, T.A., Hofbauer, A., Pflugfelder, G.O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S., and Buchner, E. 1996. Invertebrate synapsins: A single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* **16:** 3154–3165.

Koh, Y.H., Gramates, L.S., and Budnik, V. 2000. *Drosophila* larval neuromuscular junction: Molecular components underlying synaptic plasticity. *Micros. Res. Tech.* **49:** 14–25.

Littleton, J.T., Bellen, H.J., and Perin, M.S. 1993. Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. *Development* **118:** 1077–1088.

Neuser, K., Husse, J., Stock, P., and Gerber, B. 2005. Appetitive olfactory learning in *Drosophila* larvae: Testing for effects of training amount, reinforcer intensity, age, gender, assay type, and memory span. *Anim. Behav.* **69:** 891–898.
Preat, T. 1998. Decreased odor avoidance after electric shock in *Drosophila* mutants biases learning and memory tests. *J. Neurosci.* **18:** 8534–8538.
Scherer, S., Stocker, R.F., and Gerber, B. 2003. Olfactory learning in individually assayed *Drosophila* larvae. *Learn. Mem.* **10:** 217–225.
Silva, A.J., Rosahl, T.W., Chapman, P.F., Marowitz, Z., Friedman, E., Frankland, P.W., Cestari, V., Cioffi, D., Sudhof, T.C., and Bourtchuladze, R. 1996. Impaired learning in mice with abnormal short-lived plasticity. *Curr. Biol.* **6:** 1509–1518.
Stewart, B.A., Schuster, C.M., Goodman, C.S., and Atwood, H.L. 1996. Homeostasis of synaptic transmission in *Drosophila* with genetically altered nerve terminal morphology. *J. Neurosci.* **16:** 3877–3886.
Sudhof, T.C. 2004. The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27:** 487–507.
Zhang, S.D. and Odenwald, W.F. 1995. Misexpression of the white (w) gene triggers male–male courtship in *Drosophila*. *Proc. Natl. Acad. Sci.* **92:** 5525–5529.
Zinsmaier, K.E., Hofbauer, A., Heimbeck, G., Pflugfelder, G.O., Buchner, S., and Buchner, E. 1990. A cysteine-string protein is expressed in retina and brain of *Drosophila*. *J. Neurogenet.* **7:** 15–29.
Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N., and Benzer, S. 1994. Paralysis and early death in cysteine string protein mutants of *Drosophila*. *Science* **263:** 977–80.

Received July 22, 2004; accepted in revised form March 22, 2005.

Chapter I.

I.3. Memory trace localization

Michels B, Tanimoto H, Engmann O, Saumweber T, Gerber B

INTRODUCTION

Associative, predictive learning is an essential function of the brain, enabling animals to for example use food-predicting stimuli to guide their food-search behaviour. Given the fierce competition for food sources in natural environments, such ability is of obvious selective advantage. Clearly, identification of the molecular machinery underlying these learning processes, and the neuronal circuits in which these molecules act, is essential to understand this ability. The fruit fly *Drosophila melanogaster* is a suitable organism in this regard, as it allows experimental access on the molecular, cellular, and behavioural level.

On the behavioural level, *Drosophila* can manage a range of well-defined learning tasks, both as larva and as adult fly. The most frequently used learning tasks for adult flies are visual learning, heat-box conditioning and odour-shock associative learning (reviewed in Heisenberg and Gerber, 2008). For visual learning, individual flies are tethered to a torque meter such that they can control their visual surround by their yaw torque (Heisenberg and Wolf, 1984, 1988; Brembs and Heisenberg, 2000), in other words, the flies can choose their 'flight direction' relative to visual landmarks. If in such a set-up particular flight directions are associated with heat punishment, the flies will subsequently avoid these flight directions. In heat-box conditioning, flies are trained to avoid one side of a narrow chamber by associating it with heat punishment, such that subsequently they restrict their movement to one chamber-half (Putz and Heisenberg, 2002; Putz et al., 2004; Diegelmann et al., 2006a). In odour-shock associative learning, groups of about 100 flies receive presentations of one odour paired with electroshock, whereas another odour is presented without shock. Subsequently, flies avoid the previously punished odour in a T-maze (Tully and Quinn, 1985). In the larva, the previously published odour-shock associative learning paradigm for *Drosophila* larvae (Aceves-Pina and Quinn, 1979; Heisenberg et al., 1985 but see Forbes, 1993 for unsuccessful attempts of replication) was in the last few years complemented by a robust odour-taste learning paradigm (Scherer et al., 2003; Hendel et al., 2005; Neuser et al., 2005; Schipanski et al., 2008; Niewalda et al., 2008) as well as a visual learning paradigm (Gerber et al., 2004a) which also uses tastants as reinforcer.

On the cellular level, the circuits underlying these learning tasks are amenable to fine-grained analyses, thanks to the Gal4-UAS method (Brand and Perrimon, 1993): The yeast transcription factor Gal4, whose spatial and temporal expression is controlled by a flanking genomic enhancer, determines the cellular pattern of effector gene expression, whereas the

UAS element determines the kind of effector transgene expressed. The transgenically expressed proteins then can e.g. block neurotransmitter release (*shibire*^{ts1} [*shi*^{ts1}]: a temperature-sensitive dominant-negative Dynamin blocking endocytosis during synaptic vesicle recycling [Kitamoto, 2001, 2002]; *tetanus toxin light chain* [*TNT*]: a protease specifically cleaving neuronal synaptobrevin [Sweeney et al., 1995; Martin et al., 2002]) or induce cell death (*reaper* [*rpr*]: induces apoptosis by activating the caspase proteolytic cascade [White and Steller, 1995; Bergmann et al., 2003; Hay et al., 2004]; *diphtheria toxin A* [*DTA*]: an inhibitor of protein synthesis by ribosylating elongation factor-2 [Wilson and Collier, 1992]). Additionally, to induce the effector gene specifically in the adult, a temperature-sensitive mutant of *Gal80* (*Gal80ts*) has been generated to control the Gal4/UAS gene expression system temporally (McGuire et al., 2003). Finally, using the binary Gal4/UAS expression system, RNAi-inducing transgenes can be used to target gene inactivation to specific cell types.
With this array of techniques available, expression or suppression of any protein or peptide in any cell group and at any time point is feasible, and can be used to unravel learning and memory processes.

On the molecular and genetic level, earlier mutagenesis screens used feeding of ethyl methanesulfonate (EMS) and characterization of strains defective in learning and memory. By such screens, mutations in genes such as *dunce* (Byers et al., 1981), *turnip* (Duerr and Quinn, 1982), *Ddc* (Livingstone and Tempel, 1983; Tempel et al., 1984)and *rutabaga* (Livingstone et al., 1984) were discovered. One common denominator of many of these mutants is that they are part of the AC-cAMP-PKA pathway (Byers et al., 1981; Livingstone et al., 1984). It has been proposed that the type I adenylyl cyclase (AC) acts as a molecular coincidence detector in associative learning. In flies the adenylyl cyclase is encoded by the *rutabaga* gene; in analogy to what has been found in *Aplysia* (Dudai et al., 1988; Abrams et al., 1998), the working model is that the Rutabaga cyclase is activated by the joint presentation of odour and electric shock: The odour leads to Ca^{2+} influx and activation of calmodulin, whereas the shock leads to an activation of dopaminergic neurons (Riemensperger et al., 2005) and the activation of a G-protein coupled dopamine receptor (Hauser et al., 2006). Only the joint activation of these two pathways is thought to be sufficiently activating the Rutabaga cyclase, such that the cAMP-PKA cascade can be triggered, the respective effector proteins be phosphorylated, and hence ultimately the input-output characteristics of the neuron be altered. Notably, the role of the AC-cAMP-PKA cascade for neuronal plasticity has subsequently been confirmed for a

variety of organisms throughout the animal kingdom (Yin and Tully, 1996; Kandel, 2001); however, in most cases the downstream processes, that is: the actual effector proteins which are phosphorylated by PKA, remain to be discovered.

Given these possibilities of experimental access to learning and memory in *Drosophila*, one can address one of the central questions in the field, which is whether and in which sense there is a division of labour among parts of the brain with respect to memory function. More specifically, one can ask whether it is possible to localize a memory trace to specific cells in the brain. For such an endeavour, (Gerber et al., 2004b) have suggested five guide-post criteria; that is, if a memory trace is located in a specific set of cells, it should be possible to show that:

(i) neuronal plasticity occurs in these cells;
(ii) such plasticity in these cells is sufficient and
(iii) necessary for memory;
(iv) memory is abolished if these cells cannot provide output during testing;
(v) memory is abolished if these cells cannot receive input during training.

Based on these criteria, it has been suggested that, concerning odour-shock associative learning, the underlying short-term memory trace is localized to the so-called mushroom bodies. That is, olfactory sensory neurons on the *Drosophila* 3rd antennal segment and maxillary palp each express essentially a single olfactory receptor protein that determines its ligand profile. All those, and only those, sensory neurons expressing a given receptor project into a common spherical structure (‘glomerulus’) in the antennal lobe. Within this glomerulus, they connect with a network of extrinsic, modulatory interneurons, excitatory or inhibitory local interneurons, and the output elements of the antennal lobe (the projection neurons) (Sachse and Galizia, 2002; Wilson and Laurent, 2005). Notably, the input branches of individual projection neurons, as the output branches of the sensory neurons, typically are confined to a single glomerulus each (but see below). These projection neurons send their axons to two target areas: The lateral horn and the mushroom bodies (those projection neurons which collect input from multiple glomeruli bypass the mushroom bodies and project to only the lateral horn [Heimbeck et al., 2001]). Although the exact connectivity towards premotor neurons is not yet described comprehensively (Ito et al., 1998; Tanaka et al., 2008), it is commonly suggested that both the lateral horn and the mushroom bodies then provide

input to premotor areas. Thus, olfactory behaviour can be supported either by a direct lateral horn-pathway, or via a side-loop through the mushroom bodies.

Interestingly, chemically ablating the mushroom bodies has little if any effect upon olfactory behaviour in experimentally naïve flies (de Belle and Heisenberg, 1994), but disabling synaptic output from the projection neurons does impair such responses (Heimbeck et al., 2001). Thus, flies with an intact direct olfactory pathway are largely unimpaired in naïve odour responses, whereas flies in which both the direct lateral-horn-pathway and the indirect mushroom body-pathway are severed, are impaired. When, however, learned odour responses are probed in flies with ablated mushroom bodies, drastic impairments are found (de Belle and Heisenberg, 1994). This suggests that the direct lateral horn-pathway serves for innate, experimentally naïve odour responses, whereas the indirect mushroom body-pathway serves for learned odour responses. These reasonings were the starting point for asking whether the olfactory memory trace for such learned odour responses, i.e. concerning the short-term odour-shock memory trace, indeed is localized to the mushroom bodies. Following the above-mentioned criteria, this does seem to be the case:

(i), (ii) Genetic intervention at various steps of the AC-cAMP-PKA signalling cascade leads to impairments both of synaptic plasticity at the neuromuscular junction (Renger et al., 2000), and to impairments in learning ability (Davis et al., 1995; Davis, 1996). This indirectly suggests that such neuronal plasticity is required for learning. Notably, acutely restoring the AC-cAMP-PKA cascade in the mushroom body restores these learning impairments, suggesting that restoring neuronal plasticity in the mushroom bodies is sufficient to restore learning ability (Zars et al., 2000; Schwaerzel et al., 2002; McGuire et al., 2003; Thum et al., 2007).

(iii) Mushroom body expression of a dominant negative $G\alpha_s^*$ protein subunit, constitutively activating the AC-cAMP-PKA cascade and thus rendering it non-regulatable, abolishes learning (Connolly et al., 1996), suggesting a local requirement of this cascade for learning.

(iv) Blocking synaptic output from the mushroom bodies during test abolishes memory scores (Dubnau et al., 2001; McGuire et al., 2001; Schwaerzel et al., 2002).

(v) Blocking input to the mushroom bodies during training prevents flies from learning (Schwaerzel et al., 2003).

Taken to their logical extreme, collectively these arguments suggest that there is but one short-term odour-shock memory trace in the fly brain, and that it is localized to the mushroom bodies.

In contrast to this scenario with regard to odour-shock learning, the report of Thum et al. (2007) suggests that odour-sugar learning may induce a second memory trace in the projection neurons. This is because Rutabaga expression in the projection neurons reportedly can fully rescue odour-sugar learning (but, in accordance with the above scenario, not odour-shock learning).

Larval *Drospohila*

The olfactory pathways in larval *Drosophila* are in principle the same as in adult flies; however, the larvae possess much fewer neurons, actually lacking cellular redundancy across the first stages of the circuit (Fig. 1).

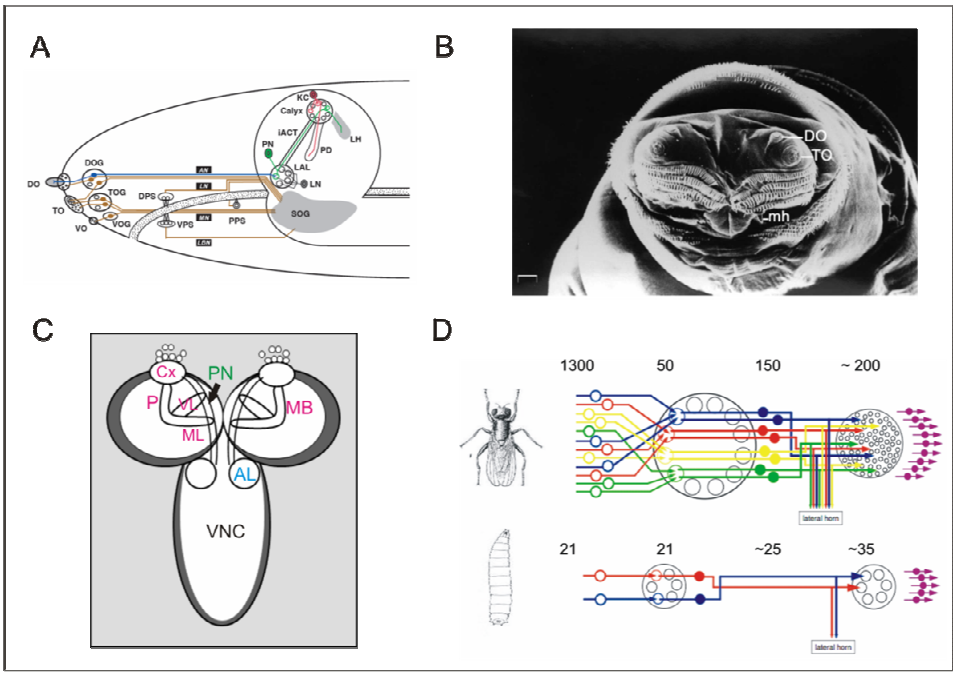


Fig. 1: The chemosensory pathway of Drosophila larva. (A) Schematic overview of the cephalic chemosensory pathways. From the 3 external chemosensory organs, the DO is a mixed structure composed of the central olfactory dome (gray) and a few putative taste sensilla (small circles). The TO with its dorsolateral and distal division (oval and small circles, respectively), the VO, as well as the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, PPS) include mainly gustatory sensilla. The cell bodies of the sensory neurons are collected in ganglia below each sense organ (DOG, TOG, VOG). Some of the neurons innervating the dorsolateral sensilla of the TO are situated in the ganglion of the DO. Odorant receptor neurons (blue) send their axon via the antennal nerve (AN) into the LAL. Local interneurons (LN) interconnect the glomeruli of the LAL. PNs (PN; green) travel in the inner antennocerebral tract (iACT) to link the LAL with theMBcalyx and the lateral horn (LH). An intrinsicMB Kenyon cell (KC) extending its process via the pedunculus (PD) into the MB lobes (not indicated) is shown in red. Axons from putative taste receptor neurons (brown) extend via the AN, the labral nerve (LN), the maxillary nerve (MN), and the labial nerve (LBN) to the subesophageal target region (SOG). The connectivity toward motor neurons is unknown, but concerning taste likely originates from the SOG and concerning olfaction likely from the LH and/or the MB lobes. The pharynx is shown stippled (from Gerber et al., 2006) (B) Frontal overview of the external chemosensory organs of the larval head (from Gerber et al., 2004) (C) Scheme of a larval brain consisting of two hemispheres and a ventral nerve court. (D) Wiring diagram of the adult versus larval olfactory system. Adult and larval olfactory pathways share the same design. However, in the adult, there are probably more primary olfactory dimensions as suggested by the number of types of ORNs (shown in different colors) and AL glomeruli. Moreover, in the adult, the different types of ORNs (open circles) and PNs (filled circles) that innervate a particular AL glomerulus occur in multiple copies, whereas larval ORNs and PNs are unique. Thus, the adult olfactory pathway is characterized by converging and diverging connectivity in the AL (ratios indicated refer to the features shown in the preceding line), whereas the larval pathway is organized without cellular redundancy. As indicated, larval ORNs, LAL glomeruli, PNs, and calyx glomeruli are related essentially in a 1:1:1:1 fashion. The larval MB calyx retains a combinatorial representation of the AL glomerular pattern, which is not obvious in the adult. For reasons of lucidity, the most nonstereotypic, highly combinatorial output of MB neurons in the calyx is not depicted. Note also that the local interneurons in the AL, which shape olfactory activity, are present in both larva and adult, but are omitted in this figure (from Gerber et al. 2006). Mouth hook (MH), ventral organ (VO), dorsal (DO) and terminal (TO) organs. Antennal lobe (AL), projection neurons (PN), mushroom body (MB), calyx (Cx), vertical lobe (VL), medial lobe (ML), peduncle (P), ventral nerve court (VNC).

That is, each olfactory receptor gene is expressed in only one of the 21 larval olfactory sensory neurons (Ramaekers et al., 2005); therefore, each glomerulus in the larval antennal lobe receives input from only one sensory neuron. Also at the level of the appr. 25 projection neurons, one can in most (16-18 projection neurons, i.e. the ones covered by the GH146-Gal4 expression pattern) cases individually identify these neurons: Because the input region of the larval mushroom body, much like the antennal lobe, is organized in a glomerular fashion, and because each projection neuron is innervating typically only one of these mushroom body glomeruli, one can individually recognize projection neurons based on the combination of antennal-lobe input and mushroom body output glomerulus (Marin et al., 2005; Ramaekers et al., 2005). Thus, the larva provides an attractive system for localizing memory traces at the cellular level.

In an early study, (Heisenberg et al., 1985) reported that late third-instar larvae with a mutation in the *mushroom body miniature* gene (*mbm*) have a strong defect in larval odour-shock associative learning. However, given problems in replicating such odour-electroshock learning in wild-type larva (Forbes, 1993), we decided to develop a novel, and apparently more robust olfactory learning paradigm, using food as reinforcer (Scherer et al., 2003; Neuser et al., 2005; Gerber and Hendel, 2006). With respect to odour-sugar learning in this paradigm, two studies may speak towards the site of the memory trace: Firstly, Kaun et al. (2007) investigated the *foraging* gene (*for*). This gene encodes a cGMP-dependent protein kinase (PKG). Natural *for* variants have subtle but significant variations in PKG activity. The rover variants (for^R) have higher *for* transcript levels and higher PKG activity compared with the sitter variants (for^S) (Osborne et al., 1997), and show significantly greater learning scores than sitters. Notably, the reduced learning scores in sitters can be increased to rover levels by boosting expression of PKG in the mushroom bodies. However, secondly, it was reported that the learning defect seen upon deletion of the *neurexin* gene ($Nrx\text{-}1^{\Delta 83}$; Zeng et al., 2007) cannot be restored by Neurexin-1 expression in the mushroom bodies. Thus, with regard to memory trace localization in larval odour-sugar learning, we largely remain at sea.

Therefore, we here venture into the first comprehensive account to localize the cellular site of odour-reward associative memory trace in larval *Drosophila*. We decided to use the presynaptic protein Synapsin for this effort. Synapsins belong to a family of highly conserved neuronal phosphoproteins that are associated with synaptic vesicles and have been implicated in the regulation of neurotransmitter release by controlling the number of vesicles available for release (Hilfiker et al., 1999; but see Sudhof [2004] for a critical discussion of Synapsin function). In insects, Synapsin is encoded by one gene only, whereas there are three distinct

genes in vertebrates (Sudhof et al., 1989; Klagges et al., 1996; Kao et al., 1999). *In vitro* studies have shown that Synapsins can bind to both synaptic vesicles, and the cytoskeletal actin meshwork (Greengard et al., 1993; Hilfiker et al., 1999; Hosaka et al., 1999). This prevents these vesicles from release, thus forming a so-called reserve pool of synaptic vesicles (Li et al., 1995; Hilfiker et al., 1999; Gitler et al., 2008). Importantly, phosphorylation of Synapsin allows the synaptic vesicles to dissociate from this reserve pool and thus to translocate to the active zone, where then these vesicles can be released upon any future activation of the cell (but see [Sudhof, 2004] for a challenge of this view of Synapsin function). The high abundance of Synapsins in the synaptic terminals, the association with synaptic vesicles, the highly conserved domains with phosphorylation sites for PKA, CamK I and IV, as well as the widespread distribution at nerve terminals, all signify Synapsins as important and evolutionarily conserved regulatory synaptic proteins. Indeed, we have shown (Michels et al., 2005) that a lack of Synapsin in the deletion mutant syn^{97} led to a 50 % reduction in odour-reward memory in the larva (in adults memory was reduced by 25% in syn^{97} flies [Godenschwege et al., 2004]). The ability to recognize the to-be-associated gustatory and olfactory stimuli, motor performance, as well as sensitivity to experimental stress, sensory adaptation, habituation and satiation, however, do not apparently differ between the wild-type and the syn^{97} mutant. This now enables us to see where in the larval brain restoring Synapsin would be sufficient to restore learning ability. We will furthermore test where a reduction of Synapsin may reduce learning ability. This allows us to map the sites of sufficiency and necessity of Synapsin for memory trace formation in larval *Drosophila*.

MATERIAL AND METHODS

Third instar feeding-stage larvae aged 5 days after egg laying were used throughout; they were kept in mass culture, maintained at 25 °C, 60- 70 % relative humidity and a 14/ 10 hour light/ dark cycle, except mentioned otherwise. 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 in all cases. The mutant investigated in Experiment 1 was syn^{97CS}, which carries a 1.4 kb deletion in the *synapsin* gene and lacks all Synapsin protein; this strain had been outcrossed to wild-type CS for 13 generations (Godenschwege et al., 2004; Michels et al., 2005) and shows an appr. 50 % reduction in larval learning scores as compared to wild-type CS (Michels et al., 2005; Fig. 3E) and will be labelled as syn^{97} for simplicity.

In cases when transgenic strains were involved, these strains all were in the w^{1118}- mutant background. To see whether the learning defect seen in syn^{97} relative to wild-type CS is also manifest within this w^{1118}- mutant background, we used w^{1118}; syn^{97} double mutants generated by classical genetics and compared them to w^{1118}.

We recombined various Gal4 driver strains (for sources see references below) into the syn^{97}- mutant background by classical genetics; as mentioned above all these transgenic strains in addition carry the w^{1118}- mutation as a 'marker', and mini-*white* rescue constructs on their respective transgenic construct(s) (see Results). The Gal4 driver strains thus obtained were (roman numerals in brackets refer to the chromosome carrying the respective Gal4 insertion):

elav-Gal4; syn^{97} [X] (called 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 strain we used the UAS-*syn; syn*^{97} [III] strain (generated on the basis of Lohr et al., 2002).
To generate experimental genotypes for the rescue experiments, three kinds of crosses were used in addition to the wild-type CS larvae which were always used as 'benchmark':
for RESCUE genotypes, we crossed a respective driver strain, e.g. *elav*-Gal4; *syn*^{97} to the UAS-*syn*; *syn*^{97} effector strain, yielding *w*^{1118}, *elav*-Gal4/*w*^{1118}; UAS-*syn*, *syn*^{97}/*syn*^{97};
for DRIVER control genotypes, we crossed e.g. the *elav*-Gal4; *syn*^{97} driver strain to *w*^{1118}; *syn*^{97} yielding *w*^{1118}, *elav*-Gal4/*w*^{1118}; *syn*^{97}/*syn*^{97};
for the EFFECTOR control genotype, we crossed the UAS-*syn*; *syn*^{97} effector strain to *w*^{1118}; *syn*^{97} yielding *w*^{1118}/*w*^{1118}; UAS-*syn*, *syn*^{97}/*syn*^{97}.

To restore Synapsin expression throughout the brain, but not in the mushroom body, a *mb247*-Gal80; UAS-*syn*; *syn*^{97} effector strain was generated (gift from S. Knapek) by classical genetics from *mb247*-Gal80 [II] (Krashes et al., 2007) and UAS-*syn*; *syn*^{97} (see above); these flies were then crossed to *elav*-Gal4; *syn*^{97}. This yielded *w*^{1118}, *elav*-Gal4/*w*^{1118}; *mb247*-Gal80/+; UAS-*syn*, *syn*^{97}/*syn*^{97}. Because Gal80 is an inhibitor of Gal4, in these animals the *mb247*-Gal80 element leads to expression of Gal80 in the mushroom body, such that in the mushroom body Gal80 can suppress Gal4 and thus prevent Synapsin expression only in the mushroom bodies. For these experiments, for DRIVER control genotypes, we crossed the *elav*-Gal4; *syn*^{97} driver strain to *w*^{1118}; *syn*^{97} yielding *w*^{1118}, *elav*-Gal4/*w*^{1118}; *syn*^{97}/*syn*^{97}. As EFFECTOR control genotype, we crossed the *mb247*-Gal80; UAS-*syn*; *syn*^{97} effector strain to *w*^{1118}; *syn*^{97} yielding *w*^{1118}/*w*^{1118}; *mb247*-Gal80/+; UAS-*syn*, *syn*^{97}/*syn*^{97}. To proof the potency of *mb247*-Gal80 an *elav*-Gal4; *mb247*-Gal80 fly strain was generated (gift from T. Saumweber) by classical genetics from *elav*-Gal4 (see above) and *mb247*-Gal80 (see above).

For a temporally-restricted 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}). These flies were then crossed to *elav*-Gal4; *syn*^{97} to yield *w*^{1118}, *elav*-Gal4/*w*^{1118}; *tub*-Gal80^{ts}/+; UAS-*syn*, *syn*^{97}/*syn*^{97}. For DRIVER control strains the *elav*-Gal4; *syn*^{97} driver was crossed to *w*^{1118}; *syn*^{97} yielding *w*^{1118}, *elav*-Gal4/*w*^{1118}; *syn*^{97}/*syn*^{97}; and for the EFFECTOR control we crossed the *tub*-Gal80^{ts}; UAS-*syn*; *syn*^{97} effector strain to *w*^{1118}; *syn*^{97} yielding *w*^{1118}/*w*^{1118}; *tub*-Gal80^{ts}/+; UAS-*syn*, *syn*^{97}/*syn*^{97}. These crosses were then cultured at 18 °C; to induce Synapsin expression, a heat-shock was applied by shifting vials for 24 hours from 18 °C to 30 °C, beginning on day 6 AEL. Then, vials were kept at room

temperature for 2 hours before experiments were performed. Thus, Synapsin expression was only induced in the RESCUE strain w^{1118}, *elav*-Gal4/w^{1118}; *tub*-Gal80^{ts}/+; UAS-*syn*, syn^{97}/syn^{97} and only when a heat shock was applied. This is because Gal80^{ts} suppresses Gal4-mediated transgene expression at 18 °C but is permissive for such transgene expression at 30 °C. As treatment-control, animals from all genotypes were treated the same, except that heat-shock was omitted.

