Associative memory in *Drosophila melanogaster*: Synapsin as a study case

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr. rer. nat.)

genehmigt durch die Fakultät für Naturwissenschaften
der Otto-von-Guericke-Universität Magdeburg

von Dipl.-Biol. (Univ.) Jörg Kleber

geb. am 13.09.1982 in Osnabrück

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eingereicht am 04.03.2016

verteidigt am 24.11.2016

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Zusammenfassung

Was bestimmt unser Handeln? Mit dieser Frage befassen sich Philosophen und Naturforscher zeitlebens. Um in einem sich verändernden Umfeld agieren und reagieren zu können, ist es für einen Organismus essentiell, Erfahrungen zu sammeln, zu speichern und darauf basierend sein Handeln anzupassen. Eine fundamentale Frage in der Neurowissenschaft ist es, wie sich auf der Basis von solchen Erfahrungen und Lernprozessen Gedächtnisse etablieren und wie sie schließlich in Verhalten umgesetzt werden. Um die Rolle des Gedächtnisses für die Verhaltenssteuerung zu verstehen, ist es wichtig die zellulären und molekularen Grundlagen des Verhaltens zu analysieren. In diesem Kontext konzentriert sich diese Arbeit auf das präsynaptische Protein Synapsin und dessen Rolle bei assoziativen Lern- und Gedächtnisprozessen bei *Drosophila melanogaster*.

Synapsin gehört zu einer Familie evolutionär konservierter Phosphoproteinen, welche mit der cytoplasmatischen Seite synaptischer Vesikel assoziiert sind. In *Drosophila* wird Synapsin von nur einem einzigen Gen kodiert und wird pan-neural im larvalen und adulten Gehirn exprimiert. Synapsin ist sowohl für die synaptische Plastizität, als auch für assoziative Lern- und Gedächtnisprozesse notwendig. Larvale, wie auch adulte Fliegen, die nicht in der Lage sind Synapsin zu exprimieren, zeigen im Vergleich zu wildtypischen Tieren eine Reduktion im assoziativen Lernen von ca. 50%.

In dieser Arbeit wurden assoziative Lernexperimente durchgeführt um die Beteiligung von Synapsin am Belohnungsgedächtnis, Bestrafungsgedächtnis und am sogenannten *Relief*-Gedächtnis näher zu untersuchen. *Drosophila* eignet sich besonders gut als Studienobjekt, da das Gehirn der Fruchtfliege aus vergleichsweise wenigen Nervenzellen besteht und sich somit neuronale Schaltkreise leichter aufdecken lassen als bei Maus, Ratte, Affe oder Mensch. Eine große Homologie vieler Gene zwischen *Drosophila* und dem Menschen, sowie ein ähnlicher Ablauf von Gedächtnisprozessen macht es möglich, die Grundlagen neuronaler Netzwerke in der Fliege zu untersuchen um somit generelle neurobiologische Prinzipien aufzudecken und später auf Vertebraten zu übertragen. Des Weiteren bietet *Drosophila* durch die einfache genetische Manipulierbarkeit mit reichhaltigen und etablierten genetischen Werkzeugen z.B. die Möglichkeit ein beliebiges Gen zellspezifisch und zu einem gewünschten Zeitpunkt zu exprimieren oder auszuschalten.

Diese Arbeit ist in zwei Kapitel gegliedert. **Kapitel I** beschäftigt sich mit der Rolle von Synapsin beim assoziativen Duft-Zucker Lernen in der *Drosophila* Larve. Hierbei lag der Fokus besonders auf der Frage, ob die auf elektrophysiologischen Daten basierende Vermutung einer Abhängigkeit zwischen dem Phänotyp von Synapsin-Nullmutanten (syn^{97}) und parametrischen Eigenschaften des assoziativen Duft-Zucker Lernens bestätigt werden kann. Um dieses Thema systematisch zu untersuchen, wurden Parameter verändert, die bekanntermaßen Einfluss auf die Gedächtnisstärke in wildtypischen Fliegenlarven haben:

Duftkonzentration, Zuckerkonzentration, sowie das Zeitintervall zwischen Training und Test. Die Ergebnisse zeigen, dass die Gedächtnisstärke der *syn*^{97} Mutanten nur dann reduziert ist, wenn hohe Duftkonzentrationen oder hohe Konzentration der Zuckerbelohnung verwendet wurden. Des Weiteren konnte gezeigt werden, dass Synapsin selektiv für das Kurzzeitgedächtnis notwendig ist. Generell ist folglich die *Drosophila* Larve auch ohne Synapsin in der Lage zu lernen und sich zu erinnern - aber Synapsin ist notwendig, um die Gedächtnisstärke bei sehr prägnanten, sogenannten „*salienten"* Lern- bzw. Erinnerungsaufgaben zu verstärken. Weiterhin konnte durch massenspektrometrische Analysen eine verstärkte Phosphorylierung von Synapsin in Abwesenheit des synaptischen Proteins (Sap47, Synapsen assoziiertes Protein von 47 kDa) nachgewiesen werden. Dadurch erscheint eine Interaktion dieser beiden präsynaptischen Proteine sehr wahrscheinlich.

Kapitel II befasst sich mit der Funktion von Synapsin beim Bestrafungs- und *Relief*-Lernen der erwachsenen Fliegen. Es wurde gezeigt, dass die Gedächtnisstärke bei Tieren denen Synapsin fehlt, sowohl im Bestrafungslernen als auch im *Relief*-Lernen reduziert ist. Dabei bleiben alle relevanten sensorischen und motorischen Fähigkeiten unbeeinflusst. Eine Schwächung der assoziativen Stärke wurde auch nach einem Synapsin-RNAi „knock-down" beobachtet. Ein lokale Expression von Synapsin im Pilzkörper von *syn*^{97}*-Drosophila* rettete die Beeinträchtigung in der Gedächtnisleistung und stellte die volle assoziative Stärke wieder her. Folglich ist Synapsin auch sowohl für die Etablierung von Bestrafungsgedächtnissen, als auch von R*elief*-Gedächtnissen relevant.

Insgesamt trägt diese Arbeit zum generellen Verständnis bei, wie sich Erinnerungen und Gedächtnisse etablieren und wie sie letztendlich in Verhalten umgesetzt werden. Diese Studie zeigt, dass bestimmte Komponenten wie das Protein Synapsin für die Entstehung von starken Gedächtnissen für besonders prägnante, ‚saliente' Informationen essentiell sind. Dies ist von besonderer Relevanz, auch aus medizinischer Sicht, um zu verstehen, warum bestimmte Sachverhalte einfacher oder schwerer zu lernen sind als andere und welche Parameter darauf Einfluss ausüben.

Summary

In order to act and react in a changing environment it is crucial for an animal to make experiences, to learn and to remember. A fundamental question in neurobiology is how such learning processes form memories and how these are turned into behavior. In this context this Thesis focuses on the role of the presynaptic protein Synapsin in associative learning tasks (reward learning, punishment learning and pain-relief learning) in larval and adult *Drosophila melanogaster* as a study case.

This thesis is divided in to two parts. In **Chapter I** I study how salient events induce strong memories. To address this topic larval *Drosophila* were trained in an odor-reward associative memory task. To systematically investigate the underlying mechanism of saliency-matched memory formation, parameters were varied which are known to affect memory scores in wild-type larvae, namely odor concentration, as well as sugar concentrations and the time interval between training and test. The results show that memory scores in mutants lacking Synapsin (syn^{97}) are lower than in wild-type animals only when higher concentrations of odors or of the sugar reward were applied. Furthermore, Synapsin is selectively required for short-term memory. Thus, without Synapsin *Drosophila* larvae in principle can learn and remember, but Synapsin is required to 'boost' memories that match in strength to high event salience. Additionally mass spectrometry analysis shows an upregulated phosphorylation status of Synapsin in the larval nervous system upon the lack of another synaptic protein Sap47 (Sap47, Synapse associated protein of 47 kDa). This result suggests a potential functional interdependence of Synapsin and Sap47.

Chapter II deals with Synapsin and its role in punishment- and pain-relief learning in the adult fly. It is shown that in flies lacking Synapsin both punishment and pain-relief memory are reduced. In contrast syn^{97} mutants are not impaired in task relevant sensory or motor abilities. A reduction in associative strength was also observed after a Synapsin-RNAi knockdown, whereas expressing Synapsin in the mushroom bodies of syn^{97} *Drosophila* could restore full associative memory.

This Thesis contributes to the general understanding of how saliency-matched memories are established and how they are translated into behavior. This study specifically finds that certain components like the presynaptic protein Synapsin are required for establishing strong memories especially for salient events or for easy to learn tasks. This is of relevance, also from the medical perspective, for the understanding of why certain tasks are easier or more difficult to learn than others and which parameters have a bearing on it.

General introduction

Why do we do what we do? In other words, what guides our actions and how does behavior come about? For adaptive behavioral control it is essential to integrate perception, motivation and expectation. These processes are strongly dependent from previous experiences and therefore our behavioral tendencies can be modulated by what we have learned and which associative memories we have established. In this sense, memories can shape our mind. When we are hungry, our choice of a restaurant is based on associations between the respective location and the food we previously ate there. Thus, learning and the resulting associative memories strongly influence our motivation and thereby our actions. This gives rise to the exciting question how learning and memory processes are translated into behavior. The organ that is capable for doing this complex job is the brain, the most complex organ that evolved during evolution. Its function is to enable the organism to behave properly in a changing environment. One of the brains most fascinating features is to allow organisms to learn and to remember and thereby modulate motivation and behavior. One major aim of neurobiology is to gain insight into these processes which are taking place during the interactions between the brain and the outside world. In particular neuroscientists are trying to understand how learning and memory come about at a cellular and molecular level. In this context my work focuses on the role of the presynaptic protein Synapsin and its potential functional partners in the presynaptic molecular network. To tackle this associative memory trace formation *Drosophila melanogaster* is used as study case.

A small animal with big advantages

Why working with the fruit fly *Drosophila*, a small insect of about 3 millimeters length, known to most of us as swarming little insects on rotten fruits? One way to trigger new developments in research is to use simple model systems. *Drosophila melanogaster* is such a simple (in terms of simple brain structure, see below) model organism that combines many advantages. William Ernest Castle introduced the fly as a study case for the first time to the scientific world (Castle, Carpenter, Clark, Mast and Barrows, 1906). He was the first to use *Drosophila* for genetic experiments. With some colleagues he did intensive studies of inbreeding and selection, which was published in 1906. Since then, during more than hundred years of *Drosophila* research, the small fly has become so popular as study case that it is impossible to enumerate all the things that have been investigated.

In the 1860's, Gregor Mendel, an Austrian monk who worked on the heritable traits of pea plants, carefully quantified the way these traits were passed on to offspring and discovered several fundamental principles of genetics (Mendel, 1866). Due to his observations he introduced some common laws of inheritance known as Mendel's laws. Interestingly, Mendel did not know the role of chromosomes, or

DNA, which were discovered much later, but he did attribute the passing of parental characteristics to their offspring to heritable 'factors'. Today, these 'factors' are known as genes. Thanks to *Drosophila* the role of genes in inheritance could be clarified and the laws of inheritance have been confirmed. A pioneer in this field was Thomas Hunt Morgan. Inspired by the work of William Castle (see above), Morgan decided to use *Drosophila melanogaster* as model organism what resulted in the Noble Prize for medicine in 1933 for the work on the role of chromosomes in heredity. Morgan used the heritable traits of the fruit fly to expand the general understanding of genetics. He was the first to demonstrate by crossing experiments that genes were located next to each other on chromosomes and that genes are the basis of heredity. He determined the order of genes on chromosomes and their distance between each other and also found that certain fruit fly traits (e.g., white vs. red eye color) are found on the same chromosome that also determine their sex.

In developmental biology it can be examined very precisely how a complex organism such as a fly develops from a fertilized egg. Furthermore, the Nobel Prize in Medicine/Physiology in 1995 for Ed Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus demonstrates the importance of *Drosophila* also for medical research: These researchers uncovered the fundamental genetic control mechanisms in the embryonic development of the fruit fly and thereby revealed some general principals of developmental biology (Nüsslein-Volhard, Wieschaus; 1980).

Today, after more than 100 years of *Drosophila* research, knowledge and tools for genetic manipulations and analysis has been gathered, which make working with *Drosophila* rather easy. The genome is relatively small, it consists of only 4 chromosomes, one sex chromosome and 3 autosomes which make it manageable and easy to handle. In 2000 the first draft version of the *Drosophila* genome has been published and since then continually revised (Adams et al. 2000). It was revealed that the entire genome comprises of about 165 million bases and contains about 14000 genes (for comparison: man has about 3.4 billion bases and 20000 genes (Lander et al. 2001), yeast only 5800 genes and 13.5 million bases (Zagulski et al. 1998). Today, there are also some other genomes sequenced including other invertebrates like the mosquito *Anopheles gambiae* (Holt et a. 2002) and the honey bee *Apis mellifera* (Weinstock et al. 2006) and vertebrates like the house mouse *Mus musculus* (Asif et al. 2002) and the human (Lander et al. 2001). These data sets may help to find out what is common to all invertebrates and vertebrates and what differentiates them from each other and thereby helping to reveal principal mechanisms of evolution. Another motivation to work with invertebrate models like *Drosophila* is the high degree of kinship to vertebrate models. Interestingly out of the about 1000 genes that are associated with human genetic disease, 77% homologue genes have been identified in *Drosophila* (Reiter et al. 2001), showing an important relation to human research. Besides this there are some more common features that link invertebrate research with that of vertebrates. A very important aspect in this regard is that *Drosophila* has a central nervous system that functions according to the "same principles" as in higher

organisms. The architecture of the *Drosophila* brain is much simpler, especially in terms of numbers of cells and connections (larva: ~10.000 and adult: ~100.000 cells). *Drosophila* shares with vertebrates the common neurotransmitters including dopamine, acetylcholine, GABA and glutamate (see review Gerber et al. 2014). Given the simple brain structure and good comparability with vertebrates brains, complex processes like learning and memory processes can be investigated more effectively and even on single cell level in the fly brain. This makes the nervous system of *Drosophila* a powerful model system for neuro-genetic studies. Further arguments to work with *Drosophila* are of practical nature. The generation cycle of *Drosophila* is only about ten days which makes it possible to generate a huge amount of progeny in a short time. This is particular useful for genetic studies and experiments. Further, flies are small and their maintenance is cheap. Further, applied entomology is beneficial for the investigation of carrier of human disease, biological pest control and even robotics. Lastly, over one century of *Drosophila* genetics does not only provide thousands of mutants, but also spearheaded the development of a rich toolbox for transgenetic manipulation.

GAL4-UAS expression system

One important example of one of the most powerful genetic tools is the GAL4-UAS expression system (Brand and Perrimon, 1993; Venken et al. 2011) and its improved derivative, the split GAL4-UAS system (Luan et al. 2006; Pfeiffer et al. 2010). These tools enable controlling cell and time specific gene expression of nearly any gene of interest.

The GAL4-UAS system is a binary expression system that was first described in 1993 by Brand and Perrimon. GAL4 is a transcription factor from yeast and is naturally not expressed in *Drosophila*. The GAL4 protein consists mainly of an activation domain (AD) and a DNA-binding domain (DBD), which recognizes specifically the so called Upstream Activation Sequence (UAS) (Fig. 1A). Any transgene of interest can be expressed under control of the UAS promoter by fusing it downstream to it. The UAS promoter needs to be activated by its transcription factor GAL4. For both constructs separate fly strains can be generated. Thus, the transgene is only expressed when both, GAL4 and UAS lines, are crossed. A defined promoter element or a defined landing site in the chromosome can be chosen to express GAL4 in a specific pattern of interest. Today thousands of GAL4 lines exist which cover many different tissues, only a couple of cells up to single cell level, making it possible to express any kind of gene exclusively in defined subsets of cells or in the larval case even in single cells (Fig. 1C-G).

Additionally, the action of the GAL4 protein can be repressed by an inhibitor of GAL4, the GAL80 protein (Fig. 1B). GAL80 can be used in combination with the GAL4 system and gives access to additional control, e.g. to restrict transgene expression both spatially and temporally. For spatial control, GAL80 can be fused to a given promoter to repress GAL4 activity in a specific region or tissue. For temporal control, one can use the temperature sensitive mutant $GAL80^{ts}$, which is active at 18 °C but does

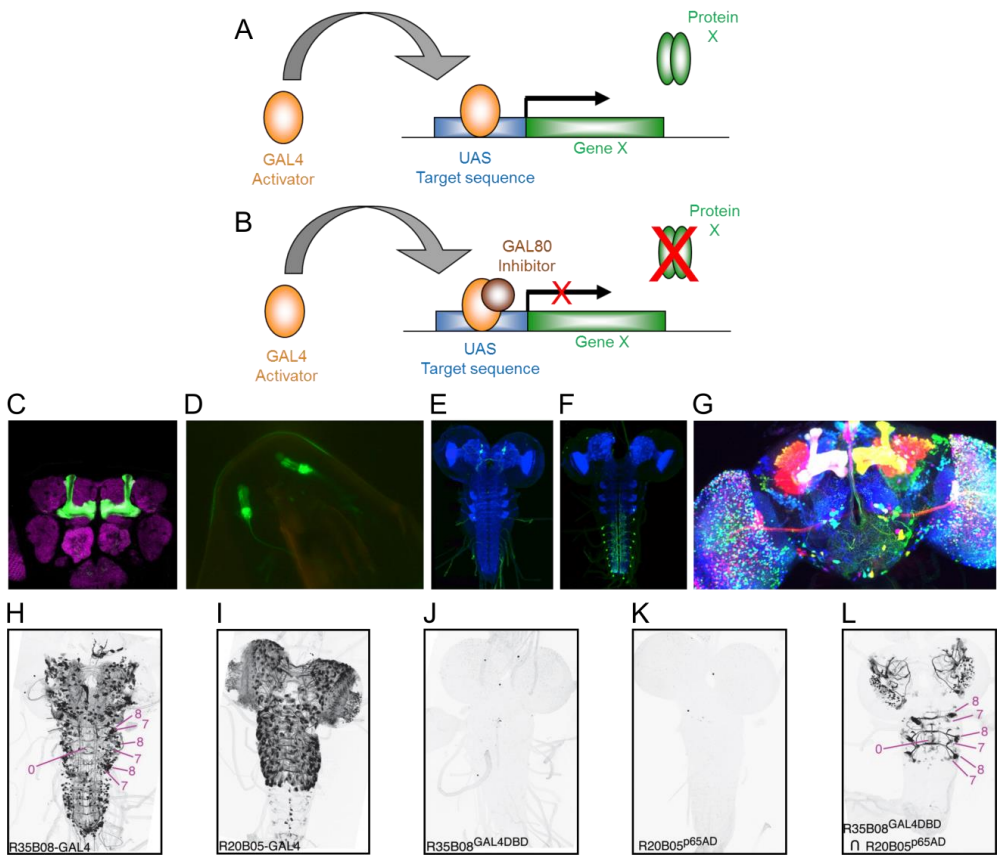


Figure 1. The GAL4–UAS and the split GAL4-UAS expression system. **(A-B)** The GAL4–UAS expression system. (A)The GAL4 transcription factor originally present only in yeast, recognizes and specifically binds to the Upstream Activation Sequence (UAS). Thereby it activates the expression of any gene that is under the control of the UAS-promotor. The *gal4* gene sequence can be fused downstream of nearly any enhancer or promotor of interest (not shown) that determines the "where", i.e. in which cells or tissue the desired transgene is expressed. Accordingly the UAS construct determines the "what" because the UAS promotor can be fused upstream of almost every gene of interest (gene X) that is most likely translated into a protein (protein X). Please note that the GAL4 and the UAS sequences are normally not present in *Drosophila* and therefore neither GAL4 nor the UAS sequence on its own have influence on further gene expression. Only if both DNA sequences are present within the same animal, GAL4 can specifically bind and activate the expression of nearly any kind of gene that lays downstream of the UAS sequence. (B) The action of the GAL4 protein can be repressed by the GAL80 protein and thereby inhibiting gene expression. The GAL80 can be used in combination with the GAL4 system and gives access to additional control, e.g. to restrict transgene expression both spatially and temporally (for more details see general introduction). **(C-G)** The GAL4-UAS system is a powerful tool that provides a big repertoire of genetic manipulations (for more details see general introduction). It allows to express almost any gene of interest in a tissue or cell specific manner. This technique can be used to generate a huge variety of specific expression pattern in the fly brain. Here, examples of cell specific GFP expression are shown, that elucidate the versatility of this powerful expression system. (C) Mushroom body specific (mb247-GAL4/ UAS-GFP) GFP expression in nearly all Kenyon cells of the adult brain and (D) GFP expression in a subset of olfactory sensory neurons (Orco-GAL4/ UAS-GFP) in a living larva. (E-F) Sparse expression in a single neuron class in (E) the larval brain or (F) in abdominal neuromeres of the ventral nerve cord of the larval brain. Figures modified after Li et al. 2014. (G) With the "brainbow" technique (Hampel et al. 2011) it is even possible to color-code different cells in a given expression pattern. This allows to identify individual cells within the same tissue and to distinguish between different neurons. This gives access to trace neural circuits and to characterize cell-cell interactions. Figure from Hampel et al. 2011. **(H-L)** Split GAL4-UAS system Two domains of the GAL4 Protein, its DNA binding domain (DBD) and its activation domain (AD) can be expressed independently of each other, in partially overlapping sets of cells to achieve intersectional effector expression. (H) and (I) show the expression pattern of two GAL4 driver lines which are partially overlapping (in parts indicated by the numbers 0, 7 and 8 in (A)). (J) Expressing the DBD domain via the enhancer used for (H) (R35B08Gal4DBD) and (K) the AD domain via the enhancer used for (I) (R20B05p65AD) and crossing both lines separately to UAS-GFP does not result in an expression of GFP because no functional GAL4 protein is established. (L) Expressing the DBD and the AD domains of the GAL4 protein, in overlapping subsets of the same animal, is necessary to activate transcription from UAS-GFP. Only those cells in which both protein domains are expressed at the same time (partially indicated by the numbers 0, 7 and 8) the functional GAL4 heterodimer is established and drives transgene expression. With this method very sparse expression pattern can be generated even down to single cell level. Figure modified after Pfeiffer et al. 2010.