To yield an RNAi-mediated knock-down of Synapsin, a 497 nt coding fragment of the *syn*-cDNA was amplified by PCR with primers containing unique restriction sites: the primer pair 5'-GAGCTCTAGAACGGATGCAGAACGTCTG-3' and 5'-GAGCGAATTCTGCCGCTGCTCGTCTC-3' was used as sense cDNA fragment and 5'-GAGCGGTACCACGGATGCAGAACGTCTG-3' and 5'-GAGCGAATTCGCCCGCTGCCGCTGCTC-3' were used as anti-sense cDNA fragment, respectively (Fig. 4A). The PCR-amplified fragments were digested with *XbaI/EcoRI* and *EcoRI/KpnI* respectively, subcloned into *XbaI/KpnI* pBluescript KSII (Stratagene, La Jolla, USA) and sequenced. 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 then was performed into a w^{1118} strain (Bestgene, Chino Hills, USA). The resulting effector strain UAS-RNAi-*syn* [III] was used for behavioural experiments and therefore crossed to a *dcr-2*; *elav*-Gal4 driver strain (generated by classical genetics from the *dcr-2* [X] strain (Dietzl et al., 2007) and the *elav*-Gal4 [III] strain, both from Bloomington stock center). This yielded KNOCK-DOWN larvae of the genotype w^{1118}, UAS-*dcr-2*/w^{1118}; *elav*-Gal4/UAS-RNAi-*syn*. For DRIVER control genotypes, we crossed the *dcr-2*; *elav*-Gal4 driver strain to w^{1118} yielding w^{1118}, *dcr-2*/w^{1118}; *elav*-Gal4/+; as EFFECTOR control genotype, we crossed the UAS-RNAi-*syn* effector strain to w^{1118} yielding w^{1118}/w^{1118}; UAS-RNAi-*syn*/+.

Western blotting

For each lane in the Western blots, 10 larval brains were homogenized in 10 µL 2 x SDS gel loading buffer; whole-larva homogenates do not yield a signal in Western blots because of insufficient protein concentration and/ or degradation by proteases. 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 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) according to the manufacturer's specifications.

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. Tissues were then incubated overnight with a 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 preparations, 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 medium (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 (which will be 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 antiserum (A6455, diluted 1:1000) (Invitrogen, Eugene, USA). After washing with PBST, samples were incubated with a secondary goat anti-rabbit antibody (Alexa Fluor 488, anti-rabbit Ig, diluted 1:100) (MoBiTech, Göttingen, Germany).

3D reconstructions of larval brain stainings were made with the ImageJ plugin ´ImageJ 3D Viewer´ (Schmid et al., 2008). (Movies 1- 5 can be found in the supplementary material: CD).

Learning experiments

Learning experiments follow standard methods (Neuser et al., 2005) (see Fig. 2 for a sketch). In brief, olfactory choice performance of groups of 30 larvae each were compared after either of two reciprocal training regimen: During one of these regimens, larvae received amylacetate (AM; Merck, Darmstadt, Germany) with a positive reinforcer and 1-octanol (OCT; Sigma-Aldrich, Seelze, Germany) without such reinforcer (AM+/ OCT); the second regimen involved reciprocal training (AM/ OCT+). Then, animals were tested for their choice between AM *versus* OCT. Associative learning is indicated by a relatively higher preference for AM after AM+/ OCT training as compared to AM/ OCT+ training. These differences in preference were quantified by the learning index (LI; see below).

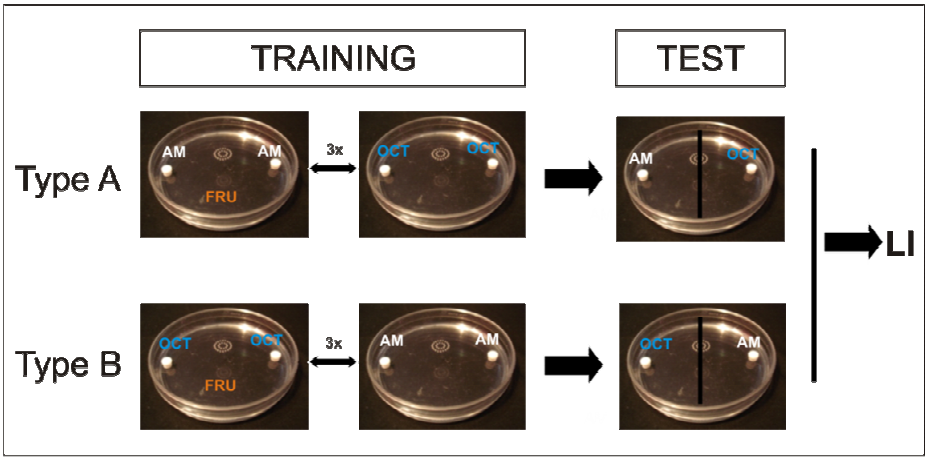


Fig. 2: Appetitive olfactory learning. (A) Scheme of the learning experiment showing the sequence of training trials. Note that for half of the cases, the sequence of training trials for the reciprocal groups is as indicated (i.e., AM+/OCT and OCT+/AM); for the other half of the cases (not shown), the sequence of training trials is reversed (i.e., OCT/AM+ and AM/OCT+). After training, animals are tested for their odour choice (AM *versus* OCT).

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

Experiments were performed 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. Then, a spoonful of food medium containing larvae was taken from the food bottle and transferred to a glass vial. Thirty animals were collected, briefly washed in tap water and as a group transferred to the assay plates for the start of training. Each training trial lasted 5 min. Immediately before a trial, two containers loaded

both with the same odour (for details see below) had been placed onto the assay plate on opposite sides of the plate, 7 mm from the edges. Within each reciprocal training condition, for half of the cases we started with AM, for the other with OCT. Thus, for half of the cases we started with an agarose plate which had FRU added to the substrate, for the other with a plate without FRU. Then, the lid was closed and the larvae were left untreated for 5 min. The larvae were thereafter transferred to a plate with the alternative odour and the respective other substrate for 5 min. This cycle was repeated three times. Fresh assay plates were used for each trial.

After such training, animals were tested for their odour choice. The larvae were placed in the middle of a fresh, pure agarose assay plate with a container of AM on one side and one of OCT on the other side. After 3 min, the number of animals on the "AM" or "OCT" side was counted. After this test was completed, the next group of animals was trained reciprocally. For both reciprocally trained groups, we then calculate an odour preference ranging from –1 to 1 as the number of animals observed on the AM side minus the number of animals observed on the OCT side, divided by the total number of animals:

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

To determine whether these preferences are different depending on training regimen, we took the PREF scores from the reciprocally trained groups and calculated a learning index ranging from –1 to 1 as:

(2) $$LI = (PREF_{AM+/\,OCT} - PREF_{AM/\,OCT+}) / 2$$

After the data for these LI values had been collected for one of the genotypes, the corresponding data for another genotype were gathered.

As behavioural data typically are not normally distributed, we displayed the LI scores using box plots, where the middle line represents the median, and the box boundaries and whiskers the 25/ 75 % and 10/ 90 % quantiles, respectively. For statistical comparisons, we used non-parametric analyses throughout. That is, comparisons of LIs against zero, i.e. random level, were performed with one-sample sign tests; comparisons of LIs between multiple genotypes were compared with Kruskal-Wallis tests. In cases of significance, this was followed by pair-wise Mann-Whitney U-tests; to retain an experiment-wide error of 5 % in cases of multiple such pair-wise tests, the significance level was adjusted by a Bonferroni correction, i.e. by dividing 0.05 by the number of these pairwise tests. For example, if three such U-tests were performed, we present statistics as $P < 0.05/ 3$. All calculations were performed with Statistica 7.1 (StatSoft Inc., Tulsa, OK, USA) on a PC.

RESULTS

Experiment 1: Prelude: Learning impairment of *syn*^{97} mutants interpretable without reference to *white*

We have shown before (Michels et al., 2005) that larvae lacking Synapsin (*syn*^{97}) show an about 50 % reduction in larval odour-sugar associative learning but show intact ability to (i) taste, (ii) to smell, and (iii) to move about the test arena; also, these mutants are not different from wild-type CS in terms of (iv) resistance to the stress of handling entailed by the training regimen, (v) olfactory adaptation which may accrue along training, and (vi) levels of motivation resulting from sugar-exposure and/ or feeding (Michels et al., 2005). This allows us to now ask where in the larval brain restoring Synapsin is sufficient to restore learning. Before venturing into this project, however, we confirm the lack of Synapsin (Fig.s 3B and D) and the learning defect of *syn*^{97} larvae: Wild-type CS show about twice as high learning indices as *syn*^{97} mutants (Fig. 3E; U-test: P< 0.05/ 4; U= 106; N= 28, 16). The same learning defect due to the *syn*^{97} mutation is uncovered when comparing *w*^{1118}; *syn*^{97} double mutants to *w*^{1118} larvae (Fig. 3E; U-test: P< 0.05/ 4; U= 44; N= 16, 13). Notably, direct comparisons do not reveal an effect of the *w*^{1118} mutation upon learning scores: Wild-type CS and *w*^{1118} larvae learn equally well (Fig. 3E; P= 0.78; U= 0.78; N= 28, 13), and *w*^{1118}; *syn*^{97} double mutants show learning scores indistinguishable from *syn*^{97} mutants (Fig. 3E; P= 0.85; U= 123; sample sizes as above) (see also Yarali et al. [in press]. This is by no means trivial, as in adult flies effects of the *w*^{1118} mutation on various behaviours, including learning, have been reported (e.g. Diegelmann et al. [2006a] for heat-box learning and Yarali et al. [in press] for associative learning between odours and electric shock in adult flies). Thus, in those cases interpretation of experiments using transgenic constructs is complicated: Transgenes typically carry a 'marker' mutation in the *white* gene. This 'marker' mutation causes white eye colour as compared to the wild-typish red eye colour; fly transgenes therefore include a mini-*white* rescue gene (supporting red eye colour), such that loss of the construct would show by white eyes.

To summarize, we confirm the odour-sugar learning defect seen in *syn*^{97} larvae (Fig. 3E; Michels et al., 2005), and show that this defect can be interpreted without reference to *white* function.

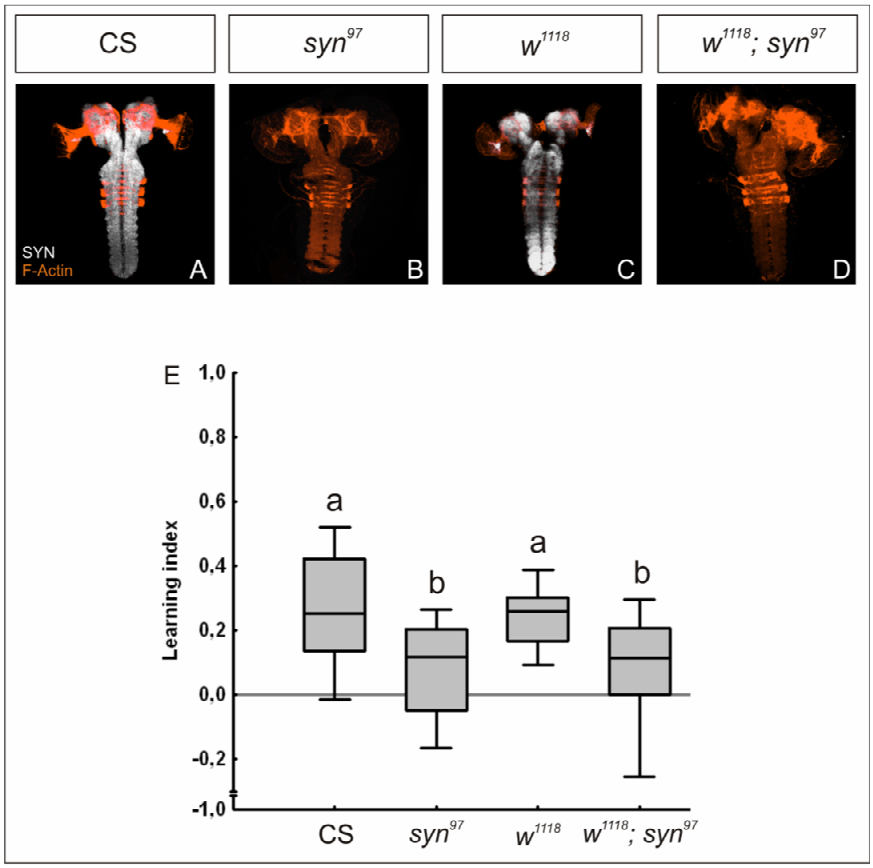


Fig. 3: Learning impairment of *syn*^{97} mutants interpretable without reference to *white*. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third-instar brains viewed under the confocal microscope. Brains of wild-type CS (A) and w^{1118} (C) show Synapsin staining, whereas brains of syn^{97} (B) and w^{1118}; syn^{97} (D) do not. (E) Learning, as measured by the learning index (LI) is reduced by half both in syn^{97} and w^{1118}; syn^{97} mutants relative to wild-type CS and w^{1118}, respectively. Additionally, there are no significant differences between wild-type CS and w^{1118} larvae, and between w^{1118}; syn^{97} and syn^{97} mutants.
Box plots marked with different letters indicate significant differences in learning ability of the corresponding genotypes (P< 0.05/ 4), box plots with the same letters indicate no significant difference (P> 0.05/ 4). Box plots represent the median as the middle line, 25 and 75 % quantiles as box boundaries, as well as 10 and 90 % quantiles as whiskers, respectively.

Experiment 2: Learning impairment upon knock-down of Synapsin by RNAi

As an independent confirmation of the necessity of Synapsin in our larval olfactory learning paradigm, we knocked down Synapsin by RNAi (Smith et al., 2000; Kalidas and Smith, 2002). Using UAS-RNAi-*syn* effector and *elav*-Gal4 as a brain-wide driver, we first performed a Western blot to test for the potential knock-down Synapsin (Fig. 4B). We moved on to use these brain-wide KNOCK-DOWN larvae (w^{1118}, UAS-*Dcr-2*/w^{1118}; *elav*-Gal4/UAS-RNAi-*syn*) for behavioural experiments, together with their proper genetic controls (brain-wide DRIVER control: w^{1118}, UAS-*Dcr-2*/w^{1118}; *elav*-Gal4/+; EFFECTOR control: w^{1118}/w^{1118}; UAS-RNAi-*syn*/+) as well as wild-type CS. Comparing learning scores between these four genotypes shows a significant difference in learning ability (Fig. 4C; Kruskal-Wallis test: P< 0.05; H= 8.00; df= 3; N= 36, 37, 34); specifically, the brain-wide KNOCK-

DOWN larvae show significantly lower learning scores when compared to EFFECTOR control larvae (Fig. 4C; P< 0.05/ 2, U= 408; sample sizes as above), and to DRIVER control larvae (Fig. 4C; P< 0.05/ 2, U= 441; sample sizes as above).

Thus, we therefore conclude that a reduction of Synapsin causes a learning impairment.

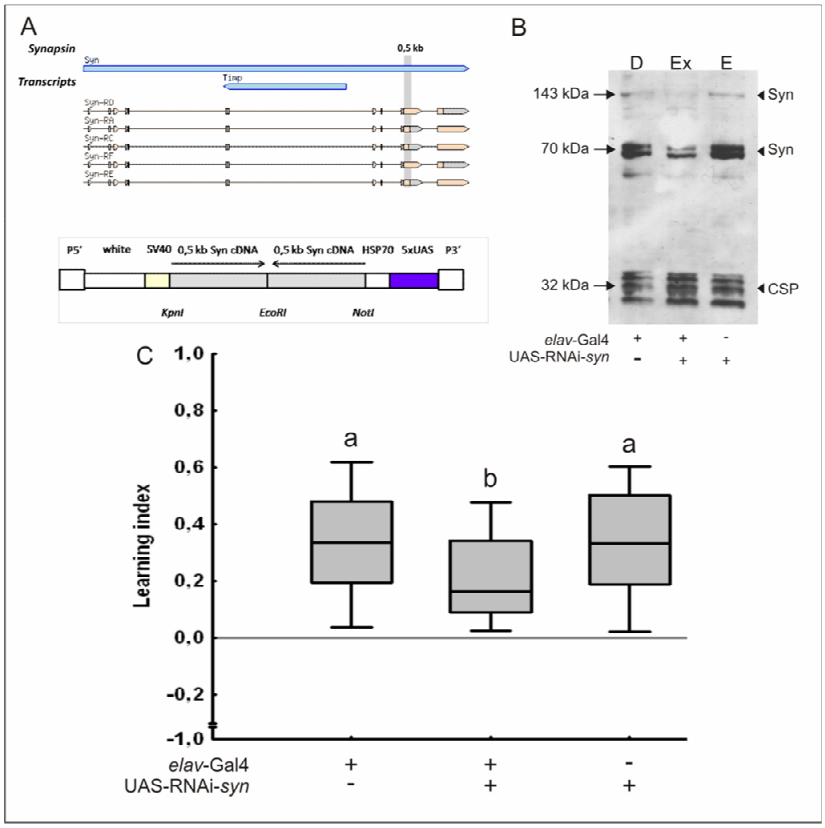


Fig. 4: Learning impairment upon knock-down of Synapsin by RNAi. (A) Genomic organization of the *synapsin* locus in *Drosophila*. The gray vertical bar in the upper panel indicates the 0.5 kb *syn-cDNA* fragment corresponding to the mRNA targeted by our RNAi-s*yn* construct (lower panel). (B) Western blot from brains of larval *Drosophila*. The blot shows staining for Synapsin and of CSP as loading control. The *left* lane was loaded from DRIVER control larvae (labelled D), the *middle* lane from brain-wide KNOCK-DOWN larvae (labelled Ex), the *right* lane from EFFECTOR control larvae (labelled E). The expected (Klagges et al., 1996; Godenschwege et al., 2004) bands at 74 and 143 kDa represent fused triple and double bands, respectively, and are reduced in the brain-wide KNOCK-DOWN larvae. (C) Learning is impaired in the brain-wide KNOCK-DOWN strain. Concerning the UAS-*dcr2* construct, please see body text.
All other details as in the legend of Fig. 3.

Experiment 3: Brain-wide rescue

For a brain-wide rescue of Synapsin (Fig. 5) we use the driver strain *elav*-Gal4; syn^{97} crossed to UAS-*syn*; syn^{97} as effector strain. The resulting offspring thus are brain-wide RESCUE larva (w^{1118}, *elav*-Gal4/w^{1118}; UAS-*syn*, syn^{97}/syn^{97}), showing strong Synapsin expression throughout the neuropil regions of the larval brain (Fig. 5B); notably, expressed Synapsin is conspicuous in the mushroom body. Similar staining patterns can be detected when GFP is expressed in *ela*v-Gal4 positive cells (Fig. 6; see also Movie 1 in supplementary material).

We use these brain-wide RESCUE larvae for behavioural experiments, together with their proper genetic controls (brain-wide DRIVER control: w^{1118}, *elav*-Gal4/w^{1118}; syn^{97}/syn^{97}; EFFECTOR control: w^{1118}/w^{1118}; UAS-*syn*, syn^{97}/syn^{97}) as well as wild-type CS as 'benchmark'. Comparing learning scores between these four genotypes shows a significant difference in learning ability (Fig. 5E; Kruskal-Wallis test: P< 0.05; H= 19.03; df= 3; N= 9, 7, 7, 10); specifically, the brain-wide RESCUE larvae learn significantly better than EFFECTOR control larvae (Fig. 5E; P< 0.05/ 3, U= 0; sample sizes as above) and than DRIVER control larvae (Fig. 5E; P< 0.05/ 3, U= 4.5; sample sizes as above). Most importantly, learning ability is restored fully in the brain-wide RESCUE larvae, i.e. they learn as well as wild-type CS larvae do (Fig. 5E; P> 0.05/ 3; U= 28; N sample sizes as above).

Thus, we conclude that brain-wide transgenic rescue of Synapsin is sufficient to fully restore the syn^{97} mutant learning defect.

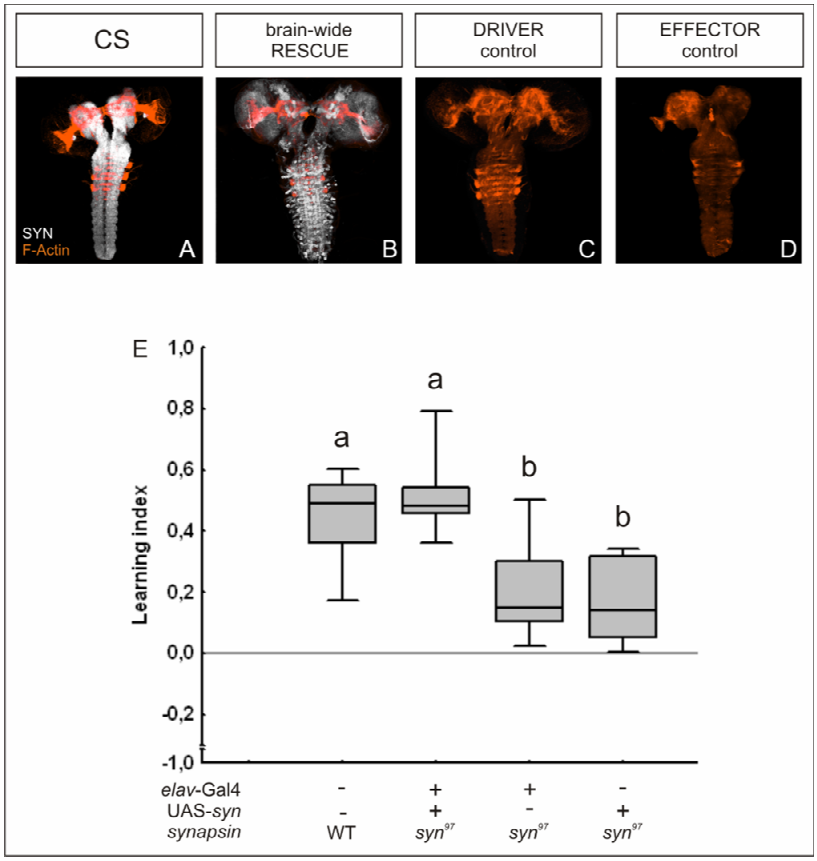


Fig. 5: Brain-wide rescue. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third-instar brains. Brains of wild-type CS (A) and the brain-wide RESCUE strain (B) show anti-Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. (E) Learning is fully rescued in the brain-wide RESCUE strain.
All other details as in the legend of Fig. 3.

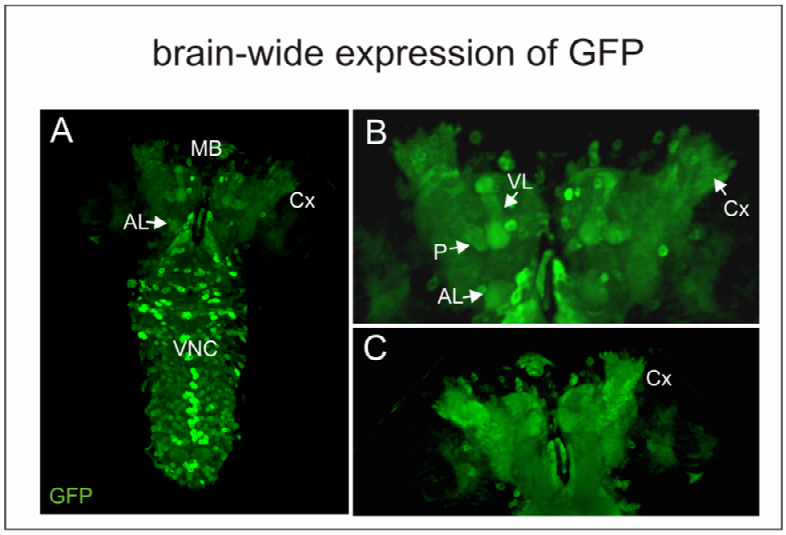


Fig. 6: Brain-wide expression of GFP. (A-C) Gal4 expressing cells in e*lav*-Gal4 monitored by UAS-*GFP* (green). 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third-instar brains were made with ImageJ 3D Viewer. The larval brain shows GFP expression throughout the neuropil regions, with strong expression in the mushroom bodies. (A) Whole larval brain. (B, C) Details of the larval brain seen in A. Mushroom body (MB), ventral nerve cord (VNC), calyx (Cx), peduncle (P), vertical lobe (VL).).

Experiment 4: Temporally-specific rescue

To see whether the learning defect upon lack of Synapsin is indeed due to an acute requirement of Synapsin, we induced its production just before the learning experiment. Therefore, a temperature-sensitive allele of Gal80 (Gal80^{ts}) was used to control transgene expression temporally (McGuire et al., 2003; Thum et al., 2006a). Gal80^{ts} has been shown to suppress Gal4-mediated transgene expression at 18 °C but to be permissive for such transgene expression at 30 °C (McGuire et al., 2003). Thus, we prepared flies carrying both the effector construct and *tub*-Gal80^{ts}, which together allowed us to control both temporally and spatially transgene expression from the UAS-*syn* construct.

When given a heat shock (HS) one day before the experiment, both wild-type CS and the temporally brain-wide RESCUE larvae (w^{1118}, *elav*-Gal4/w^{1118}; *tub*-Gal80^{ts}/+; UAS-*syn*, syn^{97}/syn^{97}), show strong Synapsin expression throughout the neuropil regions of the larval brain (Fig.s 7A and B). However, the proper genetic controls (temporally brain-wide DRIVER control: w^{1118}, *elav*-Gal4/w^{1118}; syn^{97}/syn^{97}; EFFECTOR control: w^{1118}/w^{1118}; *tub*-Gal80^{ts}/+; UAS-*syn*, syn^{97}/syn^{97}) do not show such Synapsin expression (Fig.s 7C und D). When no heat shock is applied, Synapsin is found only in the wild-type CS, but in neither of the other genotypes (Fig.s 7A´-D´).