not repress GAL4 at 29 °C or higher temperatures. Besides the GAL4-UAS system two additional binary expression systems are available: The LexA-LexAop system (Lai and Lee, 2006) and the QF-QUAS system (Potter et al. 2010). All three expression systems work according to the same principles and can be used independent from each other. Using two independent bipartite expression systems gives access to compare expression patterns, to identify cells in overlapping expression patterns, or to reconstitute e.g. the green fluorescent protein (GFP) across synaptic partners using GRASP (Diegelmann et al. 2008). To identify functional connections, formed by neurons can be achieved by activating presynaptic cells with one bipartite system, e.g. Channelrhodopsin2 as effector (see below; Nagel et al. 2003) and expression of a calcium sensor in the postsynaptic cell by using the other bipartite system (Yao et al. 2012, Pech et al. 2015).

The split GAL4-UAS system (Luan et al. 2006; Pfeiffer et al. 2010) emanates from the GAL4-UAS system and is based on the modular nature of the GAL4 transcription factor, which allows for independent expression of the GAL4-DBD and the GAL4-AD under control of different enhancers. Expressing these two motifs individually and separately from each other does not result in an activation of transcription. Only those cells in which both protein domains are expressed at the same time possess the transcription factor heterodimer to be functional to further drive transgene expression. The combined spatial and temporal specificity of these expression systems offers potential advantages in dissecting complex neural circuits and enables one to control gene expression down to the single cell level (Fig. 1H-L).

These tools provide some more highlights, like monitoring gene expression, blocking or activating cell activity or knocking down gene expression. For a gene knockdown a RNAi library for all *Drosophila* genes is available, to knock down any gene of interest (Dietzl et al.2007; e.g. Vienna Drosophila RNAi Center). The GAL4-UAS system in combination with expression of UAS-*shibire*^{ts1}, a temperature-sensitive mutation of the *Drosophila* gene encoding a Dynamin orthologue (Chen et al. 1991; van der Bliek and E.M. Meyerowitz. 1991), leads to a temperature dependent reversible block of vesicle endocytosis and thus prevents synaptic transmission at restrictive temperature. Cell specific activation can be achieved by fusing UAS with a Channelrhodopsin2 gene (*ChR2*), coding for a light activated cation channel from the single cell green alga *Chlamydomonas reinhardtii* (Nagel et al. 2003) or similar derivatives like Chrimson (Klapoetke et al. 2014) or an improved version ChR-XXL being functional even without retinal (Dawydow et al 2014). In principle these tools work similar. The original ChR2 protein contains seven trans-membrane domains and an all-trans retinal as its chromophore, and responds to light stimulation by opening an internal cation channel, resulting in generating an action potential. Accordingly, using UAS-*shibire*^{ts1} or UAS-*ChR2* gives spatial and temporal control to suppression of neurotransmitter release or activation of cells in a non-invasive way.

The GAL4-UAS system is further of huge value, because it can help to clarify some important issues for molecular questions. Creating null mutants (e.g. by creating deletion null mutants of gene *x*) always bears the risk that the observed phenotype (e.g. an impairment in memory) does not only result from the specific gene deletion and thus from the lack of the respective protein, but can be due to side effects of the genetic manipulation like silencing or enhancing effects of other genes. To exclude such side effects it is a common method to express UAS-RNAi of a given gene *x* in animals of wild-type background to see whether the down regulation of protein X can mimic the deletion phenotype of gene *x*. Please note that due to the nature of the GAL4-UAS system (see above), such an RNAi knockdown can be performed in a cell specific manner, what is in contrast to an approach with a deletion mutant. A further approach is to express protein X in the respective null mutant background via UAS-X recue construct to test if this will restore the phenotype to the wild-type status (e.g. restore full memory). The major point in this regard is to test for the necessity and sufficiency of protein X in a given subset of cells. If protein expression in a certain subsets of cells of null mutants fully rescues the respective function, protein expression in this cell compartments is sufficient. If RNAi knock down in the same cell subsets of otherwise wild-type animals can mimic the null mutant phenotype, protein expression in these cells is necessary for proper function.

To summarize, these methods and tools are of outstanding analytical power, ease, and elegance and give access to various kinds of genetic manipulations. Everything which is encoded by DNA can be artificially expressed cell-type specific at any time. The combination of these features is unique among model organisms.

How to train larvae and flies?

A common experience in our daily lives is that certain external stimuli, such as the smell of a cologne or perfume, a certain song, a specific day of the year, can result in fairly intense emotions. It is not that the emotions are caused by the smell or the song, but it is better seen that the smell or the song has been associated with, perhaps an emotional situation like an ex-boyfriend or ex-girlfriend, the death of a loved person, or maybe the day one got the doctorate. Many of our behaviors at present are influenced and formed by pairing of stimuli in the past. These associations happen all the time and often we do not even realize the power that these connections have on us, but, in fact, we have been classically conditioned. The principles of classical conditioning were discovered by the work of the Russian physiologist Ivan Petrovich Pavlov (1849-1936). In the early twentieth century, he did Nobel prize-winning work on digestion. While studying the production of saliva in dogs' digestive processes, he stumbled upon a phenomenon he labeled "psychic reflexes." Although it was mainly an accidental discovery, he had the foresight to see the importance of it. Pavlov's dogs, restrained in an experimental chamber, had their saliva collected via a surgically implanted tube in their saliva glands and were presented with meat

powder. Over time, Pavlov noticed that his dogs began to salivate before the meat powder was even presented. He speculated that it was evoked by the presence of the experimenter or merely by a clicking sound produced by the device that distributed the meat powder. Fascinated by this finding, Pavlov paired the meat powder with various stimuli such as the ringing of a bell (auditory stimulus). After the meat powder and bell were presented together (paired) several times, the bell ringing was presented alone. Pavlov's dogs, as predicted, responded by salivating to the sound of the bell without any food reward to be present. The bell previously was a neutral stimulus, i.e. the bell itself did not produce dogs' salivation. However, pairing the bell with the stimulus did produce the salivation response towards the ringing of the bell. In other words, the bell ringing acquired the ability to trigger the salivation response. Pavlov dedicated much of the rest of his career further exploring this finding. He explained his findings in technical terms: The meat powder is considered as unconditioned stimulus (US). The bell is a neutral stimulus until the dog learns to associate the bell with food. Then the bell becomes a conditioned stimulus (CS), which produces the conditioned response of salivation after repeated pairings between the bell and food. Pavlov's work describes how associations between an US and a CS (which some consider as the basic building blocks of learning) come about (Dickinson, 2001).

Conditioning a fruit fly

Also *Drosophila* can be classically conditioned, using corresponding training procedures. Relevant for this thesis are three well-established olfactory associative learning paradigms: Punishment learning, reward learning and pain relief learning. Punishment learning has been investigated since over 30 years, starting with the pioneers on this field, Tully and Quinn (1985): When flies receive paired presentations of an odor (CS) followed by an electric shock (US), they will subsequently avoid the previously punished odor. For reward learning (Tempel et al. 1983), the animals receive a paired presentation of an odor together with a sugar reward, resulting in conditioned approach towards the rewarded odor (Kleber et al. 2016).

Another type of learning, that is called pain relief learning, which also leads to conditioned approach towards a trained odor, was observed in adult flies by Tanimoto et al. in 2004. They found that if the inter stimulus interval (ISI), which is defined by the time between two stimuli (in this case odor and shock), is changed in a manner that the odor is presented after an electric shock, flies will subsequently approach this odor. That is, different from punishment learning where the flies receive paired presentations of an odor before an electric shock, such that they will subsequently avoid that odor (Tully and Quinn 1985), in pain relief learning an odor is presented after the electric shock, at a moment of relief from pain, and hence flies show conditioned approach to this odor (Tanimoto et al. 2004). Altering the ISI can result in additional types of learning. For more details see general discussion (reviewed also in Gerber et al. 2014).

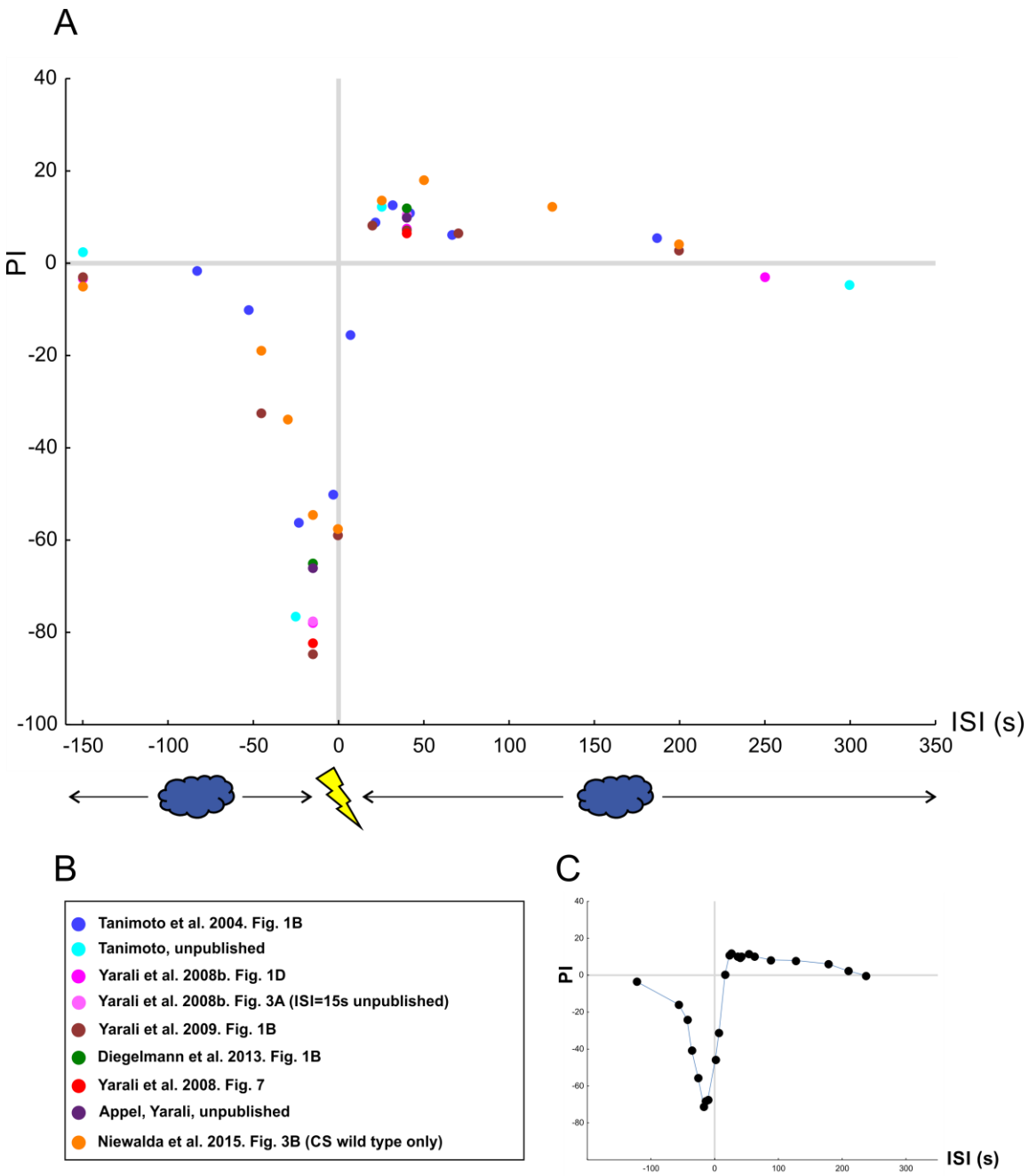


Figure 2. (A) Inter stimulus interval function of learning between odor and electric shock in adult flies. Plotted are the median performance indices (PIs) resulting from various experiments and experimenters (see B) using different inter stimulus intervals (ISI). The time point of shock application is fixed, indicated by the lightning arrow at 0 s, and the ISI varies across groups such that the odor (blue cloud) is presented at various time points before (negative ISI) or after (positive ISI) the shock. If the odor is presented before the electric shock, flies will establish punishment memory, leading to learned avoidance (negative PIs), whereas if the odor is presented after (positive ISIs) the electric shock flies will establish pain-relief memory and show learned approach towards this odor (positive PIs). Both memories are strongest if the odor is either presented shortly before the shock (for punishment memory) or shortly after the electric shock (for pain-relief memory). While the ISI is increasing both memories become weaker until they vanish because no longer associative learning can take place. Interestingly, conditioned approach is much weaker than conditioned avoidance. Please note that for all experiments a two-odor paradigm was used. **(B)** List of the origin of the data that was used to calculate the PIs shown in (A). The original data is displayed in figure S1. **(C)** Semi-schematic representation of the PIs shown in (A). For the calculation of the first PI within this semi-schematic plot the average of all PIs included in the first three ISIs was calculated, based on the calculation from (A). The second PI was calculated accordingly but this time the average of all PIs was calculated included in the second to forth ISI. In this manner all PIs were calculated. The ISIs were calculated accordingly.

Figure 2 shows the relation between the time of the odor presentation and the application of the electric shock (variation of the ISI). The time of the electric shock is fixed (indicated by the yellow lightning arrow at 0 s), and the odor either precedes the shock at different time points (punishment learning) or follows it at different time points (relief learning). Please note that conditioned approach is much weaker than conditioned avoidance in this paradigm. This means, if the odor is presented before the electric shock, it will get a predictor of punishment (or reward in case of reward learning), whereas odor presentation after the electric shock, in the moment of relief from pain (Solomon and Corbit, 1974), predicts a period of safety (Sutton and Barto, 1990). Compared to punishment learning, pain-relief learning in *Drosophila* is much less well understood, but related results are found in bees (Hellstern et al. 1998), rats and interestingly also in humans (Andreatta et al. 2010; Gerber et al. 2014). Taken together, for punishment learning animals learn to associate the odor with something bad (electric shock) and for both reward learning and pain relief learning the animals learn to associate the odor with something good (reward learning: sugar; pain relief learning: relief from pain). Interestingly, the change of sequence of odor and shock presentation or the change of the US (sugar or electric shock) results in distinct types of associative learning: punishment learning or pain relief learning and reward learning or reward-loss learning, respectively. Obviously the timing of CS and US presentation matters and is further discussed in the general discussion. For electric shock learning as well as reward learning there are well established paradigms also for the larval stage of *Drosophila* (Pauls et al 2010 a,b; Scherer et al 2003, Neuser et al 2005).

Chemosensory organs and pathways

Figure 3A gives a brief overview of the chemosensory pathways of larval *Drosophila*. The architecture of the larval olfactory pathway is similar to its adult counterpart in that it consists of the same types of neurons but is much simpler in terms of cell numbers. The larva has only 21 olfactory sensory neurons (OSNs) on each body side that usually express a single type of odorant receptor (OR); the dendrites of the OSNs harbor the ORs and extend into the dome of the dorsal organ. A given OR type, in turn, is expressed in but one OSN and can bind several different ligands (Strutz et al. 2014). Likewise, a given ligand can bind to different ORs. Accordingly, a given odor activates a specific combination of the 21 OSNs (Fig. 3 A,D) (Kreher et al. 2005 and 2008). This combinatorial activation enables the larva to differentiate between many different odors despite a relatively low number of OSNs. Each OSN projects to one glomerulus in the larval antennal lobe (LAL). The LAL consists of about 21 glomeruli receiving input from a given OSN that is specific for its OR. The OSNs also target inhibitory and excitatory local interneurons, which link many or even all glomeruli and thereby are able to shape signaling (e.g. Thum et al. 2007, Bose et al.2015, Rybak et al, 2016). From there the signal is carried forward by 25 projection