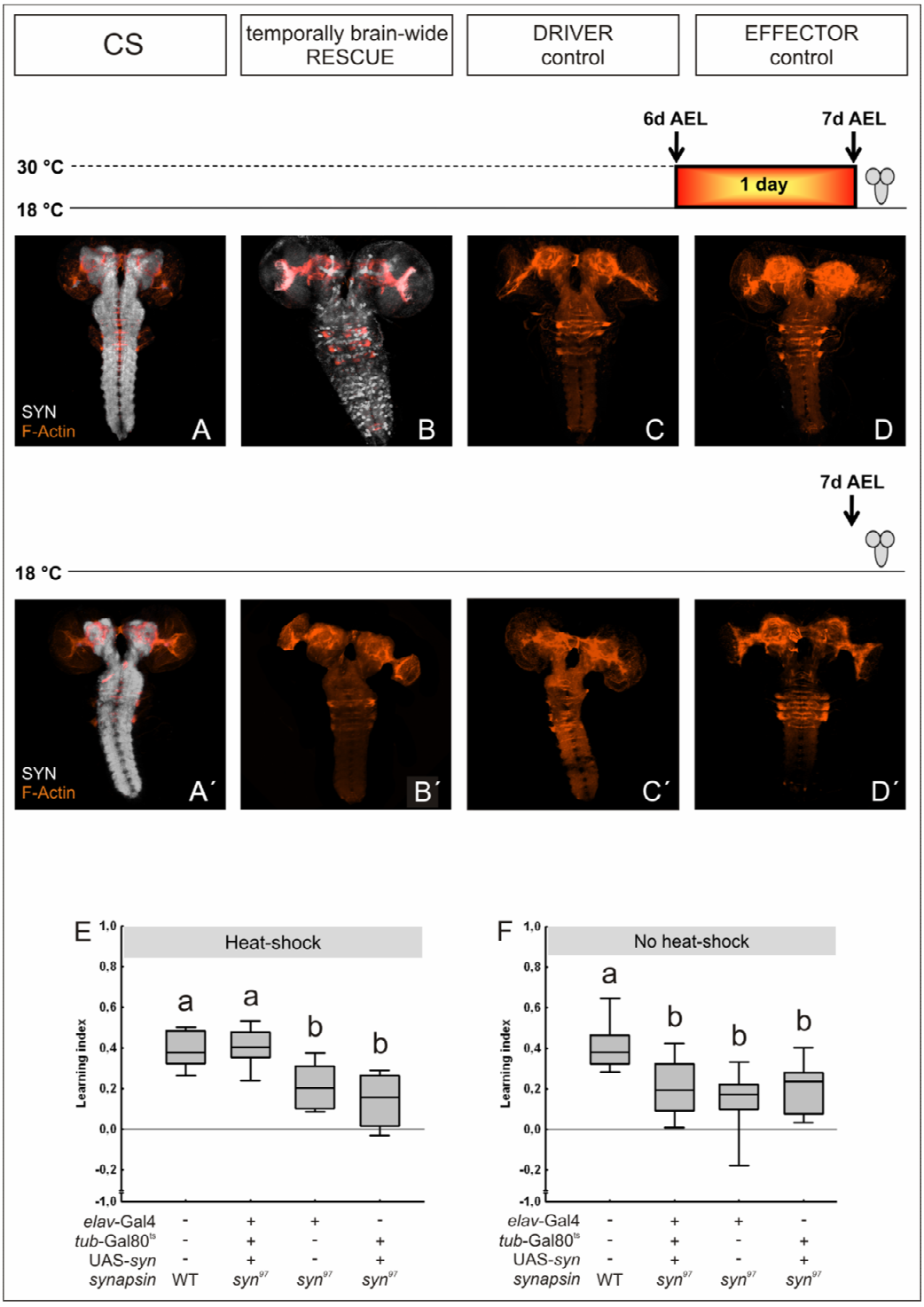


Fig. 7: Temporally rescue. (A-F, A´-D´) Temporal control of Synapsin expression in larval brains. (A-D´) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval brains. (A- D) When raised at 18 °C for 6 days AEL and shifted to 30 °C for 1 day before dissection (upper sketch), Synapsin expression is seen in wild-type CS and temporally brain-wide RESCUE larvae; however, under the same treatment condition, no Synapsin staining is detected in DRIVER control and EFFECTOR control larval brains. (A´-D´) Without heat-shock, Synapsin staining is detected only in the wild-type CS strain (A´), but neither in the induced brain-wide RESCUE larvae, nor in DRIVER or EFFECTOR control larvae. (E-F) Learning is fully rescued by induced Synapsin expression; without heat-shock, no rescue is observed.
All other details as in the legend of Fig. 3.

With regard to learning ability, these four genotypes show significant differences in learning ability after heat shock (Fig. 7E; Kruskal-Wallis test: P< 0.05; H= 18.37; df= 3; N= 8, 10, 8, 12). Importantly, temporally brain-wide RESCUE larvae show the same learning ability as wild-type CS larvae (Fig. 7E; P= 0.79; U= 37; N= sample sizes as above); also, upon heat shock the temporally brain-wide RESCUE larvae learn significantly better than EFFECTOR control larvae (Fig. 7E; P< 0.05/ 3, U= 11; sample sizes as above) and than temporally brain-wide DRIVER control larvae (Fig. 7E; P< 0.05/ 3, U= 11; sample sizes as above).
When no heat shock is given, learning scores also show a significant difference between these four genotypes (Fig. 7F; Kruskal-Wallis test: P< 0.05; H= 12.95; df= 3; N= 9, 12, 9, 8); however, after such regimen the temporally brain-wide RESCUE larvae show significantly lower learning scores than wild-type CS larvae (Fig. 7F P< 0.05/ 3; U= 16; N sample sizes as above) and do not differ from EFFECTOR control larvae (Fig. 7F; P> 0.05/ 3, U= 47; sample sizes as above) and temporally brain-wide DRIVER control larvae (Fig. 7F; P> 0.05/ 3, U= 44; sample sizes as above). Therefore, learning ability is restored fully in the brain-wide RESCUE larvae when Synapsin expression was induced one day before the experiment; this suggests an acute function of Synapsin in learning.

Experiment 5: Local rescue: Mushroom body

Given the conspicuous Synapsin expression in the mushroom bodies of the brain-wide RESCUE larvae, we next ask whether Synapsin expression in only the mushroom bodies would also restore the syn^{97}- mutant learning defect. As mushroom-body driver strain we use *mb247*-Gal4; syn^{97}, and cross it to UAS-*syn*; syn^{97} (Fig. 8). Then, we compare learning ability in the resulting mushroom-body RESCUE strain (w^{1118}/w^{1118}; *mb247*-Gal4, syn^{97}/UAS-*syn*, syn^{97}) to the DRIVER control (w^{1118}/w^{1118}; *mb247*-Gal4, syn^{97}/ syn^{97}), the EFFECTOR control (w^{1118}/w^{1118}; UAS-*syn*, syn^{97}/ syn^{97}), and to wild-type CS. Learning scores differ between these genotypes (Fig. 8E; Kruskal-Wallis test: P< 0.05; H= 21.39; df= 3; N= 10, 11, 10, 11), such that mushroom-body RESCUE larvae show learning scores indistinguishable from wild-type CS (Fig. 8E; U-test: P> 0.62; U= 48; sample sizes as above), but better than mushroom-body DRIVER control animals (Fig. 8E; P< 0.05/ 3; U= 11; sample sizes as above) and better than the EFFECTOR control larvae (Fig. 8E; P < 0.05/ 3; U= 18; sample sizes as above). We therefore conclude that Synapsin expression in the mushroom body, as covered by the *mb247*-Gal4 driver (Fig.s 8B und B´), is sufficient to fully rescue the syn^{97}- mutant learning defect.

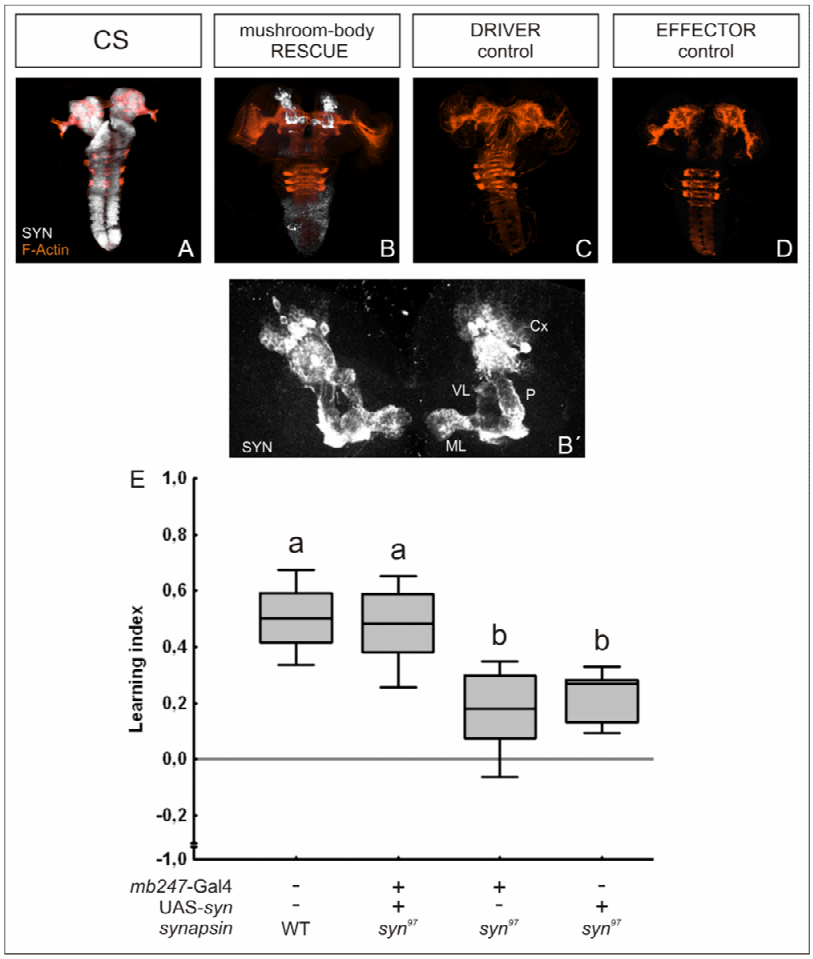


Fig. 8: Local rescue in the mushroom bodies. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third-instar brains. Brains of wild-type CS (A) and the mushroom-body RESCUE strain (B, B´) show Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. In (B´), a magnified view of the mushroom bodies from the RESCUE strain is presented; calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML). (E) Learning is fully rescued in the mushroom-body RESCUE strain. All other details as in the legend of Fig. 3.

In terms of expression pattern, *mb247*-Gal4 leads to Synapsin expression in all basic compartments of the larval mushroom body, i.e. in calyx, peduncle and lobes (Fig.s 8B und B´; see also Fig. 9 and Movie 2 for GFP expression of *mb247*-Gal4 positive cells). It covers appr. 600 larval mushroom body neurons as judged from counting GFP-positive cell bodies of w^{1118}/w^{1118}; *mb247*-Gal4/UAS-*GFP* (Saumweber et al., in prep.).

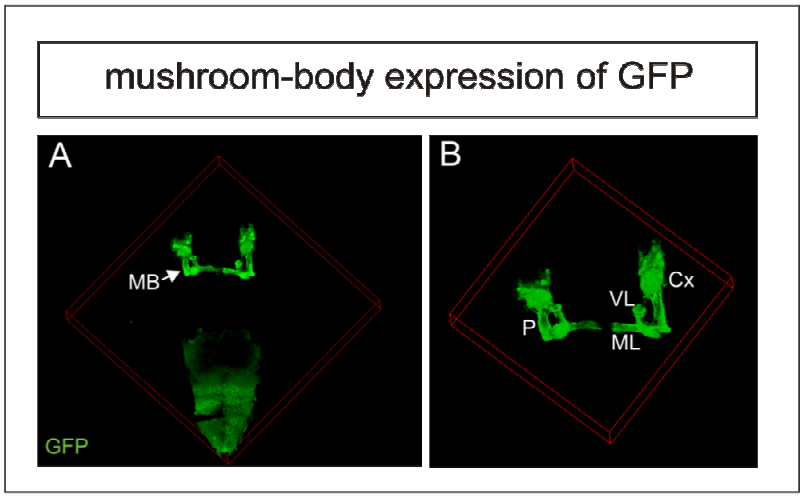


Fig. 9: Mushroom body expression of GFP. (A, B) GFP expression of *mb247*-Gal4 positive cells. 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third-instar brains. The larval brain shows strong GFP expression in the mushroom body. (A) Whole larval brain. (B) Magnified view on the mushroom body. Mushroom body (MB), calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML).

This leaves us with the question whether a comparably potent rescue of learning could also be found if drivers were used which cover fewer mushroom body neurons. To this end, we use the *D52H*-Gal4 driver (Qiu and Davis, 1993; Tettamanti et al., 1997). Crossing this driver to a UAS-*GFP* effector strain, we observe that expression is found in only very few mushroom body neurons (7 mushroom body neurons per brain hemisphere in Fig. 10). 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 (Fig.s 10B and C; and Movie 3).

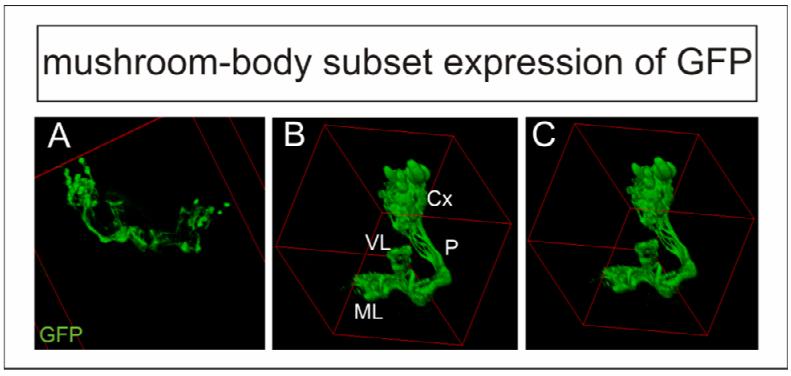


Fig. 10: Mushroom body subset expression of GFP. (A- C) GFP expression in *D52H*-Gal4 positive cells. 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third instar-brains. The larval brain shows GFP expression in the mushroom body. (A) Whole mushroom body of the larval brain. (B, C) Magnified views of a single mushroom body. Calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML).

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 UAS-*syn*; syn^{97} effector strain (Fig.s 11B and B´).

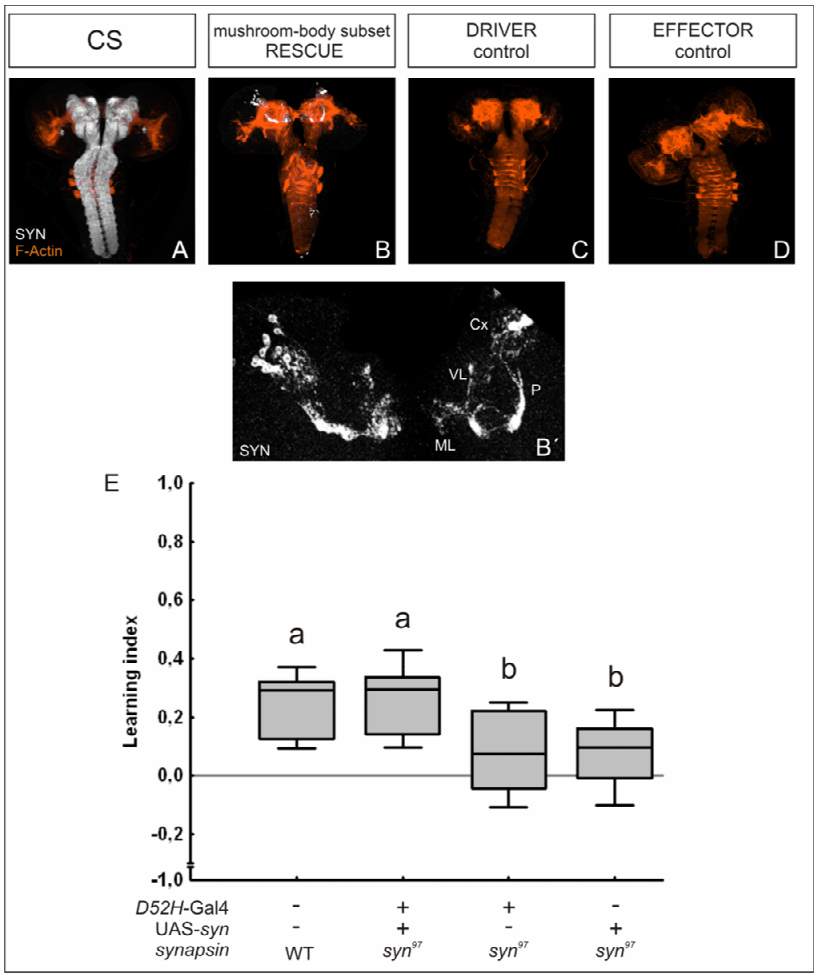


Fig. 11: Local rescue in a small subset of mushroom body neurons. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third-instar brains. Brains of wild-type CS (A) and the mushroom-body subset RESCUE strain (B, B´) show Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. In (B´), a magnified view of the mushroom bodies from the RESCUE strain is presented; calyx (Cx), peduncle (P), vertical lobe (VL), medial lobe (ML). (E) Learning is fully rescued in the mushroom-body subset RESCUE strain.
All other details as in the legend of Fig. 3.

This in-a-nutshell coverage of the mushroom body corresponds to the findings of Masuda-Nakagawa et al. (2005) who also reports that drivers which label mushroom body neurons in the larva (OK107, c739, and 201Y) cover many if not all calyx glomeruli, rather than arborizing heavily in only a small subset of them; this includes cases where drivers cover only very few mushroom body neurons (P[dnc-lacZ] flies, which express LacZ driven by the *dunce* (*dnc*) enhancer (Qiu and Davis, 1993; Kurusu et al., 2002). Actually such broad coverage of the calyx is to be expected even for fairly small subsets of mushroom body neurons: Masuda-Nakagawa et al. (2005) showed that each mushroom body neuron typically receives input in a random subset of six out of the total of appr. 36 glomeruli. Thus, if more than six mushroom

body neurons are included in a given Gal4 strain, broad regions of the calyx, and hence a fairly broad range of olfactory input space, should be covered.

In any event, using the *D52H*-Gal4 construct, we next test for learning ability in a mushroom-body-subset RESCUE strain (w^{1118}, *D52H*-Gal4/w^{1118}; UAS-*syn*, syn^{97}/syn^{97}) as compared to its genetic controls (EFFECTOR control: w^{1118}/w^{1118}; UAS-*syn*, syn^{97}/syn^{97}; mushroom-body-subset DRIVER control: w^{1118}, *D52H*-Gal4/w^{1118}; syn^{97}/syn^{97}; wild-type CS). We find that these genotypes differ in learning ability (Fig. 11E; Kruskal-Wallis test: $P< 0.05$; $H= 13.85$; $df= 3$; $N= 12, 10, 12, 12$), such that the mushroom-body–subset RESCUE larvae learn just as well as wild-type CS (Fig. 11E; $P> 0.55$; $U= 51$; sample sizes as above), whereas they learn better than either the mushroom-body-subset DRIVER control larvae (Fig. 11E; $P< 0.05/ 3$; $U= 18$; sample sizes as above) or the EFFECTOR control larvae (Fig. 11E; $P < 0.05/ 3$; $U= 21.0$; sample size as above).

Together, we must conclude that Synapsin expression in roughly to handful of mushroom body neurons, defined by expression from the *D52H*-Gal4 driver, can be sufficient to rescue the syn^{97}- mutant learning defect.

Experiment 6: No rescue in projection neurons

Given that in both bees (Hammer and Menzel, 1998; Faber et al., 1999; Farooqui et al., 2003; Peele et al., 2006) and flies (Thum et al., 2007) the projection neurons have been suggested as an additional site of an odour-sugar memory trace, we next test whether learning is restored in projection-neuron RESCUE larvae (w^{1118} /w^{1118}; *GH146*-Gal4/+; UAS-*syn*, syn^{97}/ syn^{97}) as compared to their genetic controls (projection-neuron DRIVER control: w^{1118}/w^{1118}; *GH146*-Gal4/+; syn^{97}/ syn^{97}; EFFECTOR control: w^{1118}/w^{1118}; UAS-*syn*, syn^{97}/ syn^{97}) and the wild-type CS strain. Learning scores between these genotypes are significantly different (Fig. 12E; Kruskal-Wallis test: $P< 0.05$; $H= 19.15$; $df= 3$; $N= 10, 10, 10, 10$); importantly, however, projection-neuron RESCUE larvae show learning indices significantly smaller than wild-type CS (Fig. 12E; U-test: $P< 0.05/ 3$; $U= 9$; sample size as above) and indistinguishable from either genetic control (Fig. 12E; projection-neuron RESCUE versus projection-neuron DRIVER control: U-test: $P> 0.05/ 3$; $U= 43.5$; projection-neuron RESCUE versus EFFECTOR control: U-test: $P> 0.05/ 3$; $U= 46$; sample sizes as above).

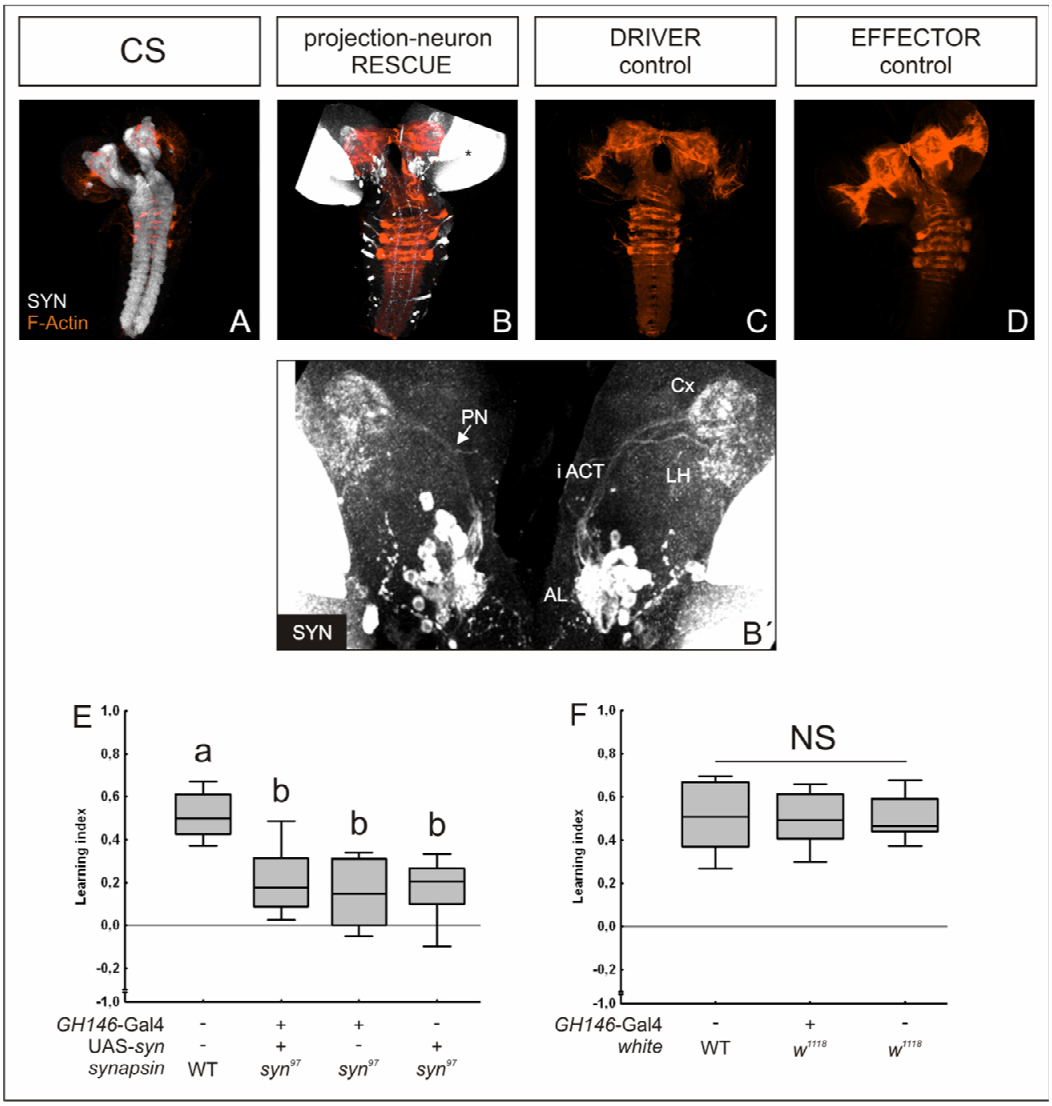


Fig. 12: No rescue in the projection neurons. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third instar brains. Brains of wild-type CS (A) and the projection-neuron RESCUE strain (B, B´) show Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. In (B´), a magnified view of the projection neurons from the RESCUE strain is presented; optic lobe anlagen (*), projection neuron (PN), antennal lobe (AL), inner antennocerebral tract (i ACT), calyx (Cx), lateral horn (LH). (E) Synapsin expression in projection neurons is not sufficient to restore learning ability. (F) No significant differences in learning ability become apparent between larvae heterozygous for the *GH146*-Gal4 construct as compared to wild-type CS and w^{1118} mutant larvae; this excludes a dominant-negative effect of the *GH146*-Gal4 driver construct.
All other details as in the legend of Fig. 3.

This may suggest that Synapsin expression in projection neurons, as covered by *GH146*-Gal4, is not sufficient for restoring learning ability in syn^{97}- mutants. However, as is the case for any observation of lack-of-rescue, a caveat needs to be filed: It may be that the driver construct has a dominant negative effect due to the site of its insertion in the genome. For example, Synapsin expression from the *GH146*-Gal4 construct may actually support a rescue of the syn^{97}- mutant learning defect, but the *GH146*-Gal4 insertion itself may cause a defect in learning, such that the rescue unwittingly remains unrecognized. Therefore, we compare larvae heterozygous for the *GH146*-Gal4 construct to wild-type CS and w^{1118} mutant larvae;

learning scores of these three genotypes are indistinguishable (Fig. 12F; Kruskal-Wallis test: P> 0.05; H= 0.04; df= 2; CS: N= 10, 10, 10). Thus, one cannot attribute the lack-of-rescue by expressing Synapsin from a *GH146*-Gal4 driver to a dominant-negative effect of the *GH146*-Gal4 construct. Thus, we conclude that expression of Synapsin in projection neurons, as covered by *GH146*-Gal4, is not sufficient for rescuing the syn^{97} mutant learning defect.

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, namely the antennal lobe, the calyx of the mushroom body, and the lateral horn (Fig.s 12B and 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 no Synapsin is expressed [marked with (*) in Fig. 12B]). As synapse formation in the lamina emerges at the earliest in the midpupal period (~55%; Frohlich and Meinertzhagen, 1982), this ectopic expression of Synapsin in the developing visual system likely is without consequence in our paradigm.

Interestingly, however, when the *GH146*-Gal4 driver is used to express GFP, we note that in addition to the expression in the projection neurons and the optic lobe Anlagen, a single mushroom body-extrinsic neuron per hemisphere is GFP-positive (Fig. 13 and Movie 4). Conceivably, this neuron also expresses Synapsin in the projection-neuron RESCUE larvae, but such expression may remain unrecognized in terms of Synapsin immunoreactivity in whole-mount larval brain preparations.

Thus, neither in the projection neurons, nor potentially in the mentioned mushroom body-extrinsic cell, a rescue of the syn^{97}- mutant learning defect is possible.

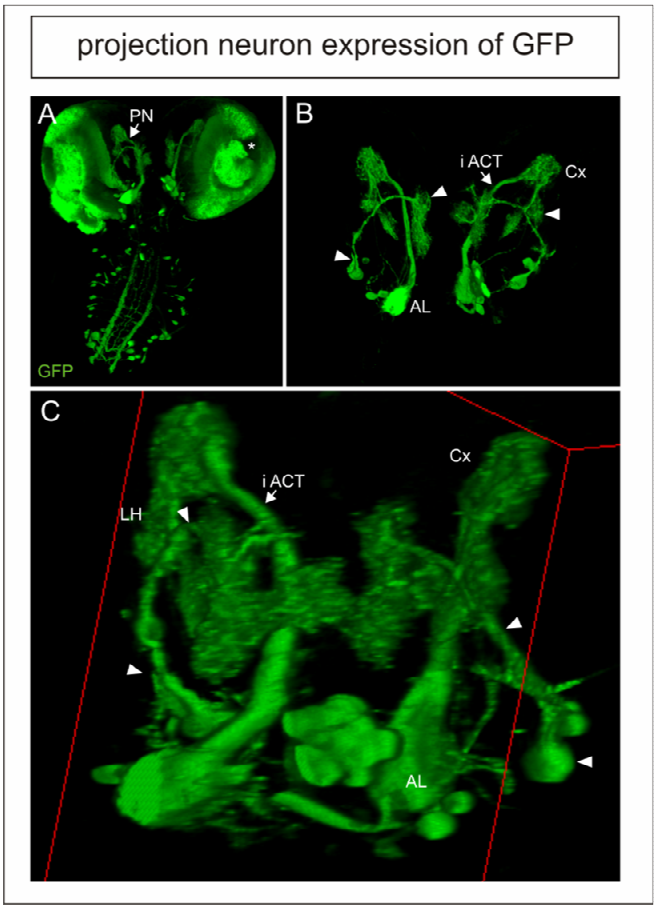


Fig. 13: Projection neuron expression of GFP in larval whole mounts. (A-C) *GH146*-Gal4 cells expressing GFP. 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third-instar brains. The projection neurons are heavily stained. Additionally, a mushroom body extrinsic neuron (►) shows strong GFP immunoreactivity as well. (A) Whole larval brain. (B, C) Magnification of projection neurons and extrinsic mushroom body neurons. Optic lobe anlagen (*), antennal lobe (AL), inner antennocerebral tract (i ACT), projection neuron (PN), calyx (Cx), lateral horn (LH).