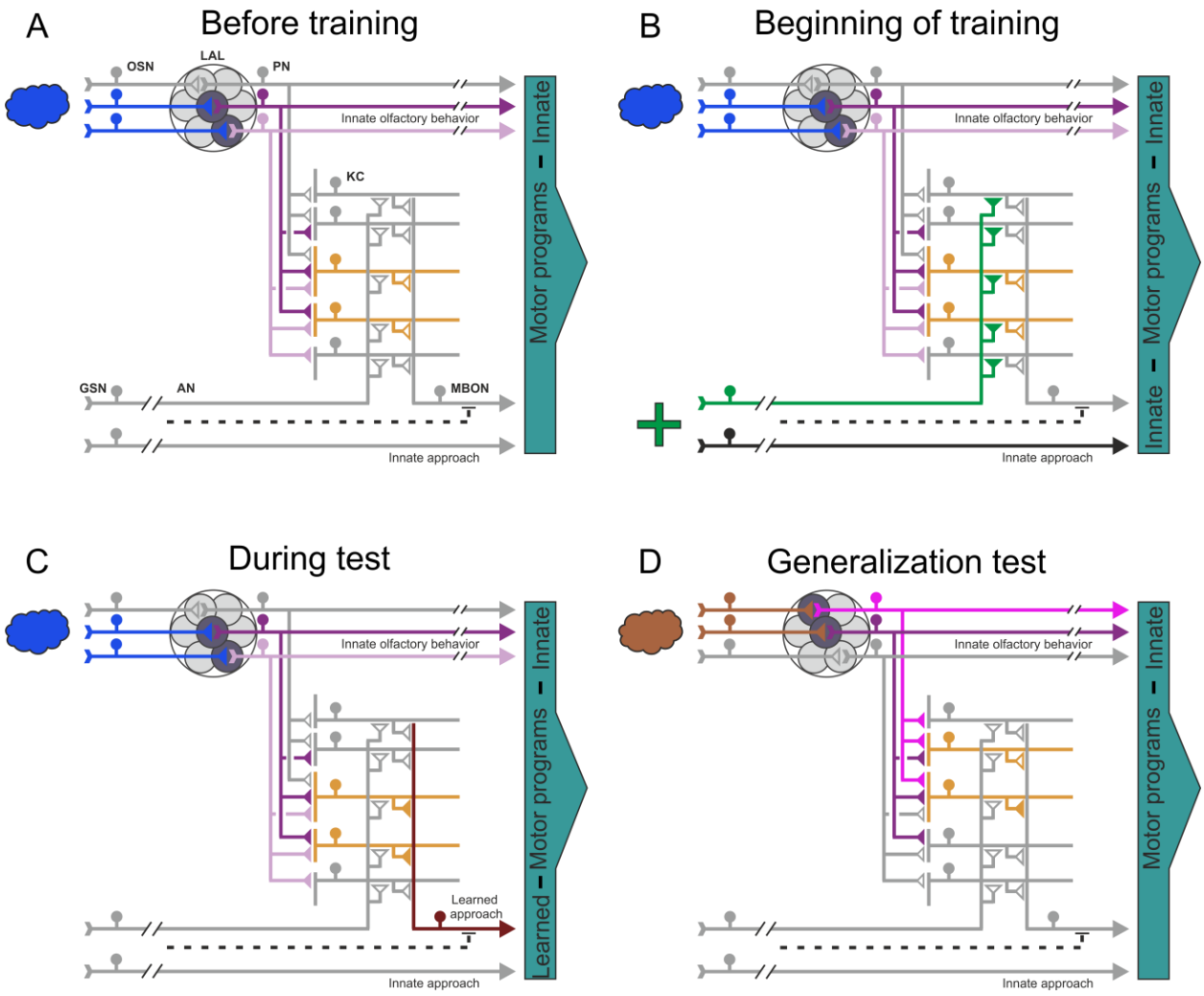


Figure 3. Overview of the chemosensory pathway, relevant for associative odor-reward learning. Please note the not every connection and synapse is shown. For detailed description see text. Colored cells are active, grey cells are inactive. **(A)** If naïve untrained animals receive an odor (blue cloud), this odor activates a specific combination of olfactory sensory neurons (OSNs) that relay the signal to the glomeruli of the larval antennal lobe (LAL). From the glomeruli the signal is carried from the projection neurons (PNs) to the Kenyon cells (KCs) of the mushroom and directly towards the motor program via the lateral horn (not shown). The direct connection to the LH and to the motor program is important for innate olfactory behavior. Each PN targets several KCs and due to their combinatorial activation, activate an odor specific pattern of KCs as they need input from more than one PN to get activated. Here, odor presentation alone is not sufficient to trigger learned approach behavior via the mushroom body output neuron (MBON). Therefore only innate olfactory behavior is expressed in experimentally naïve, untrained flies. **(B)** At beginning of training both an odor and a reward are present. The reward is detected by gustatory sensory neurons (GSNs) that forward the signal towards the motor programs and via aminergic neurons to the KCs. The direct route to the motor program is important for innate gustatory behavior. Now these two signals, the one that is odor-evoked and the reinforcement signal that is caused by the reward, arrive together at the same Kenyon cells of the mushroom body. It is assumed that nearly all KCs receive this reinforcement signal. But only in those Kenyon cells that are additionally receiving the signal from the odor at the same time, synapse strength is modulated and appetitive memory can be build up (Gervasi et al. 2010). The black stippled line indicates that there seems to be a direct connection from gustatory input to the MBON to modulate the behavioral expression of memory traces (see Schleyer et al. 2011, 2015 for details). **(C)** In a subsequent test the odor alone is now able, due to the changed in synaptic strength (filled orange triangles) to trigger the expression of learned approach via the MBON. For a closer look at the molecular mechanism see Fig. 4. **(D)** In a generalization test an odor (brown cloud) different from the trained odor is used. As mentioned above this different odor activates different patterns of OSNs, PNs and KCs compared to the odor that was used during training. Therefore, the activated KCs, if at all, overlap only partially with those KCs that were previously modulated in strength during training. Accordingly the output of these KCs does not result in learned approach.

neurons (PNs) that typically receive input in one glomerulus of the LAL. The PNs form the inner antennocerebral tract (iACT) and project the signal to two higher-order processing centers: the mushroom body (MB) and the lateral horn (LH) (Masse et al. 2009). Please note that this is not the only pathway from the AL to higher brain centres (Wang et al. 2014). The direct connection to the LH is supposed to be important for innate odor response. The input region of the MB, the so-called calyx, comprises about 30-40 relatively prominent, identifiable structures called calyx glomeruli (Marin et al. 2005; Masuda-Nakagawa et al. 2005 and 2009; Ramaekers et al. 2005). PNs innervate mostly a single, exceptionally two calyx glomeruli (Marin et al. 2005; Ramaekers et al. 2005). Many of these connections between the LAL and the KCs via the PNs were shown to be stereotypically in that a specific antennal lobe glomerulus is connected with a specific calyx glomerulus (Ramaekers et al. 2005, Masuda-Nakagawa et al. 2005 and 2009). Most of the about 600 KCs per hemisphere have their input regions in usually six, randomly selected glomeruli while some KCs are innervating a single calyx glomerulus. In other words, also the activation pattern from different sets of KCs still code for odor information. This kind of connectivity, that PNs target multiple KCs, and most KCs receive input from multiple PNs, provides a local divergence–convergence connectivity (Masuda-Nakagawa et al. 2005 and 2009; Murthy et al. 2008). This massively increases the number of possible combinatorial activation patterns and thus more information can be coded like a wide spectrum of different odors. This is especially important because, and as mentioned above the odor quality (what odor) is coded by an odor specific activation pattern of KCs.

As mentioned above, larva and adult *Drosophila* share the general organization of the central olfactory pathway. The olfactory pathway of the adult fly exhibits about 1300 OSNs that express 50 different ORs and connect to about 50 antennal lobe glomeruli (Laissue et al. 1999; Couto et al. 2005). Thus and in contrast to the larva, many OSNs host the same kind of OR, and like in the larval case all converging to a single antennal lobe glomerulus. Thus, the kind of OR an OSN expresses determines its target glomerulus (Davis 2004). About 150 PNs that have their input region in the antennal lobe glomeruli, project the signal to approximately 200 calyx glomeruli, the input region of about 2500 KCs (Gerber et al. 2009). This means, in comparison to adult flies the larval circuit exhibits some specific characteristics. Firstly, every larval ORN and probably most of the larval projection neurons are unique (Ramaekers et al. 2005). Secondly, the larva houses only 21 antennal lobe glomeruli in contrast to 50 antennal lobe glomeruli of the adults, which suggests a reduction in number of primary olfactory dimensions. Thirdly, the larval olfactory pathway lacks convergent and divergent connectivity until the mushroom bodies, since the numbers of ORNs, antennal lobe glomeruli, projection neurons and calyx glomeruli are almost the same (Ramaekers et al. 2005). Fourthly, while all larval ORNs project exclusively in ipsilateral brain regions, most of the adult ORNs are connected bilateral, targeting corresponding glomeruli in both ipsilateral and contralateral lobes (Stocker et al. 1983, 2001). Taken together, compared to the adult fly brain the larval brain exhibits fewer neurons, less OSNs what reduces

the olfactory dimensions and lack of convergent connectivity in the antennal lobe. This likely reduces the capacity for odor discrimination, but it also increases the signal to noise ratio. This makes the larva to a suitable model organism to investigate how olfactory information is processed, learnt and translated into behavior.

The neural pathways and molecular mechanisms of olfactory associative learning, using either reward or punishment, have been intensely studied (Keene and Waddell 2007; Schwaerzel et al. 2007; Gerber et al. 2009; Zars 2010; Davis 2011) including more recently also larval *Drosophila* (Michels et al. 2005 and 2011; Saumweber et al. 2011a and b; Mishra et al. 2010 and 2013, Chen et al 2011, Schleyer et al. 2011 and 2013; Chen and Gerber 2014, and others).

In short, olfactory input is detected by olfactory receptors. The odor information is carried to the antennal lobe and then processed via projection neurons to the lateral horn for innate odor response as well as to the Kenyon cells of the mushroom body (reviewed in Gerber et al 2009; see also Fig. 3 and general discussion). The reinforcement signal (e.g. sugar) is transmitted via the aminergic system onto the same Kenyon cells coincidentally with the odor signal. It is assumed that almost all Kenyon cells receive the reinforcement signal, but only in those that are additionally activated through the odor presentation, a memory trace is formed, because in only these neurons both the odor input and the reinforcement signal coincide. Such a memory trace conceivably alters the synaptic strength of the connection between the activated Kenyon cells and their mushroom body output neurons (Diegelmann et al 2013, see also Aso et al. 2014 and Hige et al 2015). If then, after training, the learnt odor is perceived again, the KC output, due to the modification in synaptic strength during training, now leads to learned behavior. In a generalization test, an odor different from the trained one is used in the test. As mentioned above, this different odor activates different patterns of OSNs, PNs and KCs compared to the odor that was used during training. Therefor the activated KCs, if at all, overlap only partially with those KCs that were previously activated and modified in strength during training. Accordingly an odor different from the trained odor is not able to generate a KC output that leads to learned approach.

The mushroom body plays a central role in olfactory memory formation and retrieval (Larva: Honjo & Furukubo-Tokunaga 2009; Pauls et al. 2010; Michels et al. 2011; adult: Schwaerzel et al. 2003; Gerber et al. 2004; Kahsai and Zars 2011; Xie et al. 2013). It was shown that blocking mushroom body output during test (McGuire et al. 2001; Dubnau et al. 2001; Schwaerzel et al. 2003), and blocking reinforcer input to the mushroom body during training (Schwaerzel et al. 2003) prevents flies from expressing or forming, respectively, any memory. Blocking olfactory input to the Kenyon cells has not been achieved so far because it is a particular technical challenge since the projection neurons, as mentioned above, do not only project to the mushroom body but also to the lateral horn. In the larva, activation of octopaminergic neurons (using TDC-Gal4 as driver) during training leads to substitution of a reward, whereas activating a large subset of dopaminergic neurons (using TH-Gal4 as driver) during

training can substitute for a punishment signal (Schroll et al. 2006). Activating another subset of dopaminergic neurons, namely the PAM neurons in adult flies or the corresponding larval pPAM neurons leads to an increase in learning performance (Adult: Burke et al 2012; larva: Rohwedder et al. 2016). In other words, dopaminergic PAM neurons mediate the positive reinforcement signal. Output from the mushroom bodies ultimately organizes learned behavior. The actual connectivity toward the motor periphery is largely unknown, but currently under investigation (adult flies: Musso et al. 2015, Owald et al. 2015, Yamagata et al. 2015, Aso et al. 2014a, 2014b, bees: Strube-Bloss et al. 2011).

The presynaptic protein Synapsin

In the associative learning and memory processes described above, the protein Synapsin likely plays a major role. Synapsins belong to a family of evolutionarily highly conserved phosphoproteins associated with the cytoplasmic side of synaptic vesicles (Greengard et al. 1993; Hilfiker et al. 1999; Hosaka et al. 1999). In *Drosophila,* Synapsin is encoded by only one gene (syn; CG 3985) that is located on the third chromosome (Klagges et al. 1996; Diegelmann et al. 2013) and is expressed in most or even all neurons in larval and adult *Drosophila*. The Synapsin null mutant (syn^{97}) is characterized by a 1400 bp deletion which removes part of the regulatory region and the first exon. Notably, in learning studies it could be shown that Synapsin is required for proper associative function. Adult flies (odor-punishment learning; Godenschwege et al. 2004; Knapek et al. 2010) as well as larvae (odor-reward learning; Michels et al. 2005) lacking Synapsin show up to 50% reductions in associative memory scores as compared to wild-type animals. Synapsin is also required for pain relief memory (Niewalda et al. 2015). Niewalda et al. showed that flies lacking Synapsin or expressing less Synapsin through RNAi also are partially impaired in punishment memory, whereas pain-relief memory is apparently fully abolished.