We next seek to confirm the lack-of-rescue in *GH146*-Gal4 using another projection-neuron RESCUE strain (w^{1118} /w^{1118}; *NP225*-Gal4/+; UAS-*syn*, syn^{97}/ syn^{97}). It turns out that all above conclusions are confirmed: Learning scores significantly differ between this projection-neuron RESCUE strain, the EFFECTOR control (w^{1118}/w^{1118}; UAS-*syn*, syn^{97}/ syn^{97}), the projection-neuron DRIVER control (w^{1118}/w^{1118}; *NP225*-Gal4/+; syn^{97}/ syn^{97}), and wild-type CS (Fig. 14E; Kruskal-Wallis test: P< 0.05; H= 16.99; df= 3; N= 10, 10, 10, 10). Also, these projection-neuron RESCUE larvae do not learn as well as wild-type CS do (Fig. 14E; U-test: P< 0.05/ 3; U= 10; sample sizes as above); actually, they perform as poorly as either genetic control (Fig. 14E; projection-neuron RESCUE versus DRIVER control: U-test: P> 0.05/ 3; U= 42; projection-neuron RESCUE versus EFFECTOR control U-test: P> 0.05/ 3; U= 40; sample sizes as above). Thus, the learning defect in syn^{97} mutants cannot be rescued using the projection-neuron driver *NP225*-Gal4.

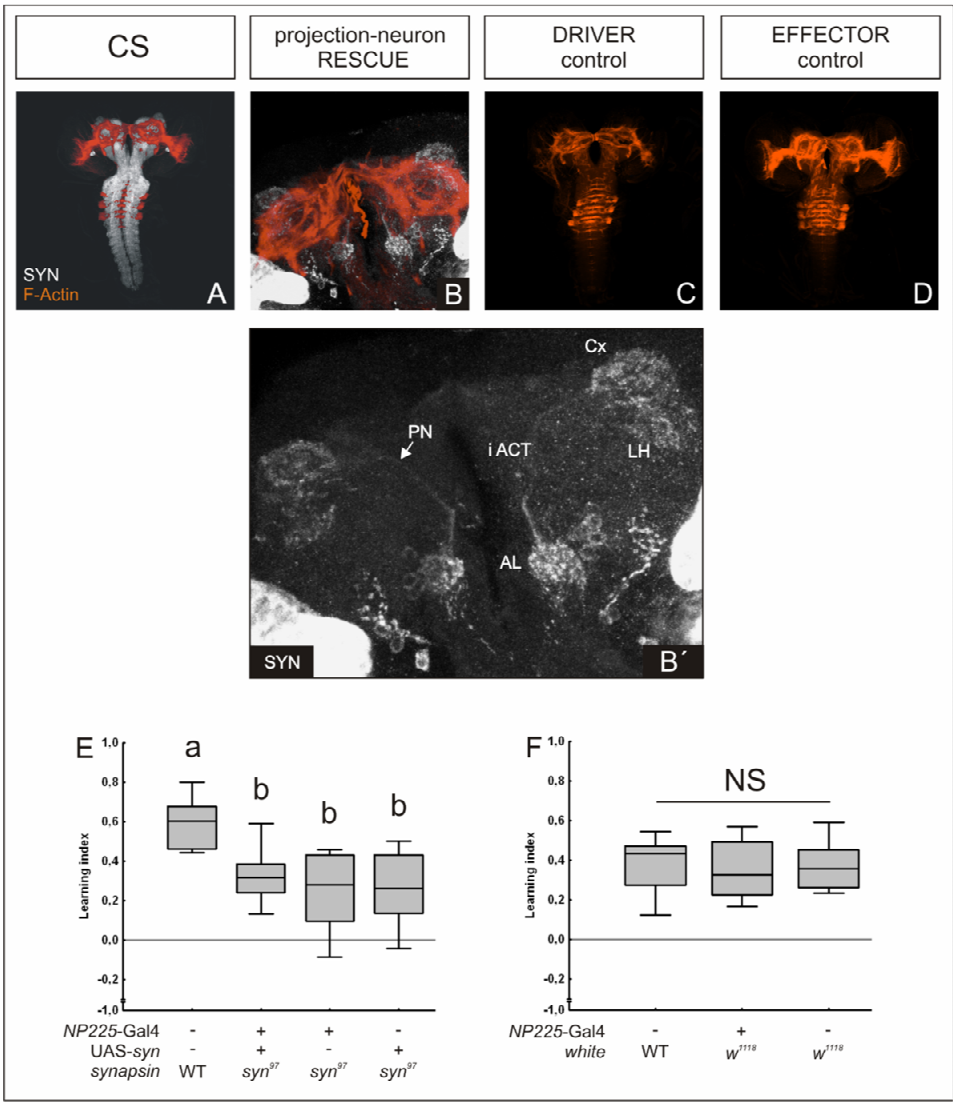


Fig. 14: No rescue in the projection neurons. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third instar brains. Brains of wild-type CS (A) and the projection-neuron RESCUE strain (B, B´) show Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. In (B´), a magnified view of the projection neurons from the RESCUE strain is presented; projection neuron (PN), antennal lobe (AL), inner antennocerebral tract (i ACT), calyx (Cx), lateral horn (LH). (E) Synapsin expression in projection neurons is not sufficient to restore learning ability. (F) No significant differences in learning ability become apparent between larvae heterozygous for the *NP225*-Gal4 construct as compared to wild-type CS and w^{1118} mutant larvae; this excludes a dominant-negative effect of the *NP225*-Gal4 driver construct.
All other details as in the legend of Fig. 3.

Again, such a lack-of-rescue calls for testing whether the *NP225*-Gal4 insertion may have a dominant-negative effect on learning; this is not the case, as heterozygous *NP225*-Gal4 larvae show learning scores indistinguishable from wild-type CS and w^{1118} larvae (Fig. 14F; Kruskal-Wallis test: P> 0.05; H= 0.15; df= 2; N= 13, 11, 11).

Regarding expression pattern, it seems that *NP225*-Gal4 labels a very similar, if not identical, set of cells as does *GH146*-Gal4; this includes expression in projection neurons, the optic lobe Anlagen, as well as a mushroom body extrinsic neuron which also in *NP225*-Gal4 is

uncovered using GFP expression (Fig. 15, Movie 5), but not Synapsin expression (Fig. 14B and B´).

Thus, despite what we believe are sincere efforts, there is no evidence to suggest that Synapsin expression in the projection neurons were sufficient to restore the odour-sugar learning defect in larval syn^{97}- mutants.

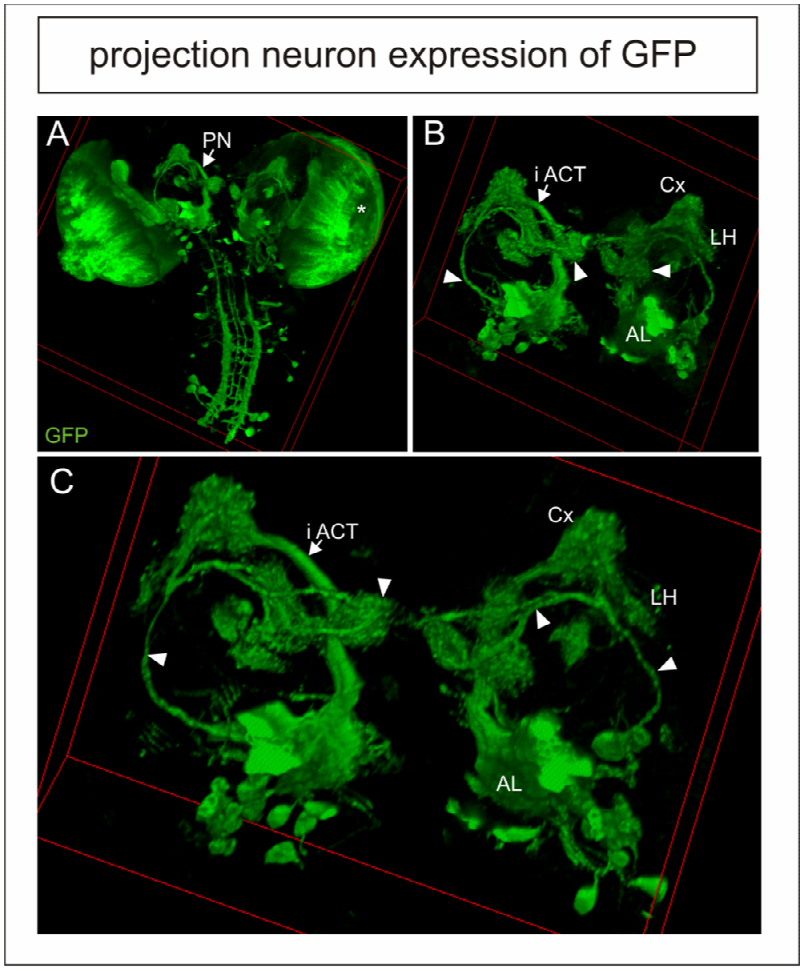


Fig. 15: Projection neuron expression of GFP. (A- C) Gal4-expressing cells in *NP225*–Gal4 monitored by UAS-*GFP*. 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third instar-brains. We find strong GFP expression in the projection neurons. Additionally, a mushroom body extrinsic neuron (►) shows strong GFP immunoreactivity as well. (A) Whole larval brain. (B, C) Magnification of projection neurons and extrinsic mushroom body neurons. Optic lobe anlagen (*), antennal lobe (AL) , inner antennocerebral tract (i ACT), projection neuron (PN), calyx (Cx), lateral horn (LH).

Experiment 7: Rescue without mushroom body expression?

The above experiments have shown that Synapsin expression in the mushroom body, but not in projection neurons, is sufficient to restore the syn^{97}- mutant defect in larval odour-sugar learning. This raises the question whether such mushroom body expression of Synapsin in turn would also be required for learning. To this end, we use a combination of the *elav*-Gal4

driver with an *mb247*-Gal80 element: While *elav*-Gal4 supports brain-wide expression including the mushroom body, the *mb247*-Gal80 element leads to expression of Gal80 in the mushroom body, such that in the mushroom body Gal80 can suppress Gal4 and thus prevent Synapsin expression.

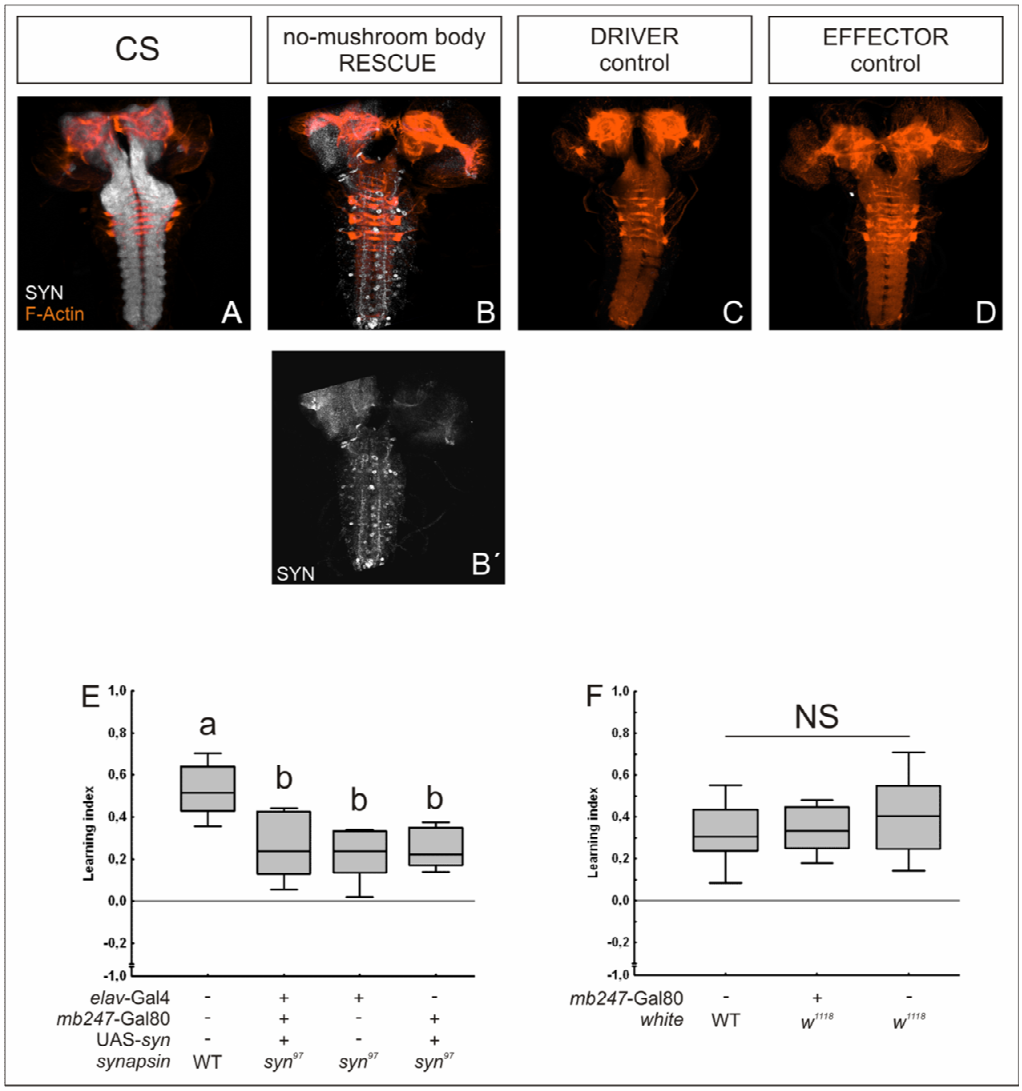


Fig. 16: No rescue by Synapsin expression outside of the mushroom bodies. (A-D) Anti-Synapsin (white) and anti-F-actin (orange) immunoreactivity of whole-mount larval third instar brains. Brains of wild-type CS (A) and the no-mushroom body RESCUE strain (B, B´) show Synapsin staining, but brains of DRIVER control larvae and EFFECTOR control larvae do not. For conveniance, (B´) shows only the Synapsin staining in the no-mushroom RESCUE strain. (E) Synapsin expression outside the mushroom bodies is not sufficient for restoring learning ability. (F) Heterozygous *mb247*-Gal80 larvae learn as well as wild-type CS and w^{1118} larvae excluding a dominant negative effect of the *mb247*-Gal80 construct.
All other details as in the legend of Fig. 3.

Comparing learning ability in such no-mushroom body RESCUE larvae (w^{1118}, *elav*-Gal4/w^{1118}; *mb247*-Gal80/+; UAS-*syn*, syn^{97}/ syn^{97}) to wild-type CS as well as to their genetic controls (no-mushroom body DRIVER control: w^{1118}, *elav*-Gal4/w^{1118}; syn^{97}/ syn^{97}; EFFECTOR control: w^{1118}, / w^{1118}; *mb247*-Gal80/+; UAS-*syn*, syn^{97}/ syn^{97}) reveals a

significant difference in learning ability (Fig. 16E; Kruskal-Wallis test: P< 0.05; H= 14.40; df= 3; N= 12, 12, 12, 12). Importantly, the no-mushroom body RESCUE larvae do not learn as well as wild-type CS (Fig. 16E; U-test: P< 0.05/ 3; U= 24; sample sizes as above); rather, learning ability is as poor as in the genetic controls (Fig. 16E; no-mushroom body RESCUE versus EFFECTOR control: U-test: P> 0.05/ 3; U= 68; no-mushroom body RESCUE versus DRIVER control: U-test: P> 0.05/ 3; U= 69.5; sample sizes as above). Such lack-of-rescue cannot be attributed to a dominant-negative effect of the *mb247*-Gal80 construct, because heterozygous *mb247*-Gal80 larvae learn as well as wild-type CS and w^{1118} (Fig. 16F; Kruskal-Wallis test: P> 0.05; H= 1.15; df= 2; N= 13, 11, 12).

In terms of Synapsin expression, the reduction of mushroom body expression can be documented clearly (Fig.s 16B and B´). However, it seems that *mb247*-Gal80 leads to a reduction of Synapsin expression also in places outside the mushroom body (Fig. 16B and B´; and see also Fig. 17 for blocking GFP-expression in the mushroom bodies).

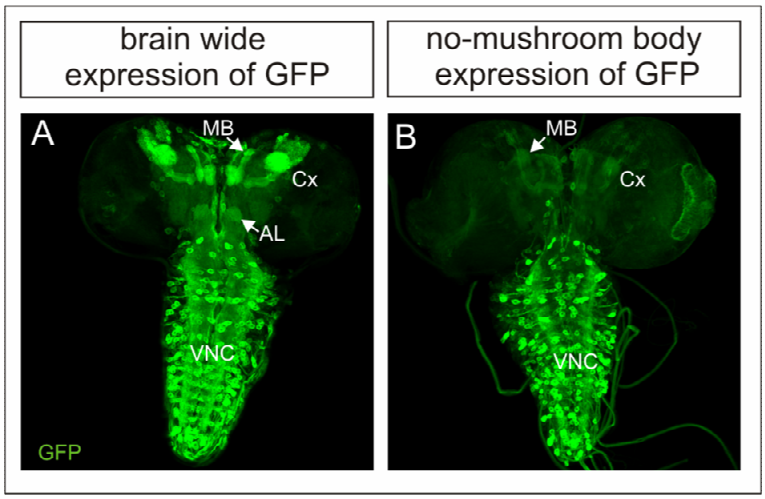


Fig. 17: No-mushroom body expression of GFP. (A) *Elav*-Gal4 and (B) e*lav*-Gal4, *mb247*-Gal80 flies were crossed to UAS-*GFP*. 3D reconstruction of anti-GFP immunoreactivity of whole-mount larval third-instar brains. (A) The larval brain shows strong GFP expression throughout the different neuropils, including strong expression in the mushroom bodies. (B) GFP expression in the mushroom body is almost completely blocked by Gal80. However, m*b247*-Gal80 apparently leads to a reduction of GFP expression also outside the mushroom bodies. Antennal lobe (AL), mushroom body (MB), ventral nerve court (VNC).

This may be due to the *mb247*-element supporting different expression patterns in the *mb247*-Gal4 strain as compared to the *mb247*-Gal80 strain, possibly due to the different insertion sites of the respective transgenes; alternatively, Gal80 may have non-cell autonomous effects and/ or may for to-be-characterized reasons reduce transgene expression in general. In any event, we can conclude that Synapsin expression outside of the coverage of *mb247*-Gal80 is not sufficient to rescue the syn^{97} mutant learning defect; in turn, those neurons which are

covered by *mb247*-Gal80 obviously do need to express Synapsin in order for normal levels of learning to be supported.

To summarize, olfactory learning is impaired upon lack of Synapsin (in the deletion mutant syn^{97}) or reduction of Synapsin expression (by means of pan-neuronal RNAi). Acute pan-neural expression of Synapsin (inducible *elav*-Gal4) and constitutive local expression in only the mushroom body (*mb247*-Gal4 as well as *D52H*-Gal4), fully restore learning, whereas no such restoration of learning is found by Synapsin expression throughout large parts of the brain excluding the mushroom bodies, or by Synapsin expression in specifically the projection neurons (*GH146*-Gal4 and *NP225*-Gal4). Thus, in order for learning to be restored, Synapsin expression in the mushroom bodies is evidently sufficient and likely necessary within the mushroom bodies of larval *Drosophila*.

DISCUSSION

We show that the learning defect in the *syn*^{97}- mutant (Michels et al., 2005) can be phenocopied by an RNAi-mediated knock-down of Synapsin (Fig. 4E), and can be rescued by acutely restoring Synapsin (Fig. 7E and F). Furthermore, locally restoring Synapsin in the mushroom bodies fully restores learning ability (Fig.s 8E and 11E), whereas such restoration in the projection neurons does not. Also, if Synapsin is restored in wide areas of the brain except for the mushroom bodies, learning ability is not restored (Fig. 16E).
We therefore conclude that a Synapsin-dependent memory trace is located in the mushroom bodies, and tentatively suggest that this is the only site where such a trace is established regarding odour-sugar short-term memory in larval *Drosophila*.

A projection neuron conundrum?

This scenario is in contrast to a recent report concerning adult flies: (Thum et al., 2007) argue that regarding the Rutabaga-dependent short-term memory trace after odour-sugar learning in adult *Drosophila*, projection neurons as well as mushroom bodies can accommodate memory traces. Before venturing into a discussion of this apparent discrepancy, however, we note that the projection neuron rescue regarding Rutabaga as reported by (Thum et al., 2007) may not actually come about by Rutabaga expression in the projection neurons. That is, Rutabaga expression was approximated indirectly, by expressing GFP from the respective projection neuron Gal4 drivers (*GH146*-Gal4 and *NP225*-Gal4). Indeed, none of the Rutabaga local-rescue studies published to-date (Zars et al., 2000; Schwaerzel et al., 2003; Akalal et al., 2006; Liu et al., 2006) reports the actual expression pattern of the Rutabaga protein (exception being McGuire et al. [2003] who report faint anti-Rutabaga immunoreactivity in the mushroom bodies of *c772*-Gal4 [loc. cit. Fig. 3]). Clearly, such indirect assessment can lead astray (Ito et al., 2003; Saper and Sawchenko, 2003); this may be particularly so with respect to the *GH146*-Gal4 and *NP225*-Gal4 driver-strains, as both strains cover, in addition to expression in the projection neurons, a prominent mushroom body-extrinsic neuron in both adult (Heimbeck et al., 2001; Thum et al., 2007) and larval *Drosophila* (Marin et al. [2005]; see also our Fig.s 13 and 15). This raises the possibility that a rescue of learning ability using these driver strains actually comes about by transgene expression in this mushroom body-extrinsic neuron. Thus, the apparent discrepancy between our present conclusion and Thum et al. (2007) may regard the function of this mushroom body-extrinsic neuron, rather than the projection neurons.

In any event, using the same Gal4-driver strains (*GH146*-Gal4 and *NP225*-Gal4), there is an apparent discrepancy between the successful Rutabaga-rescue for adult odour-shock learning on the one hand, and a lack of such rescue with respect to Synapsin in the case of larval odour-sugar learning on the other hand; this discrepancy might have various reasons:

(i) Projection neurons may house a memory trace in adult, but not larval *Drosophila*. However, despite the reduced cell number in larvae, the general layout of the olfactory system appears strikingly similar between larvae and adult (Gerber et al., 2008). We therefore hesitate to believe that major qualitative differences in functional architecture have to be reckoned with.

(ii) The projection neuron-memory trace may be Rutabaga-dependent, but Synapsin-independent. Molecularly, however, Rutabaga may rather act upstream of Synapsin. That is, the current working model of memory trace formation holds that the Rutabaga adenylyl cyclase acts as a coincidence detector for an aminergic reinforcement signal and the odour-induced activation of the mushroom body neurons (Heisenberg [2003]; this notion is largely based on the analyses of synaptic plasticity in *Aplysia* during classical conditioning: Abrams et al., 1998; Byrne and Kandel, 1996; Lechner and Byrne, 1998). The ensuing increase in cAMP levels and PKA activity then is thought to lead to phosphorylation of a number of effector proteins, potentially including Synapsin (Hilfiker et al., 1999; Fiumara et al., 2004). Such Synapsin phosphorylation then is thought to allow recruitment of synaptic vesicles from the reserve pool to the readily releasable pool (Hilfiker et al., 1999), so that upon later presentation of the learnt odour, more transmitter can be released (but see Sudhof [2004] for a critical discussion of this working model of Synapsin function in *Drosophila*). While experiments to directly verify this working model in the *Drosophila* mushroom body are still lacking, we note that such action of Rutabaga and Synapsin within the same signalling cascade is consistent with the lack of additivity of the syn^{97} and rut^{2080} mutations in adult odour-shock learning (S. Knapek, H. Tanimoto, Max-Planck Institut für Neurobiologie, Martinsried, Germany, pers. comm.). Given the apparent conservation of both adenylyl cyclase and Synaspin function across evolution, it does not appear straightforward to expect that the signalling cascade downstream of Rutabaga is different in projection neurons as compared to the mushroom bodies.

(iii) We may, concerning Synapsin, have unwittingly overlooked a projection neuron rescue. The most obvious reason for such inadvertence may be that the P-element insertions carrying *GH146*-Gal4 and *NP225*-Gal4 disrupt an endogenous gene, thereby causing dominant

learning impairments and masking an actually successful rescue. This, however, could be ruled out by showing that larvae heterozygous for these insertions learn as well as wild-type larvae (Fig.s 12F and 14F).

Alternatively, it may be that the fraction of projection neurons that express Gal4 in the larva is insufficiently low. However, *GH146*-Gal4 and *NP225*-Gal4 express Synapsin in 13-16 from a total of 21 projection neurons in the larva (~ 60 %), which about matches the fraction of projection neurons covered in the adult (~ 83 [GH146-Gal4] and ~ 70 [NP225-Gal4] from an estimated total of 150 projection neurons: Heimbeck et al., 2001; Tanaka et al., 2004).

Finally, Synapsin staining in the projection neurons is relatively faint (Fig.s 12B´ and 14B´); thus, levels of Synapsin expression in the larvae may have been too low to restore learning. Given the lack of anti-Rutabaga immunohistochemistry concerning *GH146*-Gal4 and *NP225*-Gal4, however, an educated guess about Rutabaga levels is impossible; therefore, although it remains a theoretical possibility, one can at present not reasonably compare expression levels of Synapsin versus Rutabaga. And even if this were possible, judgements as to whether the respective levels of expression are appropriate for restoring learning ability would be difficult.

(iv) The adult expression pattern of *GH146*-Gal4 and *NP225*-Gal4 may include neurons that are not covered in the larva, and/ or which are not yet functionally mature. Again, careful assessment of anti-Rutabaga immunohistochemistry is mandatory to see whether this argument holds.

Taken together, none of these lines of argument seems fully compelling to explain the apparent discrepancy between our present results and the ones presented by Thum et al al. (2007). We note, however, that mushroom-body expression of a dominant-negative form of $G\alpha_s$ ($G\alpha_s^*$) does impair adult odour-sugar learning (Thum, 2006b; loc. cit. Fig. 13), a finding that is inconsistent with a fully independent odour-sugar memory trace within the adult projection neurons. Obviously, experiments to induce a local knock-down of Rutabaga in the mushroom bodies via RNAi could help clarifying this point. Finally, it should be kept in mind that in adults, but not in larvae, the animals have to be starved before the experiment, such that any discrepancy between larvae and adult, as well as the reported discrepancies between odour-shock and odour-sugar learning within the adult stage, may partially be affected by motivational differences.

In the light of all these reasonings, it seems wise to postpone judgement as to whether there actually is a discrepancy between larvae and adult, and if so: how it may have to be explained.