For Synapsin function the mushroom body plays an important role, too (Michels et al. 2011). Regarding odor-sugar learning in larvae they demonstrated that Synapsin expression only in the MB is sufficient and most likely necessary for proper associative function. A local rescue by restoring Synapsin only in the MB of Synapsin null mutant larvae indeed led to memory performance on wild-type level. Expressing Synapsin in only a subset of MB neurons of about seven neurons per hemisphere using the mushroom body subset driver D52H-Gal4 could even rescue the impairment in memory performance. Therefore Synapsin expression in the MB, or more precisely in this subset of MB Kenyon cells, is sufficient for proper associative function. In contrast, the authors suppressed Synapsin expression only in the MB (using elav-GAL4 and mb247-$Gal80^{ts}$ in syn^{97} animals) and showed that mushroom body expression of Synapsin is required for proper associative function. These animals still showed impaired memory. However, Synapsin expression was also slightly reduced outside of the MBs and thus there remains uncertainty as to whether Synapsin expression in the MB is necessary for accurate associative

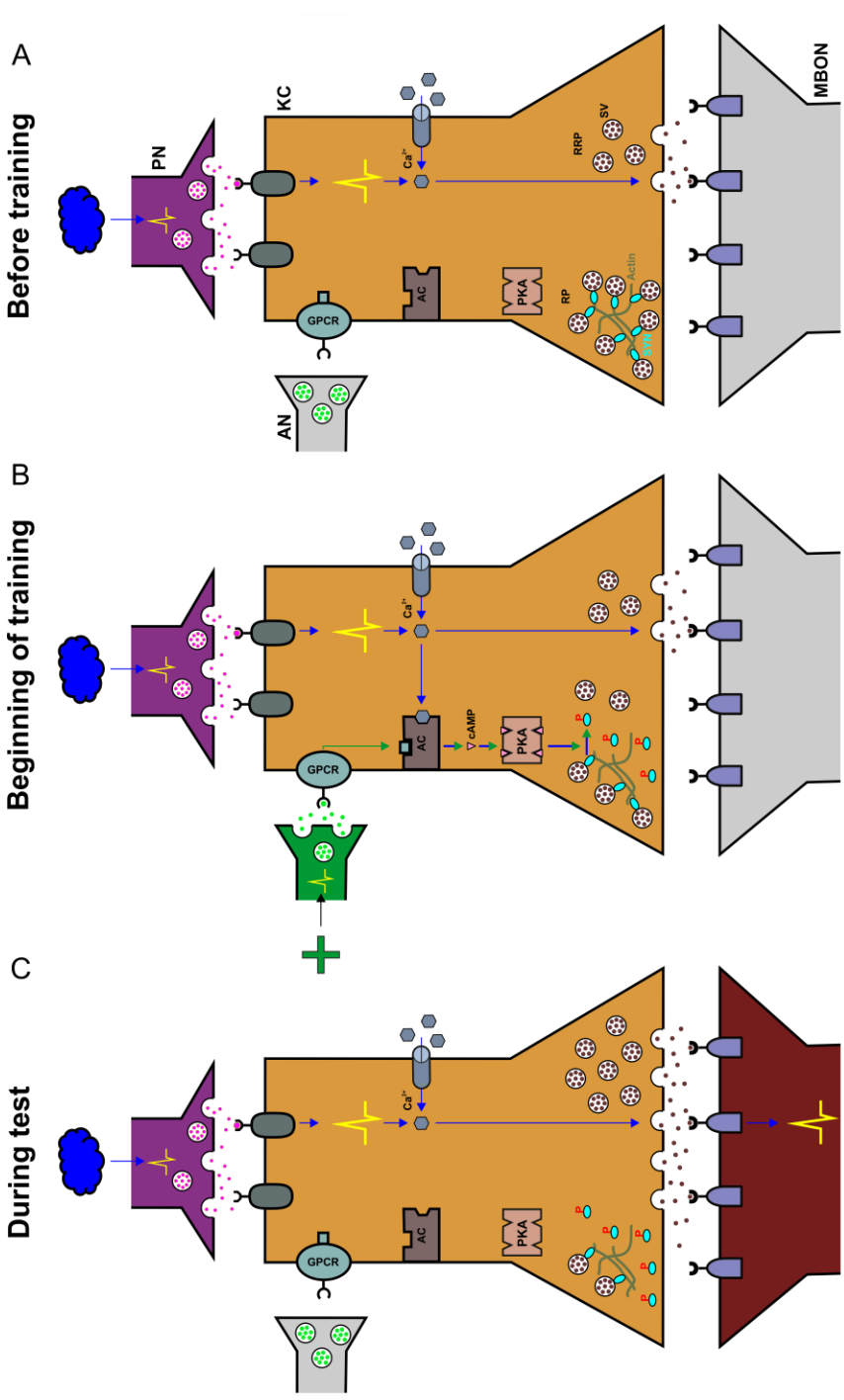


Figure 4. Proposed molecular Synapsin function in associative odor-reward learning. **(A)** In naïve untrained animals a certain odor (blue cloud) leads to activation of a subset of projection neurons (PNs) that lead to activation of a subset of Kenyon cells (KCs) followed by presynaptic calcium influx resulting in a mild neurotransmitter (NT) release from the synaptic vesicles (SVs) of the readily releasable pool (RRP). This weak NT release is not able to activate the mushroom body output neurons (MBONs). Synapsin (teal dots) is tethering SVs to the actin filaments of the cytoskeleton, thereby building up a reserve pool (RP) of vesicles. Please note that no reward is present and that an odor presentation alone is not sufficient to activate the MBONs in naïve animals. The odor evoked signal transduction is indicated by blue arrows throughout. Colored cells are active and grey cells are inactive. **(B)** At beginning of training both the odor and the reward (green plus sign) are present and both the odor signal and the reward signal are relayed onto the same KC at the same time. The odor activates the KCs according to the same activation pattern as described in (A), leading to a weak NT release, not resulting in an activation of the MBON. The reward activates an aminergic reinforcing neuron (AN) followed by an activation of a G-protein coupled receptor (GPCR). This coincidence of activated GPCR (reward-evoked) and calcium influx (odor-evoked) leads to the activation of the type I adenylyl cyclase (AC) and the AC-cAMP-PKA cascade is triggered: The AC produces cAMP that activates the protein kinase A (PKA) leading to phosphorylation (red P) of Synapsin. Please note that Synapsin is not the only substrate for PKA and that Synapsin has recognition motifs for several kinases and therefore may serve as substrate for different kinases. Phosphorylated Synapsin loses its affinity to SVs and the actin filaments, thereby the RRP is enlarged and more SVs are free for exocytosis. **(C)** During test only the odor is present but at this time point, due to the increased pool of readily releasable vesicles the odor evokes a strong release of NT resulting in an activation of the MBONs. In this sense Synapsin regulates the release of NT and thereby influences the strength of the presynapse in a phosphorylation dependent way. Please note that in this scenario the neurotransmitter of the KC is an excitatory NT and therefore activates the MBONs during test. In contrast, it has been recently reported that the response of certain MBONs to a conditioned odor was decreased and depressed (Owald et al. 2015; Hige et al. 2015; Cohn et al. 2016). This observation is discussed in the general discussion part. As mentioned above Synapsin harbours consensus motifs for several kinases (Nuwal et al. 2011; Sadanandappa et al. 2013; Niewalda et al. 2015). Therefore, the net effect of odor-reward learning on NT release is difficult to predict. In any event, the modulated output from the mushroom body neurons is thought to code the learned valence of the odor and thus is the basis for learned olfactory behavior (Séjourné et al. 2011; Plaçais et al. 2013; Aso et al. 2014a,b; Menzel, 2014). In this sense, Synapsin operates during learning to establish a memory trace, i.e. an altered functional state of an odor-specific set of mushroom body output synapses.

plasticity. Additionally the authors could show that the impairment in memory function could not been rescued by expressing Synapsin only in the projection neurons (using GH146-GAL4 or NP225-GAL4)

On the molecular level, the working hypothesis of Synapsin function (Fig. 4) proposes that Synapsin can bind to both synaptic vesicles and to the actin filaments of the cytoskeleton, thereby building up a reserve pool of vesicles (Greengard et al. 1993; Hosaka et al. 1999; Südhof, 2004; Hilfiker et al. 2005). The type I adenylate cyclase (coding gene: *rut*, CG9533) detects a coincidence of odor-induced activity in mushroom body neurons on the one hand, and of an internal aminergic reinforcement signal on the other hand, such that the cAMP-PKA cascade is activated in an odor-specific subset of mushroom body neurons (Tomchik and Davis, 2009; Gervasi et al. 2010). Arguably, Synapsin seems to be one of the target proteins of PKA (Fiumara et al. 2004; Michels et al. 2011). If Synapsin gets phosphorylated it reduces its affinity to synaptic vesicles. Due to this phosphorylation the synaptic vesicles are now detached from the cytoskeleton and therefore are available for subsequent exocytosis. Thus, when the trained odor is encountered thereafter, more synaptic vesicles will be available for release (Shupliakov et al. 2011, Michels et al. 2011). Accordingly the phosphorylation of Synapsin seems to be important and is thought to function during learning in a way that determines memory strength. Additionally, transgenically expressed Synapsin with dysfunctional PKA-consensus sites cannot rescue the defect of the syn^{97} null mutant larvae in associative function (for discussion see Michels et al. 2011).

Summary chapter I

This thesis is divided into two chapters. **Chapter I** is dealing with role of Synapsin in odor-sugar associative reward learning in larval *Drosophila*. It specifically focuses on the question in which way the Synapsin null mutant (syn^{97}) phenotype of *Drosophila* larvae depends on certain parameters of an odor-sugar associative learning experiment. To systematically investigate this topic, parameters were varied which are known to affect memory scores in wild-type, namely odor concentration (Mishra et al. 2011) as well as sugar concentration (Schipanski et al. 2008) and the time interval between training and test (adult: Knapek et al. 2010; larva: Neuser et al. 2005). The results suggest that odor-sugar memory in syn^{97} mutant larvae is statistically significant different from wild-type, but mutant larvae hardly benefit from increasing odor concentration or sugar concentration. Specifically, in the syn^{97} mutant associative memory remained at low levels across the range of tested odor and sugar concentrations. In contrast, the wild-type memory scores increased for higher concentrations of odor and sugar. Furthermore, Synapsin is selectively required for short-term memory (Knapek et al 2010). Thus, in the absence of Synapsin *Drosophila* larvae in principal can learn and remember, yet in order to benefit from an increased salience of events for establishing stronger memories Synapsin is required: without Synapsin the upper limit in mnemonic capacity is lower. Additional mass spectrometry analysis shows an upregulated phosphorylation of

Synapsin in the larval nervous system upon a lack of the protein Sap47. This suggests a functional interdependence of Synapsin and Sap47.