Mushroom body

It seems remarkable that expression of Synapsin in only a handful of mushroom body neurons is sufficient to fully restore learning scores (Fig. 11E). That is, using the *D52H*-Gal4 driver strain, a maximum of 7 mushroom body neurons (of a total of about 600 mushroom body neurons with fully developed dendritic arborizations [Lee et al., 1999]; for an estimation of a total of 1800 larval mushroom body neurons based on fiber counts in electron microscope sections, see [Technau and Heisenberg 1982]) express Synapsin; despite this low number of cells, however, the majority of the total of 36 mushroom body-glomeruli (Masuda-Nakagawa et al., 2005) appear innervated. Actually, such broad coverage of the mushroom body-input region may be expected: Masuda-Nakagawa (2005) showed that each mushroom body neuron typically receives input in a random subset of six out of the total of appr. 36 glomeruli. Thus, if more than six mushroom body neurons are included in a given Gal4 strain, fairly broad regions of the calyx, and hence a fairly broad range of the olfactory input space, should be covered (see also Murthy et al. [2008]; Turner et al. [2008]). Nevertheless, the seven mushroom body neurons covered by the *D52H*-Gal4 driver strain obviously allow for fewer combinatorial activity patterns than the whole set of mushroom body neurons (under the assumption that each neuron can only be either active or not, seven neurons allow for $2^7 = 128$ combinations of activity, whereas 600 neurons allow for 2^{600} combinations); therefore, the ability of this driver strain to support differential conditioning with relatively similar odours may be compromised. As we find, however, that wild-type larvae show hardly any generalization between the odours we have tested to date (*n*-amylacetate, 1-octanol, and 3-octanol; Y.-c. Chen, Universität Würzburg, Germany, pers. comm.), testing this hypothesis has to await the identification of perceptually sufficiently similar odour pairs (see e.g. Cobb and Domain [2000]; Boyle and Cobb [2005]; Kreher et al. [2008]). In any event, we would also like to mention that possibly the neurons covered by *D52H*-Gal4 are 'special'. That is, the *D52H*-Gal4 element includes parts of the expression pattern of Dunce, as it includes a *dunce* enhancer sequence (Qiu and Davis, 1993). The *dunce* gene codes for a cAMP-specific phosphodiesterase and is required for normal learning and memory (Tully and Quinn, 1985; Dauwalder and Davis, 1995), potentially in the larva as well (Aceves-Pina and Quinn, 1979; Tully et al., 1994). Antibodies against Dunce have been taken to suggest that the most intensely stained region in adult and larval brains is the mushroom body (Nighorn et al., 1991). Thus, although only few mushroom body neurons are labeled by *D52H*-Gal4, it may be that these neurons are particular in that they express a particular *dunce* transcript and are thus dedicated for establishing a memory trace.

Outlook into molecular network

Whereas the molecular mechanisms of coincidence detection by the type I adenylyl cyclase during training are well known and appear widely conserved across the animal kingdom (Dudai et al., 1988; Abrams et al., 1998; Renger et al., 2000; Heisenberg, 2003), the question what the downstream processes are that allow more transmitter being released at the moment of testing remain a challenge. As argued above, it seems conceivable that the PKA consensus site(s) of Synapsin are required for reserve-pool vesicle recruitment, and hence for memory trace formation and ultimately altered olfactory behaviour. Together with the efforts of the *Drosophila* research community which focus on different aspects of presynaptic function (Kim et al. [2003]; Schwaerzel et al. [2003]; Steinert et al. [2006]; Wagh et al. [2006]; Lissandron et al. [2007]), tackling these issues may eventually yield a comprehensive picture of memory trace formation not only on the cellular level, but also in terms of the underlying presynaptic network. An unbroken chain of explanation from a defined biochemical event like the phosphorylation of a given serine within a PKA consensus site to a learnt change in behaviour is not only interesting from a basic research point of view. Rather, the apparent homology of many of the molecular determinants for synaptic and behavioural plasticity suggests that such an endeavour may have relevance for biomedical research. Last but not least, understanding which specific sites along the sensory-motor circuit are altered to accommodate behavioural changes may be inspiring for the design of ‘intelligent’ technical equipment as well.

REFERENCES

Abrams TW, Yovell Y, Onyike CU, Cohen JE, Jarrard HE (1998) Analysis of sequence-dependent interactions between transient calcium and transmitter stimuli in activating adenylyl cyclase in Aplysia: possible contribution to CS--US sequence requirement during conditioning. Learn Mem 4:496-509.

Aceves-Pina EO, Quinn WG (1979) Learning in Normal and Mutant Drosophila Larvae. Science 206:93-96.

Akalal DB, Wilson CF, Zong L, Tanaka NK, Ito K, Davis RL (2006) Roles for Drosophila mushroom body neurons in olfactory learning and memory. Learn Mem 13:659-668.

Arnold C, Reisch N, Leibold C, Becker S, Prufert K, Sautter K, Palm D, Jatzke S, Buchner S, Buchner E (2004) Structure-function analysis of the cysteine string protein in Drosophila: cysteine string, linker and C terminus. J Exp Biol 207:1323-1334.

Bergmann A, Yang AY, Srivastava M (2003) Regulators of IAP function: coming to grips with the grim reaper. Curr Opin Cell Biol 15:717-724.

Boyle J, Cobb M (2005) Olfactory coding in Drosophila larvae investigated by cross-adaptation. J Exp Biol 208:3483-3491.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Brembs B, Heisenberg M (2000) The operant and the classical in conditioned orientation of Drosophila melanogaster at the flight simulator. Learn Mem 7:104-115.

Byers D, Davis RL, Kiger JA, Jr. (1981) Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. Nature 289:79-81.

Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425-435.

Cobb M, Domain I (2000) Olfactory coding in a simple system: adaptation in Drosophila larvae. Proc Biol Sci 267:2119-2125.

Connolly JB, Roberts IJ, Armstrong JD, Kaiser K, Forte M, Tully T, O'Kane CJ (1996) Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 274:2104-2107.

Dauwalder B, Davis RL (1995) Conditional rescue of the dunce learning/memory and female fertility defects with Drosophila or rat transgenes. J Neurosci 15:3490-3499.

Davis RL (1996) Physiology and biochemistry of Drosophila learning mutants. Physiol Rev 76:299-317.

Davis RL, Cherry J, Dauwalder B, Han PL, Skoulakis E (1995) The cyclic AMP system and Drosophila learning. Mol Cell Biochem 149-150:271-278.

de Belle JS, Heisenberg M (1994) Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. Science 263:692-695.

Diegelmann S, Zars M, Zars T (2006a) Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in Drosophila. Learn Mem 13:72-83.

Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblauer S, Couto A, Marra V, Keleman K, Dickson BJ (2007) A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature 448:151-156.

Dubnau J, Grady L, Kitamoto T, Tully T (2001) Disruption of neurotransmission in Drosophila mushroom body blocks retrieval but not acquisition of memory. Nature 411:476-480.

Dudai Y, Corfas G, Hazvi S (1988) What is the possible contribution of Ca2+-stimulated adenylate cyclase to acquisition, consolidation and retention of an associative olfactory memory in Drosophila. J Comp Physiol [A] 162:101-109.

Duerr JS, Quinn WG (1982) Three Drosophila mutations that block associative learning also affect habituation and sensitization. Proc Natl Acad Sci U S A 79:3646-3650.

Faber T, Joerges J, Menzel R (1999) Associative learning modifies neural representations of odors in the insect brain. Nat Neurosci 2:74-78.

Farooqui T, Robinson K, Vaessin H, Smith BH (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. J Neurosci 23:5370-5380.

Fiumara F, Giovedi S, Menegon A, Milanese C, Merlo D, Montarolo PG, Valtorta F, Benfenati F, Ghirardi M (2004) Phosphorylation by cAMP-dependent protein kinase is essential for synapsin-induced enhancement of neurotransmitter release in invertebrate neurons. J Cell Sci 117:5145-5154.

Forbes B (1993) Larval learning amd memory in Drosophila melanogaster [Diploma Thesis]. University of Würzburg, Department of Genetics and Neurobiology.

Frohlich A, Meinertzhagen IA (1982) Synaptogenesis in the first optic neuropile of the fly's visual system. J Neurocytol 11:159-180.

Gerber B, Hendel T (2006) Outcome expectations drive learned behaviour in larval Drosophila. Proc Biol Sci 273:2965-2968.

Gerber B, Tanimoto H, Heisenberg M (2004b) An engram found? Evaluating the evidence from fruit flies. Curr Opin Neurobiol 14:737-744.

Gerber B, Stocker RF, Tanimura T, Thum AS (2008) Smelling, Tasting, Learning: Drosophila as a Study Case. Springer-Verlag Berlin Heidelberg.

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, Heisenberg M (2004a) Visual learning in individually assayed Drosophila larvae. J Exp Biol 207:179-188.

Gitler D, Cheng Q, Greengard P, Augustine GJ (2008) Synapsin IIa controls the reserve pool of glutamatergic synaptic vesicles. J Neurosci 28:10835-10843.

Godenschwege TA, Reisch D, Diegelmann S, Eberle K, Funk N, Heisenberg M, Hoppe V, Hoppe J, Klagges BR, Martin JR, Nikitina EA, Putz G, Reifegerste R, Reisch N, Rister J, Schaupp M, Scholz H, Schwarzel M, Werner U, Zars TD, Buchner S, Buchner E (2004) Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. Eur J Neurosci 20:611-622.

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780-785.

Hammer M, Menzel R (1998) Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. Learn Mem 5:146-156.

Hauser F, Cazzamali G, Williamson M, Blenau W, Grimmelikhuijzen CJ (2006) A review of neurohormone GPCRs present in the fruitfly Drosophila melanogaster and the honey bee Apis mellifera. Prog Neurobiol 80:1-19.

Hay BA, Huh JR, Guo M (2004) The genetics of cell death: approaches, insights and opportunities in Drosophila. Nat Rev Genet 5:911-922.

Heimbeck G, Bugnon V, Gendre N, Keller A, Stocker RF (2001) A central neural circuit for experience-independent olfactory and courtship behavior in Drosophila melanogaster. Proc Natl Acad Sci U S A 98:15336-15341.

Heisenberg M (2003) Mushroom body memoir: from maps to models. Nat Rev Neurosci 4:266-275.

Heisenberg M, Wolf R (1984) Vision in Drosophila. Genetics of microbehavior. Springer, New York, NY.

Heisenberg M, Wolf R (1988) Reafferent control of optomotor yaw torque in Drosophila melanogaster. J Comp Physiol A

Heisenberg M, Gerber B (2008) Behavioral Analysis of Learning and Memory in Drosophila. InR Menzel (Ed), Learning Theory and Behavior Vol [1] of Learning and Memory: A Comprehensive Reference, 4 vols (JByrne Editor), pp [549-560] Oxford: Elsevier.

Heisenberg M, Borst A, Wagner S, Byers D (1985) Drosophila mushroom body mutants are deficient in olfactory learning. J Neurogenet 2:1-30.

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B (2005) The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed Drosophila larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:265-279.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354:269-279.

Hosaka M, Hammer RE, Sudhof TC (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. Neuron 24:377-387.

Ito K, Okada R, Tanaka NK, Awasaki T (2003) Cautionary observations on preparing and interpreting brain images using molecular biology-based staining techniques. Microsc Res Tech 62:170-186.

Ito K, Suzuki K, Estes P, Ramaswami M, Yamamoto D, Strausfeld NJ (1998) The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen. Learn Mem 5:52-77.

Kalidas S, Smith DP (2002) Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. Neuron 33:177-184.

Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030-1038.

Kao HT, Porton B, Hilfiker S, Stefani G, Pieribone VA, DeSalle R, Greengard P (1999) Molecular evolution of the synapsin gene family. J Exp Zool 285:360-377.

Kaun KR, Hendel T, Gerber B, Sokolowski MB (2007) Natural variation in Drosophila larval reward learning and memory due to a cGMP-dependent protein kinase. Learn Mem 14:342-349.

Kim J, Chung YD, Park DY, Choi S, Shin DW, Soh H, Lee HW, Son W, Yim J, Park CS, Kernan MJ, Kim C (2003) A TRPV family ion channel required for hearing in Drosophila. Nature 424:81-84.

Kitamoto T (2001) Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. J Neurobiol 47:81-92.

Kitamoto T (2002) Targeted expression of temperature-sensitive dynamin to study neural mechanisms of complex behavior in Drosophila. J Neurogenet 16:205-228.

Klagges BR, Heimbeck G, Godenschwege TA, Hofbauer A, Pflugfelder GO, Reifegerste R, Reisch D, Schaupp M, Buchner S, Buchner E (1996) Invertebrate synapsins: a single gene codes for several isoforms in Drosophila. J Neurosci 16:3154-3165.

Krashes MJ, Keene AC, Leung B, Armstrong JD, Waddell S (2007) Sequential use of mushroom body neuron subsets during drosophila odor memory processing. Neuron 53:103-115.

Kreher SA, Mathew D, Kim J, Carlson JR (2008) Translation of sensory input into behavioral output via an olfactory system. Neuron 59:110-124.

Kurusu M, Awasaki T, Masuda-Nakagawa LM, Kawauchi H, Ito K, Furukubo-Tokunaga K (2002) Embryonic and larval development of the Drosophila mushroom bodies: concentric layer subdivisions and the role of fasciclin II. Development 129:409-419.

Kyhse-Andersen J (1984) Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J Biochem Biophys Methods 10:203-209.

Lechner HA, Byrne JH (1998) New perspectives on classical conditioning: a synthesis of Hebbian and non-Hebbian mechanisms. Neuron 20:355-358.

Lee T, Luo L (1999) Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22:451-461.

Lee T, Lee A, Luo L (1999) Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. Development 126:4065-4076.

Li L, Chin LS, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P, et al. (1995) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9235-9239.

Lin DM, Goodman CS (1994) Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron 13:507-523.

Lissandron V, Rossetto MG, Erbguth K, Fiala A, Daga A, Zaccolo M (2007) Transgenic fruit-flies expressing a FRET-based sensor for in vivo imaging of cAMP dynamics. Cell Signal 19:2296-2303.

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L (2006) Distinct memory traces for two visual features in the Drosophila brain. Nature 439:551-556.

Livingstone MS, Tempel BL (1983) Genetic dissection of monoamine neurotransmitter synthesis in Drosophila. Nature 303:67-70.

Livingstone MS, Sziber PP, Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. Cell 37:205-215.

Lohr R, Godenschwege T, Buchner E, Prokop A (2002) Compartmentalization of central neurons in Drosophila: a new strategy of mosaic analysis reveals localization of presynaptic sites to specific segments of neurites. J Neurosci 22:10357-10367.

Marin EC, Watts RJ, Tanaka NK, Ito K, Luo L (2005) Developmentally programmed remodeling of the Drosophila olfactory circuit. Development 132:725-737.

Martin JR, Keller A, Sweeney ST (2002) Targeted expression of tetanus toxin: a new tool to study the neurobiology of behavior. Adv Genet 47:1-47.

Masuda-Nakagawa LM, Tanaka NK, O'Kane CJ (2005) Stereotypic and random patterns of connectivity in the larval mushroom body calyx of Drosophila. Proc Natl Acad Sci U S A 102:19027-19032.

McGuire SE, Le PT, Davis RL (2001) The role of Drosophila mushroom body signaling in olfactory memory. Science 293:1330-1333.

McGuire SE, Le PT, Osborn AJ, Matsumoto K, Davis RL (2003) Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302:1765-1768.

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B (2005) A role for Synapsin in associative learning: the Drosophila larva as a study case. Learn Mem 12:224-231.

Murthy M, Fiete I, Laurent G (2008) Testing odor response stereotypy in the Drosophila mushroom body. Neuron 59:1009-1023.

Neuser K, Husse J, Stock P, Gerber B (2005) Appetitive olfactory learning in Drosophila larvae: effects of repetition, reward strength, age, gender, assay type and memory span. Animal behaviour 69:891-898.

Niewalda T, Singhal N, Fiala A, Saumweber T, Wegener S, Gerber B (2008) Salt Processing in Larval Drosophila: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way. Chem Senses.

Nighorn A, Healy MJ, Davis RL (1991) The cyclic AMP phosphodiesterase encoded by the Drosophila dunce gene is concentrated in the mushroom body neuropil. Neuron 6:455-467.

Peele P, Ditzen M, Menzel R, Galizia CG (2006) Appetitive odor learning does not change olfactory coding in a subpopulation of honeybee antennal lobe neurons. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 192:1083-1103.

Putz G, Heisenberg M (2002) Memories in drosophila heat-box learning. Learn Mem 9:349-359.

Putz G, Bertolucci F, Raabe T, Zars T, Heisenberg M (2004) The S6KII (rsk) gene of Drosophila melanogaster differentially affects an operant and a classical learning task. J Neurosci 24:9745-9751.

Qiu Y, Davis RL (1993) Genetic dissection of the learning/memory gene dunce of Drosophila melanogaster. Genes Dev 7:1447-1458.

Ramaekers A, Magnenat E, Marin EC, Gendre N, Jefferis GS, Luo L, Stocker RF (2005) Glomerular maps without cellular redundancy at successive levels of the Drosophila larval olfactory circuit. Curr Biol 15:982-992.

Renger JJ, Ueda A, Atwood HL, Govind CK, Wu CF (2000) Role of cAMP cascade in synaptic stability and plasticity: ultrastructural and physiological analyses of individual synaptic boutons in Drosophila memory mutants. J Neurosci 20:3980-3992.

Riemensperger T, Voller T, Stock P, Buchner E, Fiala A (2005) Punishment prediction by dopaminergic neurons in Drosophila. Curr Biol 15:1953-1960.

Sachse S, Galizia CG (2002) Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. J Neurophysiol 87:1106-1117.

Saper CB, Sawchenko PE (2003) Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry. J Comp Neurol 465:161-163.

Saumweber T, Michels B, Bucher D, Funk N, Reisch D, Krohne G, Wegener S, Buchner E, Gerber B (in prep.) Associative learning is impaired upon lack of the presynaptic protein SAP47.

Scherer S, Stocker RF, Gerber B (2003) Olfactory learning in individually assayed Drosophila larvae. Learn Mem 10:217-225.

Schipanski A, Yarali A, Niewalda T, Gerber B (2008) Behavioral analyses of sugar processing in choice, feeding, and learning in larval Drosophila. Chem Senses 33:563-573.

Schmid B, Schindelin J, Cardona A, Heisenberg M (2008) Hardware-accelerated 3D visualization for ImageJ. Proceedings of the ImageJ User and Developer Conference, Edition1, Luxembourg.

Schwaerzel M, Heisenberg M, Zars T (2002) Extinction antagonizes olfactory memory at the subcellular level. Neuron 35:951-960.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. J Neurosci 23:10495-10502.

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407:319-320.

Steinert JR, Kuromi H, Hellwig A, Knirr M, Wyatt AW, Kidokoro Y, Schuster CM (2006) Experience-dependent formation and recruitment of large vesicles from reserve pool. Neuron 50:723-733.

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.

Sudhof TC, Czernik AJ, Kao HT, Takei K, Johnston PA, Horiuchi A, Kanazir SD, Wagner MA, Perin MS, De Camilli P, et al. (1989) Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. Science 245:1474-1480.

Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ (1995) Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14:341-351.

Tanaka NK, Tanimoto H, Ito K (2008) Neuronal assemblies of the Drosophila mushroom body. J Comp Neurol 508:711-755.

Tanaka NK, Awasaki T, Shimada T, Ito K (2004) Integration of chemosensory pathways in the Drosophila second-order olfactory centers. Curr Biol 14:449-457.

Technau G, Heisenberg M (1982) Neural reorganization during metamorphosis of the corpora pedunculata in Drosophila melanogaster. Nature 295:405-407.

Tempel BL, Livingstone MS, Quinn WG (1984) Mutations in the dopa decarboxylase gene affect learning in Drosophila. Proc Natl Acad Sci U S A 81:3577-3581.

Tettamanti M, Armstrong JD, Endo K, Yang MY, Furukubo-Tokunaga K, Kaiser K, et al (1997) Early development of the Drosophila mushroom bodies, brain centres for associative learning and memory. Development Genes and Evolution 207:242-252.

Thum AS (2006b) Sugar reward learning in Drosophila: neuronal circuits in Drosophila associative olfactory learning [PhD Thesis]. University of Würzburg, Department of Genetics and Neurobiology.

Thum AS, Jenett A, Ito K, Heisenberg M, Tanimoto H (2007) Multiple memory traces for olfactory reward learning in Drosophila. J Neurosci 27:11132-11138.

Thum AS, Knapek S, Rister J, Dierichs-Schmitt E, Heisenberg M, Tanimoto H (2006a) Differential potencies of effector genes in adult Drosophila. J Comp Neurol 498:194-203.

Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant Drosophila melanogaster. J Comp Physiol [A] 157:263-277.

Tully T, Cambiazo V, Kruse L (1994) Memory through metamorphosis in normal and mutant Drosophila. J Neurosci 14:68-74.

Turner GC, Bazhenov M, Laurent G (2008) Olfactory representations by Drosophila mushroom body neurons. J Neurophysiol 99:734-746.

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Durrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E (2006) Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in Drosophila. Neuron 49:833-844.

White K, Steller H (1995) The control of apoptosis in Drosophila. Trends Cell Biol 5:74-78.

Wilson BA, Collier RJ (1992) Diphtheria toxin and Pseudomonas aeruginosa exotoxin A: active-site structure and enzymic mechanism. Curr Top Microbiol Immunol 175:27-41.

Wilson RI, Laurent G (2005) Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe. J Neurosci 25:9069-9079.

Yarali A, Krischke M, Michels B, Saumweber T, Mueller MJ, Gerber B [In Press] Genetic Distortion of the Balance between Punishment and Relief Learning in Drosophila. J Neurogenet:1-16.

Yin JC, Tully T (1996) CREB and the formation of long-term memory. Curr Opin Neurobiol 6:264-268.

Zars T, Fischer M, Schulz R, Heisenberg M (2000) Localization of a short-term memory in Drosophila. Science 288:672-675.

Zeng X, Sun M, Liu L, Chen F, Wei L, Xie W (2007) Neurexin-1 is required for synapse formation and larvae associative learning in Drosophila. FEBS Lett 581:2509-2516.

Zinsmaier KE, Eberle KK, Buchner E, Walter N, Benzer S (1994) Paralysis and early death in cysteine string protein mutants of Drosophila. Science 263:977-980.

Zinsmaier KE, Hofbauer A, Heimbeck G, Pflugfelder GO, Buchner S, Buchner E (1990) A cysteine-string protein is expressed in retina and brain of Drosophila. J Neurogenet 7:15-29.

Chapter II.

II.1. Common involvement of Synapsin in predictive learning about the presence and absence of shock in adult flies

Niewalda, T, Yarali, A., Michels B, Gerber, B.

Blackwell Publishing
EJN European Journal of Neuroscience

Common Involvement of Synapsin in Predictive Learning about the Presence and Absence of Shock

Journal:	*European Journal of Neuroscience*
Manuscript ID:	draft
Manuscript Type:	Short Communication
Date Submitted by the Author:	n/a
Complete List of Authors:	Gerber, Bertram; University of Wuerzburg, Genetics and Neurobiology Niewalda, Thomas; University of Wuerzburg, Genetics and Neurobiology Michels, Birgit; University of Wuerzburg, Genetics and Neurobiology Yarali, Ayse; University of Wuerzburg, Genetics and Neurobiology
Key Words:	olfaction, Drosophila, memory, pain

Receiving Editor: M. Sarter

Common Involvement of Synapsin in Predictive Learning about the Presence and Absence of Shock

Thomas Niewalda, Ayse Yarali, Birgit Michels & Bertram Gerber*

All authors at:

University of Würzburg

Biocenter, Am Hubland

Department of Genetics and Neurobiology

D 970 74 Würzburg

* Corresponding author

Phone +49 931 888 4483

Fax +49 931 888 4452

Email bertram.gerber@biozentrum.uni-wuerzburg.de

Running title: Synapsin and Predictive Learning

Pages 14	Figures 3	Tables 0	Equations 4
Words	In MS[1)]~3200	In Abstract ~170	In Introduction ~430

[1)] Exclusive of Abstract, Acknowledgements, Figure Legends

Key Words: Olfaction, Memory, *Drosophila*, Pain

Abstract

Painful experiences can mould future behaviour, but it is relatively little recognized that in addition to the canonical 'negative' memories for those stimuli that had preceded a painful event, there are 'positive' memories for those stimuli occurring right after it. Indeed to date nothing is known about the molecular underpinnings for this latter kind of learning. We tackle this issue for predictive associative learning between odour and electric shock in *Drosophila*. We show that an odour is avoided after odour ➔ shock training because it predicts shock, whereas after shock ➔ odour training the odour is approached because it signals no-shock. Do these learning processes, which have antagonistic effects on behaviour, share molecular determinants? We show that a lack of Synapsin, a presynaptic protein regulating synaptic output, leads to reduced learning about both shock-predicting and no-shock predicting stimuli, but leaves all critical sensory-motor faculties intact. These are the first results, in any experimental system, concerning how these antagonistic behavioural consequences of shock come about molecularly.

Introduction

Painful, traumatic experiences can have a moulding influence on behaviour. In terms of associative learning, research is largely concerned with the 'negative' memories that such experiences induce: Those stimuli perceived right before the painful event become predictors of danger and consequently will be avoided when encountered again. While in principle danger-predictions are adaptive, they may under unfavourable conditions also contribute to maladaptive behaviours and undesired psychological states (e.g. panic, anxiety, stress); thus, any means to counteract these effects may be of value.

We look at the 'backside' of painful events by investigating memories related to stimuli perceived after a painful event. Interestingly, these stimuli will be approached when subsequently encountered: In fruit flies, odour ➔ shock training leads to conditioned avoidance of the odour during subsequent test, whereas repeated shock ➔ odour training leads to conditioned approach (Tanimoto et al. 2004; Yarali et al. 2006). Thus, regarding the same painful, traumatic experience there are two kinds of memory, one 'negative' for preceding events which supports conditioned avoidance, and one, typically much less strong, 'positive' memory for following events which supports conditioned approach (Tanimoto et al. 2004; Yarali et al. 2006) (see Hellstern et al. 1998 for related results in bees). This makes sense, as due to odour ➔ shock training the odour predicts danger, whereas shock ➔ odour training turns the odour into a predictor of a period of safety (Sutton & Barto 1990; Chang et al. 2003) and/ or of relief from shock (Solomon & Corbit, 1974, Wagner 1981). We call these opposing behavioural shock effects *shock-learning* and *no-shock learning*. Despite the rich literature on shock-learning, to our knowledge nothing is known about the molecular determinants of no-shock learning, which is the topic of the present contribution.

We focus on the *synapsin* gene, which codes for an evolutionarily conserved presynaptic phosphoprotein expressed throughout the flies' nervous system (Godenschwege et al. 2004; Michels et al. 2005). Synapsin tethers reserve-pool vesicles to the cytoskeleton and regulates their recruitment to the readily-releasable pool in a phosphorylation-dependent way, thus contributing to the regulation of synaptic output (review by Hilfiker et al. 1999). Correspondingly, Synapsin null mutant flies (Fig. 1A) are impaired in shock-learning (Godenschwege et al. 2004; Fig. 1B), spatial learning in the heat-box paradigm (Godenschwege et al. 2004), and in odour-sugar learning in larval *Drosophila* (Michels et al. 2005) (concerning vertebrates see Silva et al. 1996; Garcia et al. 2004; Gitler et al. 2004). We therefore chose to ask whether no-shock learning depends on Synapsin as well.

Materials and Methods

We compare wild-type Canton-S flies to the deletion mutant syn^{97CS}, which had undergone 13 outcrossing steps to ensure identical genetic background (Michels et al. 2005). Flies are kept in mass culture at 25 °C, 60-70 % humidity and a 14/10 hour light/ dark cycle. One to five-day old flies are collected and kept at 18°C until the following experimental day.

Experiments are performed at 22-25 °C and 75-85 % relative humidity. We use flies in groups of about 150 (learning experiments, SHOCK- and ODOUR-EXPOSURE controls) or 50 (shock avoidance and naïve odour avoidance). Loading of the flies to the experimental set-up as well as training is performed in dim red light to allow sight for the experimenter; all tests are run in darkness. Electric shock is applied via an electrifiable grid, covering the inner side of the training tubes. A vacuum pump ensures removal of odour-saturated air. As odorants, 80 µl benzaldehyde (BA; Fluka, Steinheim, Germany) and 110 µl limonene (LM; Sigma-Aldrich, Steinheim, Germany) are applied in Teflon containers of 5-mm or 7-mm diameter, respectively.

For learning experiments, flies receive 6 training trials. At time := 0 min, flies are loaded to the experimental set-up, which takes appr. 1 min. After an additional accommodation period of 3 min, the control odour is presented for 15 s. For shock-learning, the to-be-learned odour is presented from 7:15 to 7:30 min. At 7:30 min, the electric shock is delivered, consisting of 6 pulses of 100 V, each 1.2 s long and followed by the next pulse after an onset-onset interval of 5 s. At 12:00 min, flies are transferred back to food vials for 16 min until the next trial, or the test, starts. For no-shock learning, all parameters are identical, except that the to-be-learned odour is presented from 8:20 to 8:35 min.

For the test, animals are loaded to the set-up, and at 5:00 min, are transferred to the choice point of a T-maze, where they can choose between the control odour and the learned odour. After 2 min, the arms of the maze are closed and the number of animals (denoted #) within each arm is determined. A preference index (PI) is calculated to yield positive values if the learned odour is prefered:

(1) $$PI = (\#_{\text{learned odour}} - \#_{\text{control odour}})*100 / \#_{\text{total}}$$

In half of the cases, flies receive LM as control odour and BA as to-be-learned odour; in the other half of the cases, flies are trained reciprocally. PIs of two reciprocally trained sets of flies are then averaged to obtain a 'odour-balanced' learning index (LI). Positive LIs thus indicate conditioned approach, negative LIs conditioned avoidance:

(2) $LI = (PI(BA) + PI(LM)) / 2$

To test for shock avoidance, one arm of the maze can be electrified. Flies are loaded to the experimental set-up and after an accommodation period of 2 min are transferred to the choice point where they can enter either arm of the maze; ten seconds later, shock is applied as specified above. Ten seconds after the onset of the last shock pulse, the maze is closed and flies are counted. A preference index (PI) is calculated to provide negative values for avoidance of the electrified arm:

(3) $PI = (\#_{Electrified} - \#_{Non\text{-}Electrified})*100 / \#_{Total}$

To assess odour responses, flies are loaded to the experimental set-up and at 5:00 min are brought to the choice point of the T-maze where they can choose between a blank arm with air only and the other arm with odour (either BA or LM); after 2 min, the maze is closed and the flies are counted. A preference index (PI) is calculated to yield positive values for odour preference:

(4) $PI = (\#_{Odour} - \#_{Air})*100 / \#_{Total}$

For the ODOUR EXPOSURE and SHOCK EXPOSURE controls, flies receive the same treatment as if they were trained, except that either the shock or the odours, respectively, are omitted. Then, the responses towards BA and LM are measured as described above.

Mann –Whitney U-tests are used to compare two groups of flies. To test for differences from zero, we use one-sample sign-tests. Significance level is $P< 0.05$. For multiple comparisons against zero, P-levels are adjusted by a Bonferroni correction to maintain the experiment-wide error-rate at 5 % and are reported as '$P< 0.05$/ number of single-genotype comparisons'.

For immunohistochemistry, brains are dissected in saline and fixed for 2 h in 4 % formaldehyde with PBST as solvent (phosphate-buffered saline containing 0.3% Triton X-

100). After a 1.5 h incubation in blocking solution (3 % normal goat serum [Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA] in PBST), brains are incubated overnight with the monoclonal anti-Synapsin mouse antibody SYNORF1 (diluted 1:20 in PBST, gift of E. Buchner, Universität Würzburg). This primary antibody is then detected after an overnight co-incubation with Alexa488 goat anti-mouse Ig (diluted 1:250 in PBST) (Invitrogen Molecular Probes, Eugene, OR, USA). All incubation steps are followed by multiple PBST washes. Incubations with antibodies are done at 4 °C; all other steps are performed at room temperature. Brains are mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA), containing propidium iodide for counterstaining of DNA and examined under a confocal microscope.

Results

We first confirm the defect of syn^{97CS} flies in 'normal' shock-learning (Godenschwege et al. 2004), which tests for conditioned odour avoidance after odour ➔ shock training. Conditioned avoidance in our plots is indicated by negative learning indices (LI; see Methods). Such confirmation seems mandatory, given the parametric differences in the training regime between our study and the one by Godenschwege et al. (2004) (see Methods). We find that indeed syn^{97CS} flies show less shock-learning than Canton-S wild-type flies (Fig. 1B; U-test: U= 3.0, $P< 0.05$; N= 12, 12 for Canton-S wild-type and syn^{97CS}, respectively). Both genotypes show significantly negative learning indices (Fig. 1B; one-sample sign tests: $P< 0.05/2$ in both cases; sample sizes as above). Thus, in flies lacking Synapsin, shock-learning is impaired, but not abolished.

When the sequence of odour and shock presentation during training is reversed (i.e. flies receive shock ➔ odour training), learning indices for the Canton-S wild-type strain are higher than for syn^{97CS}, indicating stronger conditioned approach in the wild-type (Fig. 1C; U-test: U= 72.0, $P< 0.05$; N= 16, 16 for Canton-S wild-type and syn^{97CS}, respectively). Canton-S wild-type flies show significantly positive, yet small, learning indices (Fig. 1C; one-sample sign test: $P< 0.05/2$; sample size as above) indicating conditioned approach towards the trained odour; in contrast, learning indices for syn^{97CS} flies are not different from zero (Fig. 1C; one-sample sign test: $P> 0.05/2$; sample sizes as above). Thus, no-shock learning is intact in Canton-S wild-type flies, but is abolished in Synapsin null mutants.

To test whether these common learning defects for shock-learning and no-shock learning in syn^{97CS} are secondary to any sensory or motor impairment, we test whether the responsiveness of naïve animals towards the to-be-associated stimuli may be impaired. This is not the case: There is no between-genotype difference in shock avoidance (Fig. 2A; U-test, U= 104.5, $P> 0.05$; N= 16, 16 for Canton-S wild-type and syn^{97CS}, respectively); obviously, flies do avoid the shock as demonstrated by significantly negative scores (Fig. 2A; one-sample sign test, $P< 0.05$; N= 32 for the dataset pooled across genotypes). With respect to the odours, Canton-S wild-type and syn^{97CS} do not differ in their responses towards benzaldehyde (BA) (Fig. 2B; U-test, U= 116.5, $P> 0.05$; N= 19, 16 for Canton-S wild-type and syn^{97CS}, respectively) which is an avoidance response (Fig. 2B; one-sample sign test, $P< 0.05$; N= 35 for the pooled dataset). The same pattern of results is found for limonene (LM), the other odour used (Fig. 2C; U-test: U= 158.5, $P> 0.05$; N= 20, 16 for CS and syn^{97CS}, respectively; one-sample sign test, $P< 0.05$; N= 36 for the pooled dataset). Together, naïve avoidance of the shock as well as to both odours used is indistinguishable between both genotypes.

Although these kinds of control procedure have been state of the art since the early days of fly learning-mutant research (Dudai et al. 1976), it has been argued (Preat, 1998; Michels et al. 2005) that they may not be sufficient to prove a genuine learning defect. That is, testing the responsiveness in naïve animals argues that at the beginning of the experiment the mutants are normal in stimulus responsiveness and motor ability- whether these faculties are still intact at the moment of the test, however, remains open. For example, the potentially stressful handling during the experiment may disrupt the mutants´ odour responses at test; similarly, exposure to odours during training can alter odour responses (Boyle & Cobb 2005), as can exposure to shock (Preat 1998). If the mutant would differ from wild-type in its susceptibility to handling, odour exposure, or shock exposure in such a way that the mutant but not the wild-type is rendered unresponsive to odour at the moment of test, this may feign a learning phenotype (indeed, for isoamylacetate and 1-butanol, H. Tanimoto, Universität Würzburg, found that Canton-S wild-type and syn^{97CS} can differ in odour responses after odour exposure; pers. comm.). To test for this possibility concerning the odours used here, we run two kinds of "sham training" control: animals were handled just as in normal training, but either the shock was omitted (ODOUR EXPOSURE) or the odours were omitted (SHOCK EXPOSURE). After this kind of treatment, we test whether Canton-S wild-type and syn^{97CS} flies differ in their responsiveness to BA and LM. We do not find any between-genotype differences in these tests (Fig. 3A: BA responses after ODOUR EXPOSURE: U-test: U= 165.0, $P> 0.05$; N= 21, 20 for Canton-S wild-type and syn^{97CS}, respectively; Fig. 3B:

LM responses after ODOUR EXPOSURE: U= 163,5, $P> 0.05$; N= 21, 19 for Canton-S wild-type and syn^{97CS}, respectively; Fig. 3C: BA responses after SHOCK EXPOSURE: U-test: U= 113.0, $P> 0.05$; N= 16, 16 for Canton-S wild-type and syn^{97CS}, respectively; Fig. 3D: LM responses after SHOCK EXPOSURE: U-test: U= 120.0, $P> 0.05$; N= 16, 16 for Canton-S wild-type and syn^{97CS}, respectively). For the datasets pooled across genotypes, we find that flies, after either exposure regime, still avoid both odours (Fig. 3A: BA responses after ODOUR EXPOSURE: one-sample sign test: $P< 0.05$; N= 41; Fig. 3B: LM, ODOUR EXPOSURE: one-sample sign test: $P< 0.05$; N= 40; Fig. 3C: BA, SHOCK EXPOSURE: one-sample sign test: $P< 0.05$; N= 32; Fig. 3D: LM, SHOCK EXPOSURE: one-sample sign test: $P< 0.05$; N= 32). We conclude that, also at the moment of test, those sensory and motor abilities which are required to show odour responses are not defective in syn^{97CS}.

Discussion

We report common defects of Synapsin null mutants in shock-learning and no-shock learning (Fig. 1); these learning defects do not reflect any sensory or motor impairment (Fig. 2), even if most stringent control procedures are applied which go beyond the state-of-the-art in fly learning-mutant research (Fig. 3). Thus, predictive associative learning concerning the presence and the absence of shock share molecular determinants.

We find that shock-learning is only partially abolished in syn^{97CS} flies, whereas no-shock learning is completely absent. The partial defect in shock-learning is in agreement with Godenschwege et al. (2004), who reported a 25 % decrement in learning; a somewhat stronger yet still partial defect (50 %) is seen also in larval odour-sugar learning (Michels et al. 2005). We cannot decide whether the full abolishment of no-shock learning in this study means that indeed this type of learning is not possible without Synapsin, or that a residual, Synapsin-independent memory is undetectable due to the low learning scores in no-shock learning. The observation that the amount of learning is much larger for shock-learning than for no-shock learning matches the reports by Tanimoto et al. (2004) and Yarali et al. (2006), as well as predictions of psychological theory (Wagner 1981).

Given that one and the same molecule is involved in establishing memories which eventually bring about opposite effects in behaviour, i.e. conditioned avoidance in shock-learning or conditioned approach in no-shock learning, two questions arise: first, do both kinds of learning utilize Synapsin within the same cells and if so, within the same subcellular

compartments? Current evidence suggests that the mushroom body Kenyon cells, a third-order olfactory brain region, house a memory trace which underlies short-term memory in shock-learning (reviews by Heisenberg 2003; Gerber et al. 2004), but the site(s) of the memory trace(s) underlying no-shock memory are currently unknown. Second, do the same molecular modifications of Synapsin underlie both kinds of learning? That is, the recruitment of the reserve-pool vesicles for release is thought to depend on the phosphorylation-dephosphorylation balance of Synapsin. For neither shock-learning nor no-shock learning do we know whether and which of the phosphorylation site(s) of the protein (Hilfiker et al. 1999) is relevant and it may be that shock-learning and no-shock learning differ in this regard. Answering these questions should allow to see how the fly brain is organized on the cellular, subcellular and/ or molecular level to establish associative memories between odour and shock which lead to opposite predictions concerning shock (i.e. its presence *versus* absence) and support opposite behaviour (i.e. conditioned avoidance *versus* approach). Such knowledge may, eventually, help reducing the impact of traumatic experience in man.

Acknowledgements

Supported by the Deutsche Forschungsgemeinschaft via Sonderforschungsbereich 554 *Arthropode Behaviour* (to B.G.), International Graduate Training Program 1156 *From Synaptic Plasticity to Behavioural Modulation in Genetic Model Organisms* (to B.G. and B.M.), and a Heisenberg Fellowship (to B.G.). A.Y. is supported by a PhD fellowship of the Boehringer Ingelheim Fonds.

The continuous support of the members of the Department of Genetics and Neurobiology, especially M. Heisenberg, E. Buchner and H. Tanimoto, is gratefully acknowledged; many thanks to R. Menzel (Freie Universität Berlin) and H. Lachnit (Universität Marburg) for critical discussions.

Abbreviations

BA: benzaldehyde

LM: limonene

LI: learning index

PI: performance index

SYNORF1: monoclonal anti-synapsin antibody

AL: antennal lobe

C: mushroom body calyx

E: ellipsoid body

F: fan shape body

L: lobula

LP: lobular plate

M: medulla

ML: mushroom body medial lobes

P: pedunculus

PB: protocerebral brigde

SOG: subesophageal ganglia

VL: mushroom body vertical lobes

References

Boyle, J. & Cobb, M. (2005) Olfactory coding in *Drosophila* larvae investigated by cross-adaptation. *J. Exp. Biol.* **208**, 3483-3491.

Chang, R.C., Blaisdell, A.P. & Miller, R.R. (2003) Backward conditioning: Mediation by the context. *J. Exp. Psychol. Anim. Behav. Process.* **29**, 171-183.

Dudai, Y., Jan, Y.N., Byers, D., Quinn, W.G. & Benzer, S. (1976) *dunce*, a mutant of *Drosophila* deficient in learning. *Proc. Natl. Acad. Sci. U S A* **73**, 1684-1688.

Garcia, C.C., Blair, H.J., Seager, M., Coulthard, A., Tennant, S., Buddles, M., Curtis, A. & Goodship, J.A. (2004) Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy. *J. Med. Genet.* **41,** 183-186.

Gerber, B., Tanimoto, H. & Heisenberg, M. (2004) An engram found? Evaluating the evidence from fruit flies. *Curr. Biol.* **14**, 737-744.

Gitler, D., Takagishi, Y., Feng, J., Ren ,Y., Rodriguiz, R.M., Wetsel, W.C., Greengard, P. & Augustine, G.J. (2004) Different presynaptic roles of synapsins at excitatory and inhibitory synapses. *J. Neurosci.* **24**, 11368-11380.

Godenschwege, T.A., Reisch, D., Diegelmann, S., Eberle, K., Funk, N., Heisenberg, M., Hoppe, V., Hoppe, J., Klagges, B.R., Martin, J.R., Nikitina, E.A., Putz, G., Reifegerste, R., Reisch, N., Rister, J., Schaupp, M., Scholz, H., Schwarzel, M., Werner, U., Zars, T.D., Buchner, S. & Buchner, E. (2004) Flies lacking all synapsins are unexpectedly healthy but are impaired in complex behaviour. *Eur. J. Neurosci.* **20**, 611-622.

Heisenberg, M. (2003) Mushroom body memoir: From maps to models. *Nat. Rev. Neurosci.* **4**, 266-275.

Hellstern, F., Malaka, R. & Hammer, M. (1998) Backward inhibitory learning in honeybees: A behavioral analysis of reinforcement processing. *Learn. Mem.* **4**, 429-444.

Hilfiker, S., Pieribone, V.A., Czernik, A.J., Kao, H.T., Augustine, G.J. & Greengard, P. (1999) Synapsins as regulators of neurotransmitter release. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **354**, 269-279.

Michels, B., Diegelmann, S., Tanimoto, H., Schwenkert, I., Buchner, E. & Gerber, B. (2005) A role for Synapsin in associative learning: the *Drosophila* larva as a study case. *Learn. Mem.* **12**, 224-231.

Preat, T. (1998) Decreased odor avoidance after electric shock in *Drosophila* mutants biases learning and memory tests. *J Neurosci.* **18**, 8534-8538.

Silva, A.J., Rosahl, T.W., Chapman, P.F., Marowitz, Z., Friedman, E., Frankland, P.W., Cestari, V., Cioffi, D., Sudhof, T.C. & Bourtchuladze, R. (1996) Impaired learning in mice with abnormal short-lived plasticity. *Curr. Biol.* **6**, 1509-1518.

Solomon, RL., Corbit, JD. (1974) An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol. Rev*. **81(2)**, 119-145

Sutton, R.S. & Barto, A.G. (1990) Time-derivative models of Pavlovian reinforcement. In M. Gabriel & J. Moore (eds), *Learning and computational neuroscience: Foundations of adaptive networks.* MIT Press, Cambridge, MT, pp. 497-537.

Tanimoto, H., Heisenberg, M. & Gerber, B. (2004) Event-timing turns punishment to reward. *Nature* **430**, 983.

Wagner, A.R. (1981) SOP: A model of automatic memory processing in animal behavior. In N. E. Spear & R. R. Miller (eds), *Information processing in animals: memory mechanisms.* Erlbaum, Hillsdale, NJ, pp 5-47.

Yarali, A., Niewalda, T., Chen, Y.-c., Tanimoto, H., Duerrnagel, S. & Gerber, B. (2006) Predictive associative learning about the absence of shock: Parametric analyses in the fruit fly. *Learn Mem*. **Under Review**.

Figure Legends

Fig. 1: Common impairment of *syn*^{97CS} in shock-learning and no-shock learning.

A. Synapsin immunoreactivity is absent in *syn*^{97CS}. Anti-Synapsin staining (green) with monoclonal anti-Synapsin antibody SYNORF1, and DNA-counterstaining with propidium iodide (magenta) in frontal optical sections (0.9 µm) of Canton-S wild-type (left column) and *syn*^{97CS} (right column). Synapsin immunoreactivity is seen in the neuropile regions of Canton-S wild type, where it does not co-localize with DNA-counterstaining. In *syn*^{97CS}, no Synapsin immunoreactivity can be found. In western blots of Canton-S wild-type, SYNORF1 recognizes all predicted isoforms of Synapsin; in *syn*^{97CS}, none of these isoforms is seen, neither in adult flies nor in larvae (Godenschwege et al. 2004; Michels et al. 2005).

AL: antennal lobe, C: mushroom body calyx, E: ellipsoid body, F: fan shape body, L: lobula, LP: lobular plate, M: medulla, ML: mushroom body medial lobes, P: pedunculus; PB: protocerebral brigde, SOG: subesophageal ganglia, VL: mushroom body vertical lobes.

B. Shock-learning: Both Canton-S wild-type and *syn*^{97CS} flies show conditioned aversion towards the trained odour (negative learning indices); this shock-learning is significantly less strong in *syn*^{97CS} than in Canton-S wild-type.

C. No-shock learning: Only Canton-S wild-type flies show conditioned approach towards the trained odour (positive learning indices); in contrast, no-shock learning cannot be observed in *syn*^{97CS} flies, which show learning indices indistinguishable from zero and significantly smaller than Canton-S wild-type flies.

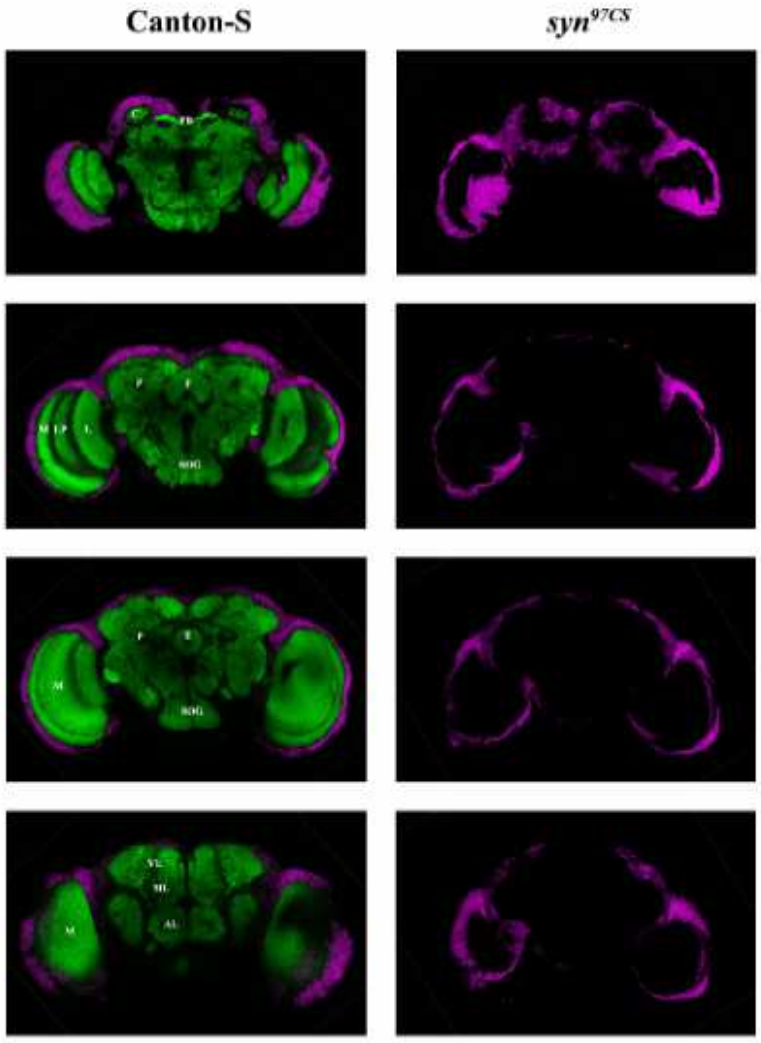
*: $P< 0.05$; NS: $P> 0.05$, except for the comparison of each genotype against zero, where *: $P< 0.05/2$ and NS: $P> 0.05/2$ are used to maintain the experiment-wide error at 5 % (Bonferroni-correction). LI: Learning Index. The middle line represents the median, the boundaries of the box the 25 % and 75 % quantiles, and the whiskers the 10 % and 90 % quantiles, respectively. The sketches represent the experimental procedure, BA and LM indicating the used odours benzaldehyde and limonene.

Fig. 2: Naive responses towards the to-be-associated stimuli are normal in *syn*^{97CS}.

Responses to the shock (A) and the odours (B: BA; C. LM) are not different between genotypes; flies show avoidance of the shock and of both odours. NS: $P> 0.05$. All other details as in Figure 1. The sketches represent the experimental procedure.

Fig. 3: Odour responses are normal in *syn*^{97CS} also after "sham training".

Genotypes do not differ in odour responses after either ODOUR EXPOSURE "sham training" (A: BA preference, B: LM preference) or SHOCK EXPOSURE "sham training" (C: BA preference, D: LM preference); flies show avoidance of both odours.
NS: $P > 0.05$. All other details as in Fig. 1, 2. The sketches represent the experimental procedure.



Niewalda et al. Fig 1A

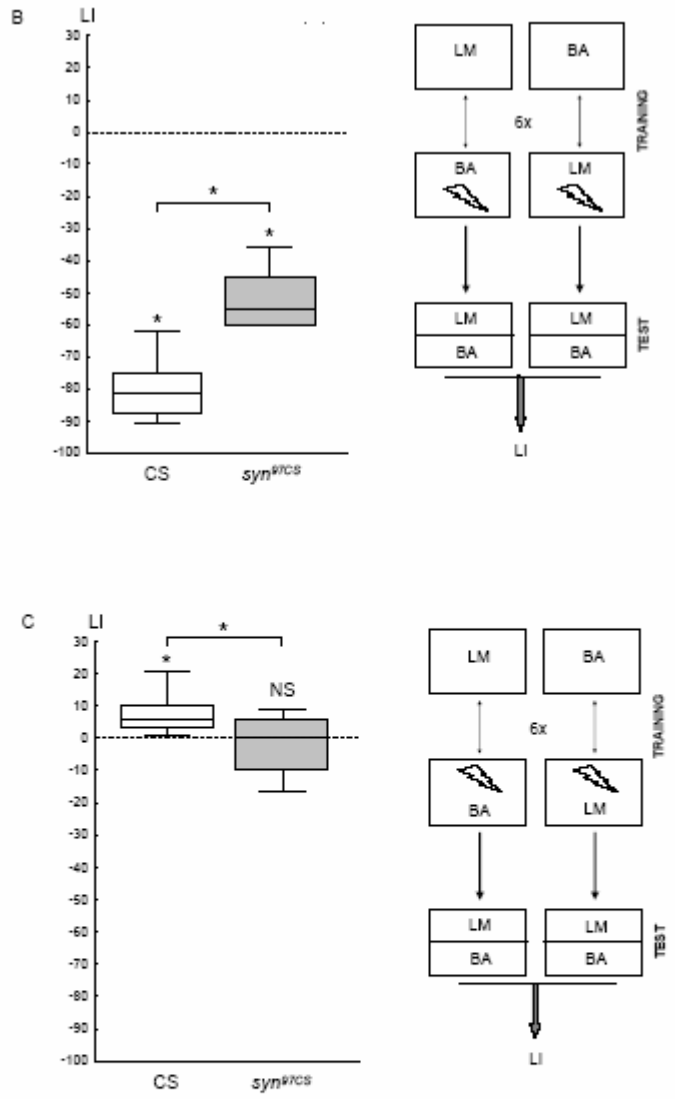


Fig. 1
B. Shock learning
C. No-shock learning

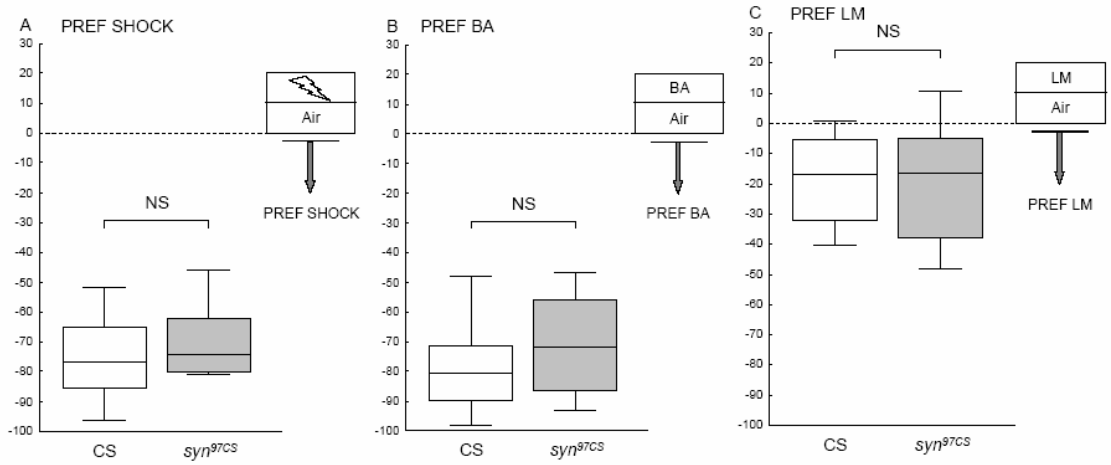


Fig. 2
A. Shock preference
B. Naïve BA preference
C. Naïve LM preference

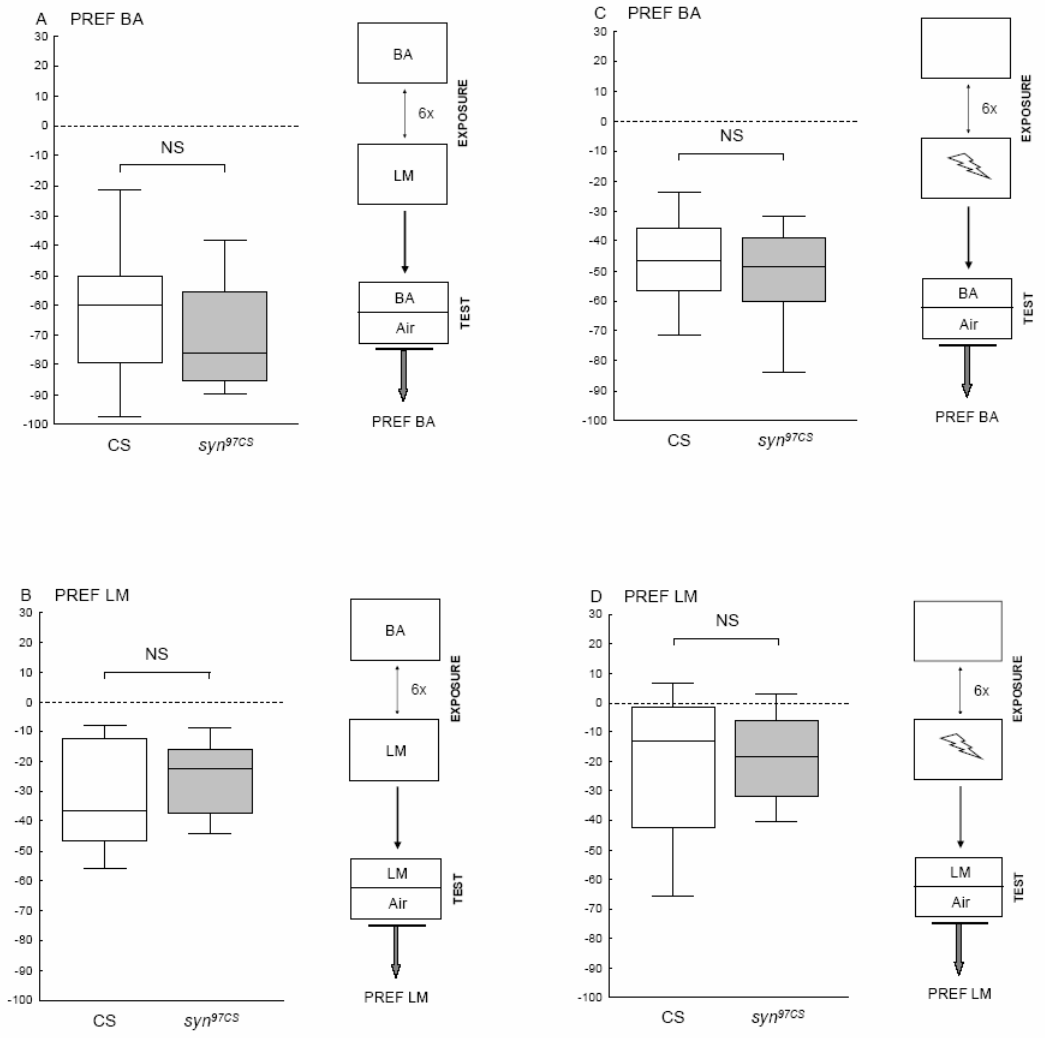


Fig. 3
A. Odour exposure, BA preference
B. Odour exposure, LM preference
C. Shock exposure, BA preference
D. Shock exposure, LM preference

Chapter II.