Summary chapter II

Chapter II addresses the role of Synapsin in punishment- and relief learning in adult flies. It is shown that in animals lacking Synapsin both punishment memory and pain-relief memory are reduced. In contrast syn^{97} mutants are not impaired in task relevant sensory or motor abilities. The observations exclude that the impairment in memory is due to non-associative side effects like handling, adaptation, habituation, or sensitization, and therefore reflect a true lessening of associative memory strength. A reduction in memory strength was also observed after Synapsin RNAi knockdown whereas expressing Synapsin in syn^{97} animals brain wide or only in the mushroom bodies could fully restore associative memory performance. These observations suggest the Synapsin is required for establishing both punishment memory and pain-relief memory and that these two forms of memory in this sense share genetic and molecular determinants.

I Chapter I

Synapsin function in larval odor-sugar memory*

*Based on Kleber J, Chen YC, Michels B, Saumweber T, Schleyer M, Kähne T, Buchner E, Gerber B. 2015. Synapsin is required to 'boost' memory strength for highly salient events

Introduction

One of the brain's more fascinating features is that it allows the organism to learn and to remember. Learning and memory fine-tune the way an animal can act in its environment, e.g. in the search for food. Using odor-sugar reward associative learning in larval *Drosophila* as a study case, we investigate the role of the Synapsin protein in learning and memory (Scherer et al. 2003; Neuser et al. 2005; Saumweber et al. 2011; for reviews see Gerber et al. 2009; Diegelmann et al. 2013).

Synapsins constitute a family of evolutionarily conserved phosphoproteins. They are associated with the cytoplasmic side of synaptic vesicles and tether vesicles to the cytoskeleton, thus forming a reserve pool (Greengard et al. 1993; Hosaka et al. 1999; Südhof, 2004; Hilfiker et al. 2005). In *Drosophila*, Synapsin is encoded by only one gene and is expressed in most if not all neurons of both the larval and adult nervous system (coding gene: *syn*, CG 3985: Klagges et al. 1996; Michels et al. 2005). Both adult and larval *Drosophila* lacking Synapsin show associative memory scores that are reduced by about half as compared to wild-type animals, as do animals upon an RNAi-mediated knock-down of Synapsin (adult odor-punishment memory: Godenschwege et al. 2004; Knapek et al. 2010; Walkinshaw et al. 2015; Niewalda et al. 2015; larval odor-reward memory: Michels et al. 2005; Michels et al. 2011). Corresponding phenotypes in learning and memory tasks were reported throughout the animal kingdom, including man (Silva et al. 1996; Garcia et al. 2004; Südhof, 2004; Gitler et al. 2008; Greco et al. 2013).

In both larval and adult *Drosophila*, animals lacking Synapsin exhibit normal task-relevant sensory-motor performance as indicated by normal naïve responsiveness to odors, sugar-reward, and electric shock punishment as well as normal odor detection after training-like exposure to these stimuli (Michels et al. 2005; Knapek et al. 2010; Niewalda et al. 2015). The memory impairment of Synapsin null mutant larvae could be rescued by acute transgenic Synapsin expression locally in the mushroom bodies but not by expression in the projection neurons that convey olfactory input to them (Michels et al. 2011) (acute mushroom body expression rescues memory scores for the association of odors and electric shock punishment in adult *Drosophila*, too: Niewalda et al. 2015). Thus, a Synapsin-dependent odor-reward memory trace in larval *Drosophila* arguably is local to the mushroom bodies, a third-order 'cortical' brain region of the insects (Tomer et al. 2010).

Notably, phosphorylation seems to be important in the mode of operation of Synapsin (Angers et al. 2002; Fiumara et al. 2004; Giachello et al. 2010; Michels et al. 2011; Sadanandappa et al. 2013). The working hypothesis of Synapsin function is that the type I adenylate cyclase (coding gene: *rut*, CG9533) detects a coincidence of odor-induced activity in mushroom body neurons on the one hand, and of an internal aminergic reinforcement signal on the other hand, such that the cAMP-PKA cascade is activated in an odor-specific subset of mushroom body neurons (Tomchik and Davis, 2009; Gervasi et al. 2010). Arguably, Synapsin is one of the target proteins of PKA (Fiumara et al. 2004; Michels et al. 2011) such

that upon phosphorylation of Synapsin its affinity to the cytoskeleton is reduced and reserve-pool vesicles can be recruited. Thus, when the trained odor is encountered thereafter, more synaptic vesicles will be available for release (Shupliakov et al. 2011). It should be noted that Synapsin harbours consensus motifs for other kinases as well (Nuwal et al. 2011; Sadanandappa et al. 2013; Niewalda et al. 2015). Therefore, the net effect of odor-reward learning on the balance between reserve-pool and releasable vesicles and on synaptic transmission is difficult to predict. In any event, the modulated output from the mushroom body neurons is thought to code the learned valence of the odor and thus is the basis for learned olfactory behavior (Séjourné et al. 2011; Plaçais et al. 2013; Aso et al. 2014a,b; Menzel, 2014). In this sense, Synapsin operates during learning to establish a memory trace, i.e. an altered functional state of an odor-specific set of mushroom body output synapses.

Based on electrophysiology as well as behavioral analyses it has been suggested that the regulation of synaptic transmission via Synapsin may be particularly important to maintain high levels of transmission upon continuous, heavy demand (Godenschwege et al. 2004; Bykhovskaia 2011; Vasin et al. 2014). Regarding our odor-reward learning paradigm, we therefore predicted that Synapsin is particularly critical for forming memories of highly salient events. To put this to test, we parametrically vary odor as well as sugar salience (both affect memory scores in wild-type larvae: Schipanski et al. 2008; Mishra et al. 2013) and ask whether Synapsin is selectively involved to form stronger memories for high concentrations of odor and/or reward.

In odor-punishment memory of adult *Drosophila*, Synapsin is specifically required for short-term but not longer term memory (Knapek et al. 2010). Considering the above-mentioned working hypothesis of Synapsin function this is conceivably because the changes in the phosphorylation pattern of Synapsin are transient. Regarding the present larval odor-sugar learning task, we therefore decided to test memory at various retention intervals to see whether Synapsin is selectively necessary for short-term and/or longer term memory.

As mentioned above memory scores in Synapsin null mutants typically are not abolished but reduced to about half, a finding that we confirm in the present study. The same partial memory defect we have observed in null-mutants of another presynaptic protein, namely Sap47 (Saumweber et al. 2011). The **s**ynapse **a**ssociated **p**rotein of **47** kDa (coding gene: *sap47*, CG 8884) has been identified by a monoclonal antibody from the Wuerzburg hybridoma library (Reichmuth et al. 1995; Funk et al. 2004; Hofbauer et al. 2009). Within this study, we ask whether Synapsin and Sap47 work in different, parallel pathways, or in series. Towards this end, we test for additive defects in memory of Synapsin/Sap47 double mutants. The rational is that no additivity should be observed if Synapsin and Sap47 operate in series, i.e. within the same process.

Lastly, for adult *Drosophila* several phosphorylation sites of Synapsin have been identified by mass spectrometry (Nuwal et al. 2011; Niewalda et al. 2015). We therefore decided to determine the

phosphorylation status of Synapsin in larvae as well. In addition we ask for differences in the pattern of Synapsin phosphorylation between wild-type and Sap47 null mutant larvae, as such differences would be indicative of a functional interdependence of Synapsin and Sap47.

Results

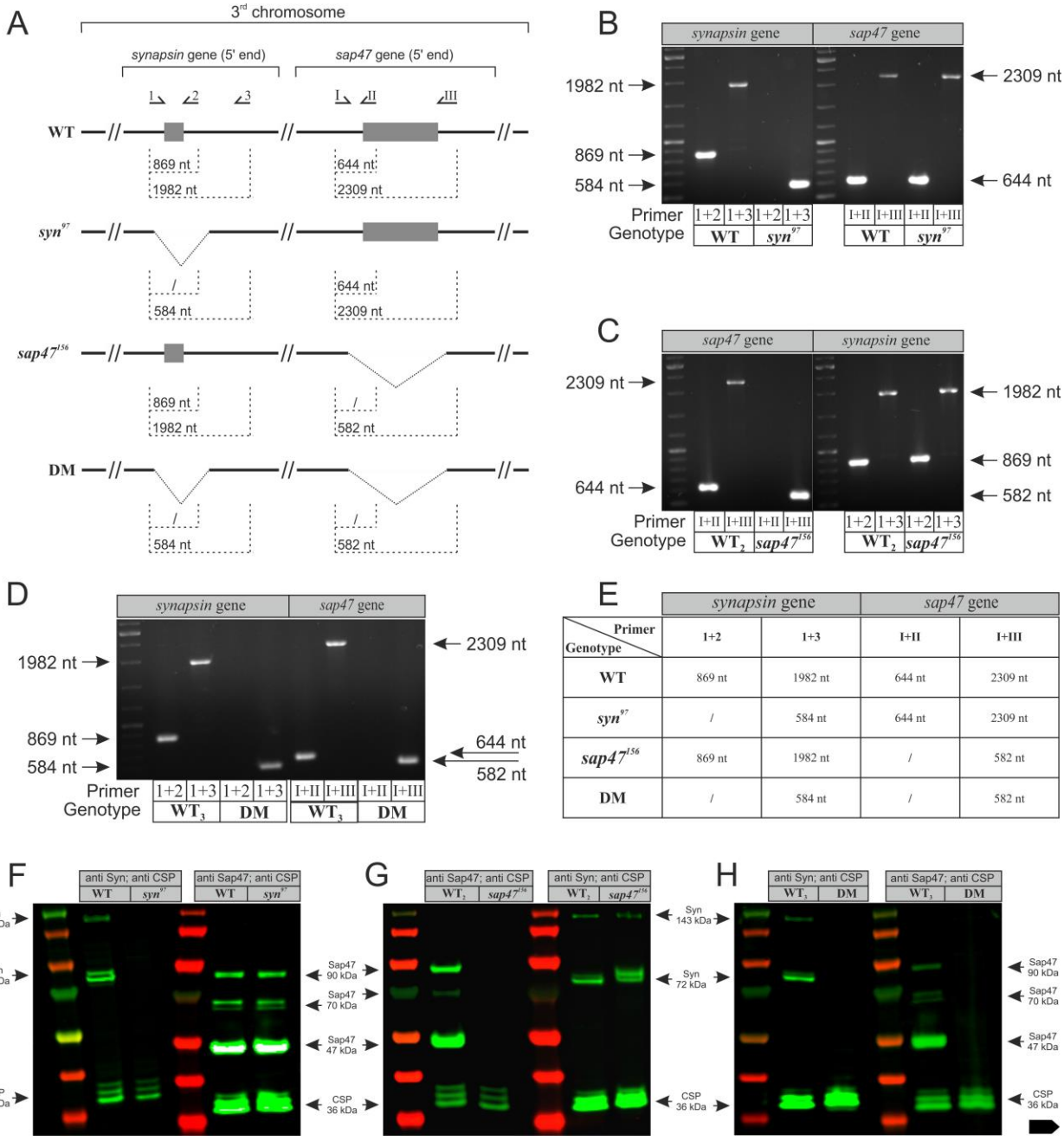
Genetic and molecular status

Using PCR, Western blotting and whole mount brain preparations we probed all strains used in this study for the status of the *synapsin* and *sap47* genes and the expression of their Synapsin and Sap47 protein products in the larva (Fig. 5).