II.2. A role for White on olfactory associative learning in larval and adult *Drosophila*?

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

J. Neurogenetics, 2008, 1–16, iFirst article

ISSN: 0167-7063 print/1563-5260 online
DOI: 10.1080/01677060802441372

informa
healthcare

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

Ayse Yarali,[1] Markus Krischke,[2] Birgit Michels,[1] Timo Saumweber,[1] Martin J. Mueller,[2] and Bertram Gerber[1]

[1]Universität Würzburg, Biozentrum, Lehrstuhl für Genetik und Neurobiologie, Würzburg, Germany
[2]Universität Würzburg, Biozentrum, Lehrstuhl für Pharmazeutische Biologie, Würzburg, Germany

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 the loss of *white*-function, the balance between these two kinds of learning is distorted in favor 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 apparently unaffected, both in adult and larval *Drosophila*. Prompted by the proposed function of the White protein as the transporter for biogenic amine precursors, we probed the brains of $white^{1118}$ mutants for the amounts of biogenic amines (octopamine, tyramine, dopamine, and serotonin) by using high-pressure liquid chromatography coupled to mass spectrometry. Using this method, we found, however, no difference between $white^{1118}$ and wild type flies 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 the ''marker gene'' in behavior-genetic analyses of any sort.

Keywords: *Drosophila*, punishment learning, relief learning, olfaction, *white*, biogenic amine levels

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 & Sullivian, 1975), the lack of which 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 the 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 overexpression of White induces male-to-male courtship (Zhang & Odenwald, 1995; Hing & 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). Further, $white^{1118}$ mutant flies are impaired in heat-reinforced place learning, whereas in associative odor-shock learning, they perform better than the wild type (Diegelmann et al., 2006). How does the *white*-gene affect such a broad spectrum of behavioral phenotypes? We note that in neurons,

Received 12 August 2008; Accepted 29 August 2008
Address correspondence to either author:
Ayse Yarali, Gènes et Dynamique des Systèmes de Mémoire, CNRS UMR 7637 ESPCI, 10 rue Vauquelin, 75005 Paris, France. E-mail: ayse.yarali@biozentrum.uni-wuerzburg.de
Bertram Gerber, Biozentrum, Lehrstuhl für Genetik und Neurobiologie, Universität Würzburg, 97074 Würzburg, Germany. E-mail: bertram.gerber@biozentrum.uni-wuerzburg.de

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, 2006], aggression [Dierick & Greenspan, 2007], and 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 (NO) (reviewed by Koshimura et al., 2000). Dopamine, apart from signaling 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, NO is an atypical neurotransmitter in the synapses of the olfactory, visual, and mechanosensory systems, as well as at the neuromuscular junction (reviewed by Bicker, 2001). Thus, the roles of White in behavior may, conceivably, come about by its effects on serotonin, dopamine, and/ or NO signaling.

Here, following up on Diegelmann et al. (2006), we analyzed how the loss of *white*-function in the $white^{1118}$ mutant affects olfactory associative learning. We did 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 (i.e., punishment learning), whereas those odors that follow shock are learned as signals for *relief* and are subsequently approached (i.e., relief learning) (Tanimoto et al., 2004; Yarali et al., 2008). In addition, we tested whether $white^{1118}$ larvae are altered in associating an odor with a sugar reward. In order to offer an explanation for any potential behavioral alterations, we provide an analysis of the brain levels of biogenic amines (i.e., octopamine, tyramine, dopamine, and serotonin) by using high-pressure liquid chromatography coupled to mass spectrometry (HPLC-MS/MS).

MATERIALS AND METHODS

Flies

Drosophila melanogaster were reared in mass culture at 25°C, at 60–70% relative humidity, under a 14-10-hour light-dark cycle. The Canton-Special wild type strain was used as a control for the White-null $white^{1118}$ strain, which was 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., 2006; Hoyer et al., 2008).

Adult Behavior

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

For electric shock–reinforced learning (Figure 1A), flies received 6 training cycles. Each cycle started by loading the flies into the experimental setup (0 minutes). From 4 minutes on, a control odor was presented for 15 seconds. From 7.5 minutes on, electric shock was applied as 4 pulses of 100 V; each pulse was 1.2 seconds long and was followed by the next with an onset-to-onset interval of 5 seconds. In different groups, a to-be-learned odor was presented at different times relative to this shock; thus, the interval between the to-be-learned odor and the shock (i.e., the interstimulus interval; ISI) was varied between groups. Negative ISIs indicate first-odor-then-shock presentation, whereas positive ISIs indicate first-shock-then-odor presentation. At 12 minutes, flies were transferred out of the setup into food vials, where they stayed for 16 minutes until the next training cycle started. At the end of the sixth training cycle, after the usual 16-minute break, flies were loaded back into the setup. After a 5-minute accommodation period, they were transferred to a T-maze, where they could choose between the two odors that they had encountered during training. After 2 minutes, the arms of the maze were closed and flies on each side were counted. A preference index (PREF) was calculated, as shown in Equation 1:

$$PREF = (\#_{Learned\ odor} - \#_{Control\ odor}) \times 100/\#_{Total} \quad (1)$$

In this equation, # indicates the number of flies found in the respective maze arm. For each ISI, two subgroups of flies were trained and tested in parallel (Figure 1A): For one of these, OCT was the control odor and BA was to be learned; the second group was trained reciprocally (i.e., the roles of these two odors were switched). A learning index (LI) was calculated, based on the PREF values from the two reciprocal measurements, as shown in Equation 2:

$$LI = (PREF_{BA} + PREFO_{CT})/2 \quad (2)$$

Subscripts of PREF (BA or OCT) indicate the learned odor in the respective subgroups of flies. Positive LIs indicate conditioned approach to the learned odor, whereas negative values reflect conditioned avoidance.

To test for the immediate, reflexive shock response, flies were transferred to the choice point of a T-maze 5 minutes after being loaded into the setup. Then, 10

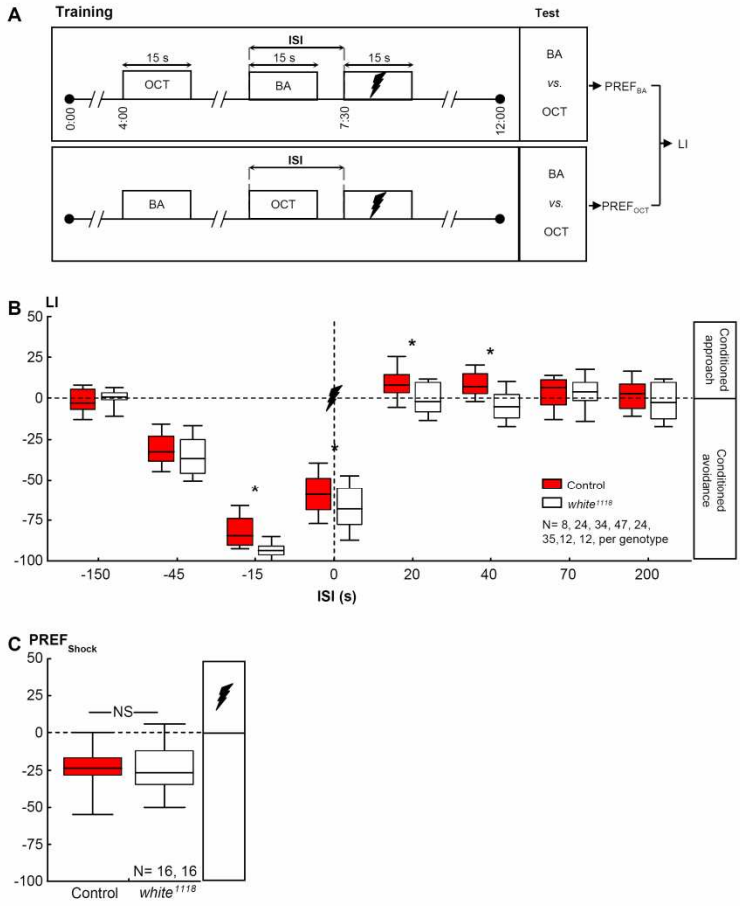


Figure 1. Memory of shock was, overall, more ''negative'' for *white*^{1118} mutants. (**A**) Adult flies were trained with two odors and pulses of electric shock. Between the groups, we varied the interval between the as-yet-to-be-learned odor and the shock (interstimulus interval; ISI). Negative ISIs 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**).

seconds later, one of the maze arms was electrified with four 1.2-second-long pulses of 100-V shock with 5-second interpulse intervals. Then, 10 seconds after the onset of the last pulse, the arms of the maze were closed and flies on each side were counted. A preference index for the electrified arm ($PREF_{Shock}$) was calculated, as shown in Equation 3:

$$PREF_{Shock} = (\#_{Electrified\ arm} - \#_{Nonelectrified\ arm}) \times 100/\#_{Total} \quad (3)$$

Again, # indicates the number of flies found in the respective maze arm, and negative $PREF_{Shock}$ values indicate avoidance of the shock.

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

Larval Behavior

Larval learning experiments followed the mass assay described in Neuser et al. (2005). Larvae, aged 5 days after egg laying, were assayed in groups of 30, under a fume hood at 24–28°C, in regular daylight. One day before the experiments, Petri dishes (Sarstedt, Nümbrecht, Germany), each with an 85-mm inner diameter, were 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 the sugar reward, 2 M of fructose (FRU, purity: 99%; Sigma, Steinheim, Germany) was added to the agarose 10 minutes after boiling. During the experiments, the regular lids of the Petri dishes were replaced by lids perforated in the center by ~60 1-mm holes to improve aeration. The odor, *n*-amylacetate (AM; Merck, Darmstadt, Germany),

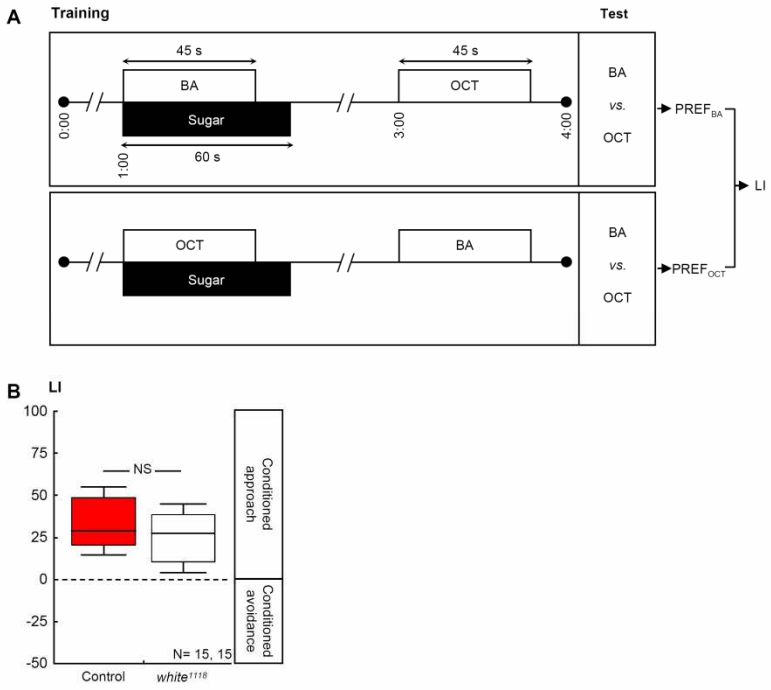


Figure 2. Loss of *white*-function did not affect olfactory reward learning in adult *Drosophila*. (**A**) Adult flies were successively exposed to an as-yet-to-be-learned odor in the presence of sugar and to a control odor without any sugar. Two subgroups were trained reciprocally, that is, with switched roles for the odors 3-octanol (OCT) and benzaldehyde (BA). Both subgroups were then given the choice between the two odors; a learning index (LI) was calculated based on their odor preferences (PREFs). Positive values indicate a conditioned approach toward the learned odor. (**B**) Control flies and $white^{1118}$ mutants performed equally well in such reward learning. Details are as in Figure 1C.

was diluted 1:1600 in paraffin oil (Merck, Darmstadt, Germany) and applied in custom-made Teflon containers placed in each Petri dish on opposite sides, 7 mm from the edges; these containers were of a 5-mm inner diameter and closed with a lid with seven 0.5-mm holes.

To start training, 30 larvae were 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 (Figure 3A). Larvae were left to crawl in this Petri dish for 5 minutes and were then transferred into another Petri dish filled with agarose only and with two empty containers. Also, in this Petri dish larvae remained for 5 minutes. We repeated this training cycle three times, each time using fresh Petri dishes. At the end of training, we placed 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 was alternated for every other set of larvae). After 3 minutes, the number of animals on each side was counted. For each group of larvae thus trained (i.e., ''AM+/Empty,'' as in this example; note that in half of the cases, training was in reversed order, i.e., ''Empty/AM+''), another group of larvae was trained reciprocally as ''Empty+/AM'' (or, in half of the cases as ''AM/Empty+''; Figure 3A). An LI was then calculated, as detailed above for adult learning.

Quantification of Biogenic Amine Amounts

We quantified the amounts of octopamine, tyramine, dopamine, and serotonin in the brains of adult fruit flies by using HPLC coupled to a tandem mass spectrometer (HPLC-MS/MS). For the non-specialist reader, we will 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 will present the technical particulars.

Principle of Method

Extracts of fruit fly brain homogenate were loaded onto a liquid chromatography column that contained silica

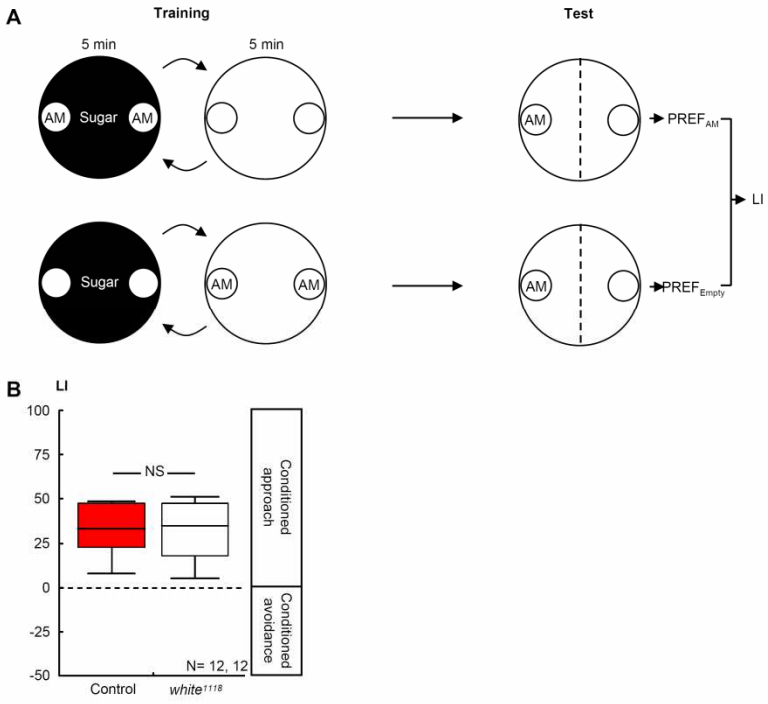


Figure 3. Loss of *white*-function did not affect olfactory reward learning in larval *Drosophila*. (**A**) Larvae were successively exposed to the odor *n*-amylacetate (AM), in the presence of sugar and to a no-odor situation (Empty) without any sugar. Another group of larvae was trained reciprocally. Both groups were then tested for their response to AM; a learning index (LI) was calculated based on their AM preferences (PREF). Positive LI values indicate appetitive learning. (**B**) Control larvae and $white^{1118}$ mutant larvae performed equally well in such reward learning. Details are as in Figure 1C.

particles coated with C18 hydrocarbon chains. Biogenic amines, along with other organic molecules, were retained by this column material. By increasing the proportion of the organic solvent in the aqueous mobile phase, molecules were gradually separated and eluted before they entered the MS. Retention times of the molecules on the column depended largely on their lipophilicity (i.e., polar, hydrophilic compounds eluted early, while hydrophobic molecules elute late). Hence, molecules of interest reached the MS at different and characteristic retention times (RTs). As the molecules entered the MS, they became ionized through protonation (i.e., became positively charged). Molecule ions characterized by their specific mass-per-charge (*m*/*z*) ratios were physically separated by this first MS. Next, selected molecular ions were broken by collision-induced dissociation (CID) into a series of compound-specific fragments, which were then physically separated by a second MS that also recorded the ion intensities of the derived fragments. In the multireaction monitoring mode (MRM), even fragments from several molecules coeluting from the HPLC column (i.e., molecules with the same RT) can be sorted and analyzed within some hundreds of milliseconds. Hence, molecules were 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 (e.g., RT, CID-energy, and *m*/*z* values) could be obtained by analyzing authentic reference compounds. Moreover, the technique allowed the use of internal standards labeled with stable isotopes that were added to the tissue prior to extraction. These standards displayed the same physical-chemical properties as the target molecules and only differed by their mass. Hence, compound losses occurring during sample preparation and processing were proportional for standard and target molecules. To quantify e.g., the amount of serotonin, a known amount of deuterated serotonin ([D4]serotonin) was added to the brain homogenate. Labeled and endogenous serotonin then were simultaneously extracted and purified by HPLC. The endogenous ''light'' serotonin and the heavier [D4]serotonin could be separated by the MS, according to their different *m*/*z* values, and the intensities of the ions could 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 unextracted sample could be calculated. To validate the method, for example with respect to serotonin, we initially prepared a series of samples; each sample contained 5 ng of [D4]serotonin and a certain known amount of unlabeled, light serotonin, varying between 5 and 1,000 pg. The amount of serotonin in each sample was 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 Figure 6A-A′ (for the other amines, see the Supplemental Figs.). When isotopically labeled standards are used, the slope of the linear fit is usually one, as in the case of octopamine (Supplemental Figure 1A-A′). Sometimes, however, the ionization and fragmentation efficiencies differ between the isotopically labeled heavy standard and the unlabeled, light molecule, resulting in a slope that is different from one; in such cases, a *correction factor* is employed (e.g., as in the case of serotonin [Figure 6A-A′], tyramine [Supplemental Figure 2A-A′], and dopamine [Supplemental Figure 3A-3A′]).

Chemicals

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

Sample Preparation

Each sample contained 5 female and 5 male brains (2–3 days old) from either $white^{1118}$ mutant or Canton Special wild type flies. Brains were dissected in ice-cold Ringer's solution and directly placed into 50 µl of ice-cold 50-mM citrate-acetate buffer (pH 4.5), which in addition contained 5 ng of each internal standard. Once 10 brains were collected (which took ~30 min), they were homogenized in this solution on ice with a Teflon pestle. After centrifugation at 14,000 rpm for 5 minutes at room temperature, 10 µl of the supernatant was 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, Massachusetts, USA), was used. Liquid chromatography was performed by using an Agilent Eclipse XDB-C18 column (150 mm × 4.6 mm, 5-µm particle size; Agilent Technologies, Waldbronn, Germany). The column was eluted with a linear mobile-phase gradient (0.6 ml/min flow rate), starting from water containing 0.1% formic acid at 0 minutes to an acetonitrile:water:formic acid mixture (50:50:0.1, v/ v/ v) at 10 min.

For MS, ionization was achieved by using electrospray in the positive ionization mode (ESI^+) with a

capillary voltage of 2.5 kV. The temperature of the source block was set at 120°C, and nitrogen was 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 analogs, standard solutions were directly infused into the mass spectrometer and the cone voltage was adjusted to maximize the intensity of the protonated molecular species. CID of each compound was performed by using argon as collision gas with a flow rate of 0.3 ml/min and a pressure of 3.0×10^{-3} mBar; collision energy (eV) was adjusted to optimize the signal for the most abundant fragment ions, which were subsequently used for 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 were analyzed by using nonparametric statistics and are reported as box plots, showing the median as the midline and 10, 90, and 25%, 75% quantiles as whiskers and box boundaries, respectively. For comparing values of each group to zero, we used one-sample sign tests. To compare values between two groups, we used a Mann-Whitney U-test. When multiple tests were performed within a single experiment, we adjusted the experiment-wide error rate to 5% by Bonferroni correction; that is, we divided the critical $P < 0.05$ by the number of tests. For example, if 8 such comparisons were made, we report the P-level as $P < 0.05/8$. To compare more than two groups with each other, we used Kruskal-Wallis tests. Sample sizes are mentioned within the figures. All statistical analyses were performed on a PC, using Statistica (Statsoft, Tulsa, Oklahoma, USA).

RESULTS

white-Function and Olfactory Associative Learning

Regarding wild type control flies, conditioned behavior depended on the relative timing of odor and shock (red displays in Figure 1B: Kruskal-Wallis test; control flies: $H = 168.96$, $df = 7$; $P < 0.05$): If, during training, the odor had been presented either long before (Figure 1B: one-sample sign test; control: ISI = −150 seconds; $P > 0.05/8$) or long after shock (Figure 1B: one-sample sign tests; control: ISI = 70 and 200 seconds; $P > 0.05/8$ each), flies did not show any conditioned behavior. If the odor had shortly preceded or overlapped with shock during training, it was avoided in the test (i.e., punishment learning) (Figure 1B: one-sample sign tests; control: ISI = −45, −15, and 0 seconds; $P < 0.05/8$ each). Contrarily, if the odor had shortly followed shock during training, wild-type flies later on approached it (i.e., relief learning) (Figure 1B: one-sample sign tests; control: ISI = 20 and 40 seconds; $P < 0.05/8$ each). These results conform to the previous reports of Tanimoto et al. (2004) and Yarali et al. (2008).

Next, we compared *white*^{1118} mutants' learning to the the wild type. For very long ISIs, which did not support learning in the wild type to begin with, we found no difference between the two genotypes (Figure 1B: U-tests: ISI = −150 seconds: U = 28.00; ISI = 70 seconds: U = 70.00; ISI = 200 seconds: U = 58.00; $P > 0.05/8$ each). In contrast, using short ISIs, which did support learning in the wild type flies, the loss of *white*-function did have an effect: Namely, regardless of the sequence of the odor and the shock during training, the learning scores of the *white*^{1118} mutants were shifted ''southward'' that is, toward stronger conditioned avoidance (Figure 1B: U-tests: ISI = −15 seconds: U = 183.00; ISI = 0 seconds: U = 745.00; ISI = 20 seconds: U = 157.00; ISI = 40 seconds: U = 226.00; $P < 0.05/8$ each; note, however, that

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.

for the −45-second ISI, U = 239.00; $P = 0.32$). Thus, the ''take-home message'' from the shock episode, overall, was more negative for the *white*^{1118} mutants than for wild type flies.

Is this effect 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 found that wild-type control flies and *white*^{1118} mutants avoided shock to the same extent (Figure 1C: U-test: U = 123.5; $P > 0.05$; one-sample sign test for the pooled data set: $P < 0.05$). Further, loss of *white*-function left olfactory discrimination ability, in principle, intact, as odor-reward learning remained unaffected: After odor-sugar training (Figure 2A), learning scores did not differ between genotypes (Figure 2B: U-test: U = 82.00; $P > 0.05$); when pooled, they reflected a conditioned approach (one-sample sign test for the pooled data set: $P < 0.05$). Also, *white*^{1118} mutant larvae were not different from the wild type with respect to odor-sugar learning (Figure 3B: U-test: U = 71.00: $P > 0.05$).

No Effect of the Loss of *white*-Function on Whole-Brain Amounts of Biogenic Amines

Next, we probed the *white*^{1118} mutants' brains for abnormalities in the levels of the biogenic amines: octopamine, tyramine, dopamine, and serotonin. This was 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.

Using HPLC-MS/MS, we did 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 (Figure 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.

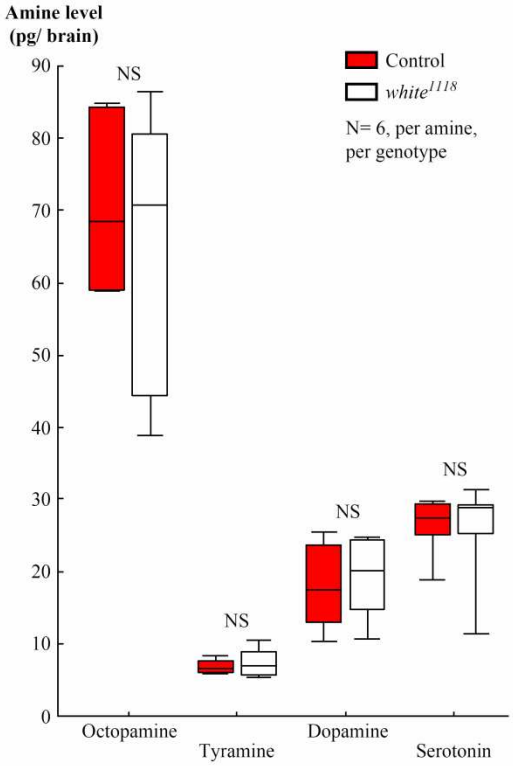


Figure 4. Loss of *white*-function did not affect the adult brain amounts of biogenic amines. High-pressure liquid chromatography, coupled to tandem mass spectrometry, revealed no difference between wild type controls and *white*^{1118} mutants in terms of the brain amounts of octopamine, tyramine, dopamine, or serotonin. From samples that each included 10 brains, we report amine levels as pg per single brain. NS, $P > 0.05$. Box plots are as in Figure 1B.

DISCUSSION

In this paper, we report an effect of the loss of *white*-function on what fruit flies remember about a shock episode (Figure 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., 2006) 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 (Figure 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 toward painful stimuli, as well as toward the stimuli associated with them. Distortion of the balance between these opponent processes in man are conceivably implicated in psychiatric conditions (anxiety: Vincent & Kukstas, 1998; addiction: Koob & Le Moal, 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 probed for the brain levels of the biogenic amines, octopamine, tyramine, dopamine, and serotonin. The amounts of these substances, in the present analysis, appeared indistinguishable between $white^{1118}$ mutants and the wild type (Figure 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 Figure 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 (de Belle & Heisenberg, 1996). Second, the 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 & Hirsh, 2006; compare red triangles vs. red circles in Figure 5): Levels of, for example, dopamine are much higher in the head than in the brain, conceivably because some dopamine is contained in the cuticle (Wright, 1987). Third, sample purification, detection, and quantification differ across studies. Most studies cited in Figure 5 coupled HPLC to an electrochemical detector (HPLC-ECD), with two exceptions: 1) the present study, for all amines, employed HPLC-MS/MS and 2) for measuring dopamine in unpurified head extracts, Sitaraman et al. (2008) used an enzyme immunoassay. Electrochemical detection has the drawback that oxidizable phenols and catechols in the sample, which comigrate through the HPLC column with biogenic amines, may accidentally yield ECD signals, potentially resulting in overestimations of amine levels. Therefore, methods relying on HPLC-ECD have to be carefully evaluated, especially when unpurified samples from nonstandard biological sources, potentially including unknown metabolites of the target trace-amount molecules, are analyzed. A similar caveat may

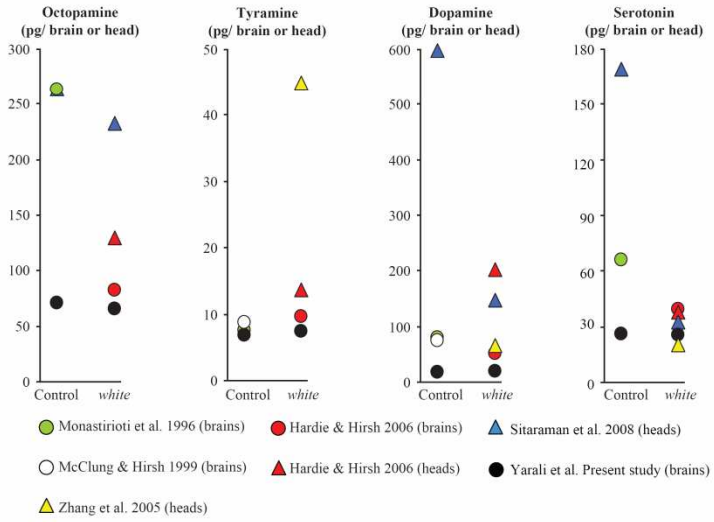


Figure 5. Meta-analysis of amine amounts. We compared various high-pressure liquid chromatography (HPLC)-based studies (color coded) in terms of the biogenic amine amounts they found in whole-head (triangles) or only-brain (circles) homogenates from wild type control or $white^{1118}$ mutant flies. We plotted mean values throughout in pg/brain or head to enable comparison between studies. Please note the different Y-axes for each amine.

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 unpurified samples are measured. In any event, both of these two methods do not employ isotopically labeled internal standards, which help to compensate for variable extraction efficiencies, chemical degradation (i.e., auto-oxidation), 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 are specifically detected and quantified by MS, arguably, seem preferable. MS/MS, as used in this study, adds two further dimensions of physical separation of molecules (i.e., the separation of the molecular ions in the first MS and the separation and quantification of specific fragment ions in the second MS). In addition, the ionization 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.

With such methodology, the current study did not detect a difference between $white^{1118}$ mutant and wild-type brains in terms of 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 by using the present method. As can be seen in Figure 5, a number of independent reports, including the present one, agree upon the amount of serotonin per *white*-mutant head and brain. As compared to this ''consensus level'' of serotonin in the *white*-mutant, Sitaraman et al. (2008) found 5–6-fold more serotonin in wild type heads. Could our method have measured such a high serotonin amount? In Figure 6A-A′, the dynamic range of the present measurement, with respect to serotonin, can be seen. To reveal this dynamic range, we analyzed, by HPLC-MS/MS, a series of samples, each containing 5 ng of labeled [D4]serotonin and known amounts of unlabeled serotonin, ranging from 5 to 1000 pg. We plotted, 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 corresponded well. Within this dynamic range, the total amount of serotonin in a homogenate of 10 brains, as found in this study (Figure 6A-A′: black arrow) fell 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 a 4-fold higher serotonin level 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 the detection of serotonin within the brain homogenate possible with the same specificity as over the solvent background? We compared chromatograms obtained over a solvent background, on the one hand (Figure 6B), with the measurements over a brain-homogenate background, on the other hand (Figure 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 Supplemental Figs.).

In turn, it may be that the sample treatment in the current report unwittingly led to a degradation of serotonin, such that overall serotonin levels were too low to allow for between-genotype differences to be detected. As shown in Figure 6A-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 could not detect between-genotype differences in serotonin levels because of a ''floor-effect'' does not seem to be valid—unless one would assume that, for as-yet-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 Supplemental Figs.).

With all these reasonings in mind, including the principle caveats of interpreting lack-of-difference results, we note that the present study did not find an abnormality of biogenic amine levels in the brains of $white^{1118}$ mutants and hence could not 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 & Menzel, 1998; Farooqui et al., 2003; Vergoz et al., 2007; cricket: Unoki et al., 2005, 2006). In other words, both the mentioned amines and *white*-can matter for learning, but these effects, based on the present data, appear independent of each other.

A Role for NO Signaling?

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 NO synthesis (reviewed by Koshimura

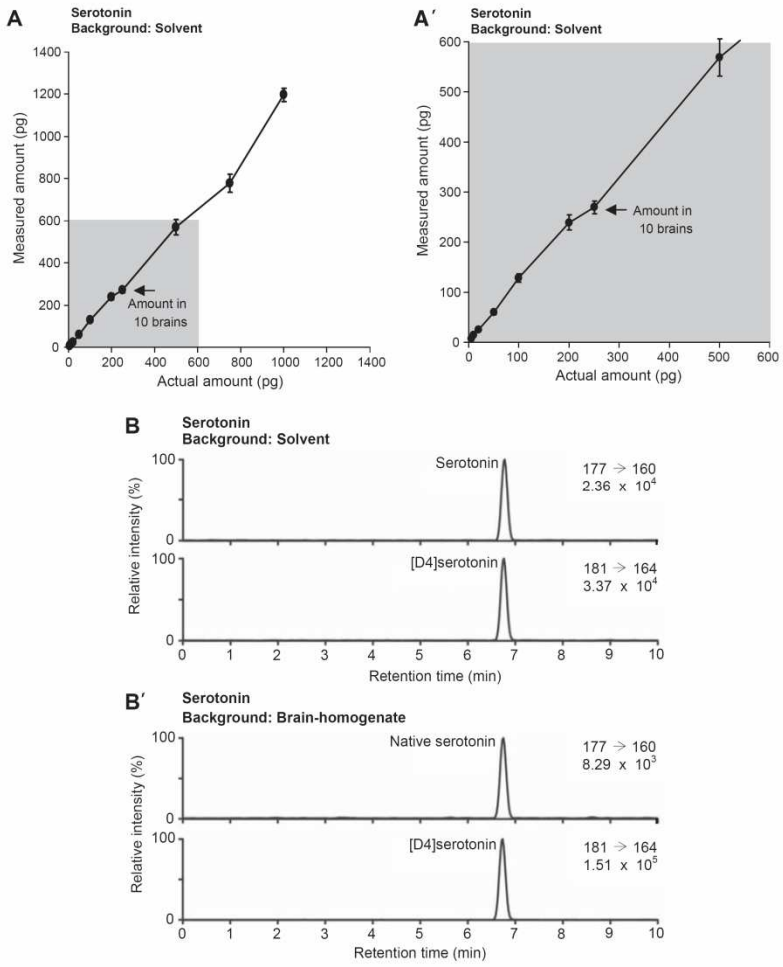


Figure 6. Assessment of serotonin measurement. (**A**) Using high-pressure liquid chromatography coupled with tandem amss spectrometry (HPLC-MS/MS), we analyzed a series of samples, each containing 5 ng of [D4]serotonin and a known amount of unlabeled serotonin, ranging from 5 to 1000 pg. For each sample, we plot the measured amount of unlabeled serotonin against the actual, known amount. Mean ± standard deviations were obtained from three independent measurements. The black arrow marks the mean total amount of serotonin we found in a homogenate of 10 wild type brains (i.e., we multiplied the single-brain value from Figure 4 by 10). (**A′**) Close-up on the lower range of (**A**). (**B**) Example HPLC-MS/MS chromatograms for unlabeled serotonin (top) and labeled [D4]serotonin (bottom) over a solvent ''background.'' As expected, their retention times were equal. (**B′**) Example HPLC-MS/MS chromatograms obtained by analyzing a homogenate of 10 wild type brains, added with isotope-labeled serotonin (5 ng). Both unlabeled, endogenous serotonin (top) and labeled [D4]serotonin (bottom) are clearly detectable. As expected, their retention times were the same. Note that the signal-to-noise ratio for the measurements over the solvent background in (**B**) does not apparently differ from the measurements over the brain-homogenate background. On the top-right corner of (**B**) and (**B′**), the first line indicates the specific MRM transitions and the second line the maximal ion current in arbitrary units.

et al., 2000). Thus, effects of the *white*-gene on NO signaling may explain its effects on learning. Indeed, NO may provide a retrograde signal at the output of the mushroom body Kenyon cells (Bicker & 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., 2004; Heisenberg & Gerber, 2008). Whether the effect of the $white^{1118}$ mutation comes about via alterations in NO signaling 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 color (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 the 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.

CONCLUSIONS

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 the wild type; thus, the balance between punishment learning and relief learning in the $white^{1118}$ mutant is distorted in favor of punishment learning. The molecular mechanisms of this distortion, in particular, regarding the role of serotonin, however, remain controversial.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft via the grants SFB 554/ A10 *Arthropode Behaviour*, SFB-TR 58/ A6 *Fear, Anxiety and Anxiety Disorders*, and a Heisenberg Fellowship (to B.G.), GK 1156 *Synaptic and Behavioural Plasticity* (to B.M.), a PhD fellowship from the Federal Excellence Initiative Grant *Graduate School Life Sciences Würzburg* (to T.S.), as well as by the Boehringer Ingelheim Fonds (PhD fellowship, to A.Y.).

The authors 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 are the collegial discussions with T. Zars (University of Missouri–Columbia).

REFERENCES

An, X., Armstrong, J. D., Kaiser, K. & O'Dell, K. M. (2000). The effects of ectopic white and transformer expression on *Drosophila* courtship behavior. *J Neurogenet*, *14*, 227–243.

Andretic, R., van Swinderen, B. & Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr Biol*, *15*, 1165–1175.

Bicker, G. (2001). Sources and targets of nitric oxide signalling in insect nervous systems. *Cell Tissue Res*, *303*, 137–146.

Bicker, G. & Hähnlein, I. (1995). NADPH-diaphorase expression in neurones and glial cells of the locust brain. *Neuroreport*, *6*, 325–328.

Bicker, G., Schmachtenberg, O. & De Vente, J. (1996). The nitric oxide/cyclic GMP messenger system in olfactory pathways of the locust brain. *Eur J Neurosci*, *8*, 2635–2643.

Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S. & Wu, R. L. (1997). Isolation and characterization of a mammalian homolog of the *Drosophila* white gene. *Gene*, *185*, 77–85.

de Belle, J. S. & Heisenberg, M. (1996). Expression of *Drosophila* mushroom body mutations in alternative genetic backgrounds: a case study of the mushroom body miniature gene (mbm). *Proc Natl Acad Sci USA*, *93*, 9875–9880.

Diegelmann, S., Zars, M. & Zars, T. (2006). Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in *Drosophila*. *Learn Mem*, *13*, 72–83.

Dierick, H. A. & Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat Genet*, *39*, 678–682.

Dreesen, T. D., Johnson, D. H. & Henikoff, S. (1988). The brown protein of *Drosophila melanogaster* is similar to the White protein and to components of active transport complexes. *Mol Cell Biol*, *8*, 5206–5215.

Farooqui, T., Robinson, K., Vaessin, H. & Smith, B. H. (2003). Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J Neurosci*, *23*, 5370–5380.

Gerber, B., Tanimoto, H. & Heisenberg, M. (2004). An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol*, *14*, 737–744.

Hammer, M. (1993). An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature*, *366*, 59–63.

Hammer, M. & Menzel, R. (1998). Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn Mem*, *5*, 146–156.

Hardie, S. L. & Hirsh, J. (2006). An improved method for the separation and detection of biogenic amines in adult *Drosophila* brain extracts by high-performance liquid chromatography. *J Neurosci Meth*, *153*, 243–249.

Hazelrigg, T., Levis, R. & Rubin, G. M. (1984). Transformation of white locus DNA in *Drosophila*: dosage compensation, zeste interaction, and position effects. *Cell*, *36*, 469–481.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat Rev Neurosci*, *4*, 266–275.

Heisenberg, M., & Gerber, B. (2008). Behavioral analysis of learning and memory in *Drosophila*. In: Menzel, R., & Byrne, J. (Eds.), *Learning and Memory: A Comprehensive Reference, Vol. 1: Learning Theory and Behavior* (pp. 549–560) Oxford: Elsevier.

Hing, A. L. & Carlson, J. R. (1996). Male-male courtship behavior induced by ectopic expression of the *Drosophila* white gene: role of sensory function and age. *J Neurobiol*, *30*, 454–464.

Hoyer, S. C., Eckart, A., Herrel, A., Zars, T., Fischer, S. A., Hardie, S. L., et al. (2008). Octopamine in male aggression of *Drosophila*. *Curr Biol*, *18*, 159–167.

Koob, G. F. & Le Moal, M. (2008). Addiction and the brain antireward system. *Annu Rev Psychol*, *59*, 29–53.

Koshimura, K., Murakami, Y., Tanaka, J. & Kato, Y. (2000). The role of 6R-tetrahydrobiopterin in the nervous system. *Prog Neurobiol*, *61*, 415–438.

McClung, C. & Hirsh, J. (1999). The trace amine tyramine is essential for sensitization to cocaine in *Drosophila*. *Curr Biol*, *9*, 853–860.

Monastirioti, M., Linn, C. E., Jr., & White, K. (1996). Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J Neurosci*, *16,* 3900–3911.

Morgan, T. H., Sturtevant, A. H., Muller, H. J., & Bridges, C. B. (1915). *The Mechanism of Mendelian Heredity*. New York: Holt Rinehart & Winston. Reprinted. Johnson Reprint Corporation, with an Introduction by Garland E. Allen, 1978.

Nakamura, M., Ueno, S., Sano, A. & Tanabe, H. (1999). Polymorphisms of the human homologue of the *Drosophila* white gene are associated with mood and panic disorders. *Mol Psychiatry*, *4*, 155–162.

Neuser, K., Husse, J., Stock, P. & Gerber, B. (2005). Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type, and memory span. *Anim Behav*, *69*, 891–898.

Nilsson, E. E., Asztalos, Z., Lukacsovich, T., Awano, W., Usui-Aoki, K. & Yamamoto, D. (2000). Fruitless is in the regulatory pathway by which ectopic mini-white and transformer induce bisexual courtship in *Drosophila*. *J Neurogenet*, *13*, 213–232.

O'Hare, K., Murphy, C., Levis, R. & Rubin, G. M. (1984). DNA sequence of the white locus of *Drosophila melanogaster*. *J Mol Biol*, *180*, 437–455.

Pajak, M. & Kanska, M. (2006). Synthesis of isotopomers of dopamine labeled with deuterium or tritium. *J Labelled Comp Radiopharm*, *49*, 1061–1067.

Riemensperger, T., Voller, T., Stock, P., Buchner, E. & Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr Biol*, *15*, 1953–1960.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., et al. (2006). Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr Biol*, *16*, 1741–1747.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. & Heisenberg, M. (2003). Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. *J Neurosci*, *23*, 10495–10502.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., et al. (2008). Serotonin is necessary for place memory in *Drosophila*. *Proc Natl Acad Sci U S A*, *105*, 5579–5584.

Solomon, R. L. & Corbit, J. D. (1974). An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol Rev*, *81*, 119–145.

Straub, R. E., Lehner, T., Luo, Y., Loth, J. E., Shao, W., Sharpe, L., et al. (1994). A possible vulnerability locus for bipolar affective disorder on chromosome 21q22.3. *Nat Genet*, *8*, 291–296.

Sullivan, D. T. & Sullivan, M.C. (1975). Transport defects as the physiological basis for eye color mutants of *Drosophila melanogaster*. *Biochem Genet*, *13*, 603–613.

Tanimoto, H., Heisenberg, M. & Gerber, B. (2004). Event timing turns punishment to reward. *Nature*, *430*, 983.

Tearle, R. G., Belote, J. M., McKeown, M., Baker, B. S. & Howells, A. J. (1989). Cloning and characterization of the scarlet gene of *Drosophila melanogaster*. *Genetics*, *122*, 595–606.

Unoki, S., Matsumoto, Y. & Mizunami, M. (2005). Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur J Neurosci*, *22*, 1409–1416.

Unoki, S., Matsumoto, Y. & Mizunami, M. (2006). Roles of octopaminergic and dopaminergic neurons in mediating reward and punishment signals in insect visual learning. *Eur J Neurosci*, *24*, 2031–2038.

Vergoz, V., Roussel, E., Sandoz, J. C. & Giurfa, M. (2007). Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS ONE*, *2*, 288.

Vincent, J. D. & Kukstas, L. A. (1998). Opponent processes and anxiety: toward a neurophysiological formulation. *Acta Psychiatr Scand Suppl*, *393*, 50–55.

Wright, T. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. *Adv Genet*, *24*, 127–222.

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S., & Gerber, B. (2008). ''Pain-relief'' learning in fruit flies. *Anim Behav, 76*, 1173–1185.

Yuan, Q., Joiner, W. J. & Sehgal, A. (2006). A sleep-promoting role for the *Drosophila* serotonin receptor 1A. *Curr Biol*, *16*, 1051–1062.

Yuan, Q., Lin, F., Zheng, X. & Sehgal, A. (2005). Serotonin modulates circadian entrainment in *Drosophila*. *Neuron*, *47*, 115–127.

Zars, T. (2000). Behavioural functions of the insect mushroom bodies. *Curr Opin Neurobiol*, *10*, 790–795.

Zhang, Y., Friedman, D., Wang, Z., Woodruff, E., Pan, L., O'Donnell, J., et al. (2005). Protein expression profiling of the *Drosophila* fragile X mutant brain reveals upregulation of monoamine synthesis. *Cell*, *107*, 591–603.

Zhang, K., Guo, J. Z., Peng, Y., Xi, W. & Guo, A. (2007). Dopamine-mushroom body circuit regulates saliency-based decision making in *Drosophila*. *Science*, *316*, 1901–1904.

Zhang, S. D. & Odenwald, W. F. (1995). Misexpression of the white (*w*) gene triggers male-male courtship in *Drosophila*. *Proc Nat Acad Sci U S A*, *92*, 5525–5529.

Summary

Animals need to adapt and modify their behaviour according to a changing environment. In particular, the ability to learn about rewarding or punishing events is crucial for survival. One key process that underlies such learning are modifications of the synaptic connection between nerve cells. This Thesis is concerned with the genetic determinants of such plasticity, and with the site of these modifications along the sensory-to-motor loops in *Drosophila* olfactory learning.

I contributed to the development and detailed parametric description of an olfactory associative learning paradigm in larval fruit flies (Chapter I.1.). The robustness of this learning assay, together with a set of transgenic *Drosophila* strains established during this Thesis, enabled me to study the role for Synapsin, a presynaptic phosphoprotein likely involved in synaptic plasticity, in this form of learning (Chapter I.2.), and to investigate the cellular site of the corresponding Synapsin-dependent memory trace (Chapter I.3.). These data provide the first comprehensive account to-date of the neurogenetic bases of learning in larval Drosophila.

The role for Synapsin was also analyzed with regard to pain-relief learning in adult fruit flies (Chapter II.1.); that is, if an odour precedes an electric shock during training, flies subsequently avoid that odour ('punishment learning'), whereas presentation of the odour upon the cessation of shock subsequently leads to approach towards the odour ('relief larning'). Such pain-relief learning was also the central topic of a study concerning the white gene (Chapter II.2.), which as we report does affect pain-relief as well as punishment learning in adult flies, but leaves larval odour-food learning unaffected. These studies regarding pain-relief learning provide the very first hints, in any experimental system, concerning the genetic determinants of this form of learning.

Zusammenfassung

Tiere müssen sich den wechselnden Umweltbedingungen anpassen und ihr Verhalten dementsprechend ändern. Insbesondere ist die Fähigkeit bestrafende und belohnende Ereignisse zu lernen wesentlich für das Überleben. Ein Schlüssel-Prozess, der solchem Lernen unterliegt, sind Veränderungen in der synaptischen Verbindung zwischen Nervenzellen. Diese Arbeit beschäftigt sich mit den genetischen Bestimmungsgrößen solcher Plastizität und mit dem Ort an, dem diese Veränderungen entlang der sensorisch-motorischen Nervenbahn des olfaktorischen Lernens in *Drosophila* stattfinden.

Ich habe an der Planung und der Festlegung der Parameter eines olfaktorischen assoziativen Lernparadigmas von *Drosophila* Larven mitgewirkt (Kapitel I.1.). Die Robustheit dieses Lernparadigmas, zusammen mit einer Anzahl an genetisch veränderten *Drosophila* Stämmen, die ich während dieser Arbeit entwickelt habe, ermöglichte es mir, die Rolle des Synapsins zu untersuchen. Synapsin ist ein präsynaptisches Phosphoprotein und wahrscheinlich an synaptischer Plastizität beteiligt, welche bei dieser Art von Lernen eine Rolle spielt (Kapitel I.2.). Außerdem ermöglichte es mir den zellulären Ort der Synapsin-abhängigen Gedächtnisspur zu untersuchen (Kapitel I.3.)
Diese Daten liefern den ersten umfassenden Wissensstand über neuro-genetische Grundlagen des larvalen Lernens.

Die Rolle des Synapsin wurde auch im „Erleichterungslernen“ in adulten Fliegen analysiert (Kapitel II.1.). Wenn während des Trainings ein Duft einem elektrischem Schock vorausgeht, vermeiden Fliegen danach diesen Duft („Beschtrafungslernen“), wohingegen die Verabreichung des Duftes nach dem Ende des Schocks anschließend zu einer Annäherung in Richtung Duft führt („Erleichterungslernen“). Solches „Erleichterungslernen“ war auch der Schwerpunkt einer Reihe von Experimenten, die sich mit dem „White“-Gen beschäftigten (Kapitel II.2.). Wie gezeigt beeinflusst dieses Gen sowohl das „Erleichterungs“- als auch das „Bestrafungs“- Lernen in adulten Fliegen; jedoch hat es keine Auswirkungen auf das Duft-Futter-Lernen in Larven. Diese Experimente, die sich mit „Erleichterungslernen“ beschäftigen, liefern die neuesten Hinweise in Systemen, die sich mit den genetischen Bestimmungsgrößen dieser Form von Lernen beschäftigen.

Curriculum vitae

Birgit Martha Michels
Ph D Student

Lehrstuhl für Genetik und Neurobiologie
Universität Würzburg
Am Hubland - Biozentrum
97074 Würzburg
Phone: ++49 931 888 4463
Fax: ++49 931 888 4452
Email: Brigit.michels@biozentrum.uni-wuerzburg.de

Surname: Michels
First name: Birgit Martha
Date of birth: 27.08.1979
Place of birth: Würzburg (Bayern) – Germany
Martial status: Single

Education:
1986-1990 Elementary school Eibelstadt, Bayern, Germany
1990-1999 High school (Gymnasium) Würzburg, Bayern, Germany
25.6.1999 Examination "Allgemeine Hochschulreife"

Undergraduate studies:
1999-2001 Intermediate examination in Biology, University of Würzburg, Bayern, Germany
2003-2004 Diploma in Biology: "Molecular bases of associative learning in the *Drosophila* Larva"
Supervisor: PD. B. Gerber

Graduate studies:
2004 (October) PhD in Biology, University of Würzburg, Bayern, Germany
"Towards localizing the Synapsin-dependent olfactory memory trace in the brain of larval *Drosophila*"
Supervisor: PD. B. Gerber

Funding:
Stipend of the Graduiertenkolleg "Von der synaptischen Plastizität zur Verhaltensmodulation in genetischen Modellorganismen"
SFB 554: Mechanismen und Evolution des Arthropodenverhaltens: Gehirn-Individuum-Soziale Gruppe

List of publication

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, Heisenberg M (2004) Visual learning in individually assayed Drosophila larvae. J Exp Biol 207:179-188.

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B (2005) The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed Drosophila larvae. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 191:265-279.

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B (2005) A role for Synapsin in associative learning: the Drosophila larva as a study case. Learn Mem 12:224-231.

Yarali A, Krischke M, Michels B, Saumweber T, Mueller MJ, Gerber B (In Press) Genetic Distortion of the Balance between Punishment and Relief Learning in Drosophila. J Neurogenet:1-16.

Acknowledgements

Danke.....

Last but not least ein paar Worte des Dankes an all die Personen, die halfen diese Arbeit entstehen zu lassen:

...Bertram Gerber für die Betreuung dieser Arbeit, für die vielen Diskussionen, Anregungen und Unterstützung. Vielen Dank für alles – ich habe gerne in deiner Arbeitsgruppe gearbeitet.

...Martin Heisenberg und Erich Buchner für die sehr angenehme Arbeitsatmosphäre im Lehrstuhl, für die Unterstützung und die hilfreichen Diskussionen.

...Timo Saumweber für die große Hilfe bei der Bearbeitung der Bilder – ohne dich hätte ich das nicht geschafft, für die nette Atmosphäre in unserem Zimmer und für alles andere...

...an meine Praktikantinnen v.a. Jana Husse und Yi-chun chen, die mir die Arbeit mit der Fliegen-Herstellung abgenommen haben.

...Katja und Katharina für das Platten-gießen.

...allen Leuten aus der AG Gerber.

...und allen Leuten vom Lehrstuhl für Genetik und Neurobiologie.

...und natürlich am Wichtigsten, meiner Familie, ohne die das alles hier sicherlich nicht möglich gewesen wäre, und es endlich mal Zeit ist DANKE zu sagen.