The syn^{97} mutant strain carries the reported 1.4 kb deletion in the *synapsin* gene, removing part of the promotor region, exon 1 and a small part of the first intron; consequentially, it lacks all Synapsin protein (Godenschwege et al. 2004; Michels et al. 2005). In the wild-type (WT) strain we confirm expected Synapsin protein isoforms between 70 and 80 kDa and a weaker and variable band at 143 kDa (Klagges et al. 1996). The *sap47* gene and the Sap47 protein isoforms, as expected, are intact in the syn^{97} mutant strain.

The $sap47^{156}$ mutant strain carries the reported 1.7 kb deletion, which removes part of the promoter region, the first exon, and a small part of the first intron; it therefore is not expressing any Sap47 protein (Funk et al. 2004; Saumweber et al. 2011). In the WT_2 strain we confirm the expected major Sap47 band at about 47 kDa (this band can sometimes be discerned as a double band, Funk et al. 2004) a group of weaker bands at about 70 kDa, as well as a higher band at about 90 kDa. Expectedly, the *synapsin* gene and the Synapsin protein are intact in the $sap47^{156}$ mutant strain. We note that in the $sap47^{156}$ mutant strain an additional band for Synapsin can be discerned at about 72 kDa (compare the two rightmost lanes of Fig. 5G).

The $syn^{97}/sap47^{156}$ double mutant strain carries the reported deletions in the *synapsin* as well as in the *sap47* gene (see above) and thus it is expressing neither the Synapsin nor the Sap47 protein. In the WT_3 strain we verified genomic status and protein expression as described above. Whole mount brain preparations confirm these conclusions (Fig. 5I-K).


A
3rd chromosome
synapsin gene (5' end)
sap47 gene (5' end)
1
2
3
I
II
III
WT
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1982 nt
644 nt
2309 nt
syn97
/
584 nt
644 nt
2309 nt
sap47156
869 nt
1982 nt
/
582 nt
DM
/
584 nt
/
582 nt
B
synapsin gene
sap47 gene
1982 nt
869 nt
584 nt
2309 nt
644 nt
Primer
Genotype
1+2 1+3 1+2 1+3
WT syn97
I+II I+III I+II I+III
WT syn97
C
sap47 gene
synapsin gene
2309 nt
644 nt
1982 nt
869 nt
582 nt
Primer
Genotype
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1+2 1+3 1+2 1+3
WT2 sap47156
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synapsin gene
sap47 gene
1982 nt
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584 nt
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644 nt
582 nt
Primer
Genotype
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WT3 DM WT3 DM
E
synapsin gene
sap47 gene
Primer / Genotype
1+2
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WT
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1982 nt
644 nt
2309 nt
syn97
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584 nt
644 nt
2309 nt
sap47156
869 nt
1982 nt
/
582 nt
DM
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584 nt
/
582 nt
F
anti Syn; anti CSP
WT syn97
anti Sap47; anti CSP
WT syn97
Syn 143 kDa
Syn 72 kDa
CSP 36 kDa
Sap47 90 kDa
Sap47 70 kDa
Sap47 47 kDa
CSP 36 kDa
G
anti Sap47; anti CSP
WT2 sap47156
anti Syn; anti CSP
WT2 sap47156
Syn 143 kDa
Syn 72 kDa
CSP 36 kDa
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Sap47 47 kDa
CSP 36 kDa

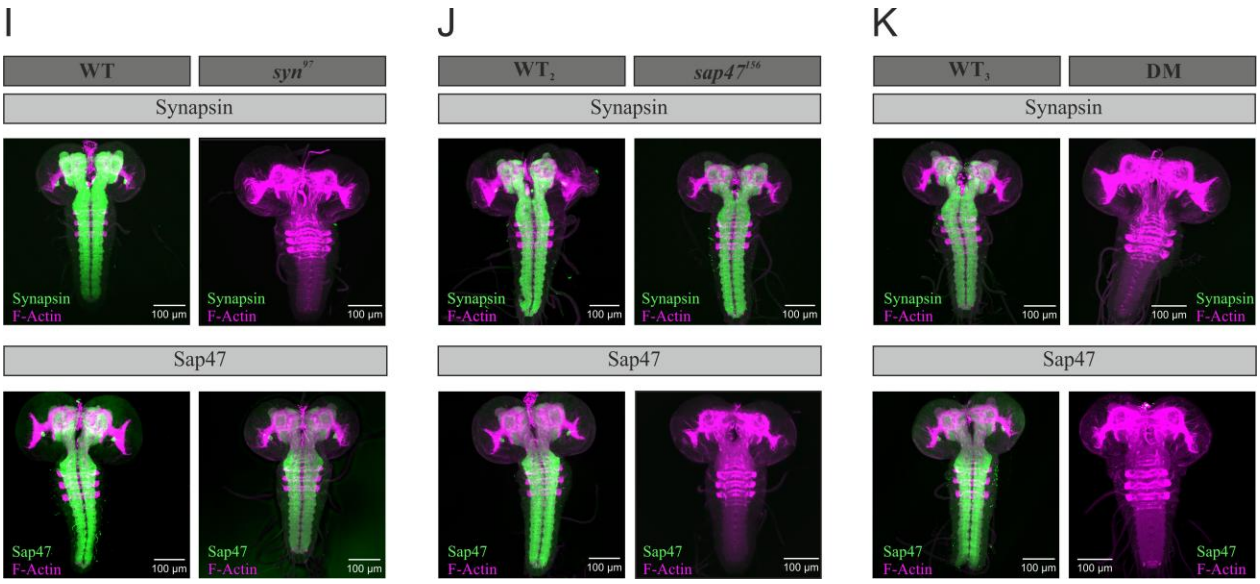

Figure 5. Validation of genetic and molecular status. **(A)** Overview of the primer binding sites and the expected PCR products with regard to the *synapsin* and the *sap47* gene. The primer binding sites were upstream (primer 1 for *syn* and primer I for *sap47*), within (primer 2 for *syn* and II for *sap47*) or downstream (primer 3 for *syn* and III for *sap47*) of the respective deletion. **(B-E)** The syn^{97}, $sap47^{156}$, and the double mutant strains carry the reported deletions. Results of the PCR show the expected products for all genotypes used in this study. **(F-H)** Western blot of larval brains. (F) In the wild-type WT strain the anti-Synapsin antibody SYNORF1 detects expected Synapsin bands, namely a double band at 72 kDa and a weaker band at 143 kDa, whereas the syn^{97} mutant is lacking all Synapsin protein (Godenschwege et al. 2004; Michels et al. 2005). The anti-Sap47 antibody nc46 labels expected Sap47 bands at 47 kDa, 70 kDa and 90 kDa in both the wild-type WT strain and the syn^{97} mutant, showing that the Sap47 protein is intact. (G) The wild-type WT_2 strain shows expected Sap47 bands, while in the $sap47^{156}$ mutant strain no Sap47 protein is expressed. The Synapsin protein is present in both wild-type WT_2 strain and the $sap47^{156}$ mutant. We note an additional anti-Synapsin band at about 72 kDa in the $sap47^{156}$ mutant. (H) The wild-type WT_3 strain shows expected Synapsin and Sap47 bands, while the $syn^{97}/sap47^{156}$double mutant is lacking both the Synapsin and the Sap47 proteins. In all blots, the first and fourth lane from the left shows the marker ladder. As loading control we used CSP as labeled by the ab49 antibody showing bands at 36 kDa for all blots (Zinsmaier et al. 1990, 1994). **(I-K)** Whole mounts of larval brains and ventral nerve cord. (I) The left two tiles show whole mount preparations from wild-type WT larvae, stained with anti F-actin for orientation plus anti-Synapsin (upper left tile) or plus anti-Sap47 (lower left tile) (magenta: anti F-actin, green: anti Synapsin or anti Sap47, respectively; the individual channels are shown in Fig. S11). Note that both the Synapsin and the Sap47 protein, if expressed, are expressed throughout the larval nervous system. The right panel of tiles shows the same as the left panel, but for the syn^{97} mutant, which lacks the Synapsin protein but expresses Sap47. (J) Same as in (I), showing that the wild-type WT_2 strain expresses both Synapsin and Sap47, while the $sap47^{156}$ mutant expresses Synapsin but lacks the Sap47 protein. (K) Same as in (I, J), showing that the wild-type WT_3 strain expresses both Synapsin and Sap47, while the double mutant (DM) lacks both these proteins. All antibodies used are the same as in F-G. Scale bar: 100 µm.

Odor-sugar memory in syn^{97} mutants is impaired only for higher odor concentrations

Using an established odor-sugar associative learning paradigm in wild-type WT larvae (Fig. 6) (Scherer et al. 2003; Neuser et al. 2005; Saumweber et al. 2011), an initial attempt to reproduce the reported syn^{97} mutant defect in odor-sugar memory failed (Fig. S2A, B). Comparing our procedures to the published ones, however, revealed that we had used a substantially lower concentration of *n*-amylacetate (AM) (a 1:1600 dilution rather than the 1:50 dilution of AM used in both Michels et al. 2005 and Michels et al. 2011). Subsequently using the higher concentration of AM (1:50), the published defect of the syn^{97} mutant did reproduce (Fig. S2C, D). This prompted us to investigate systematically whether the syn^{97} mutant phenotype depends on odor concentration.

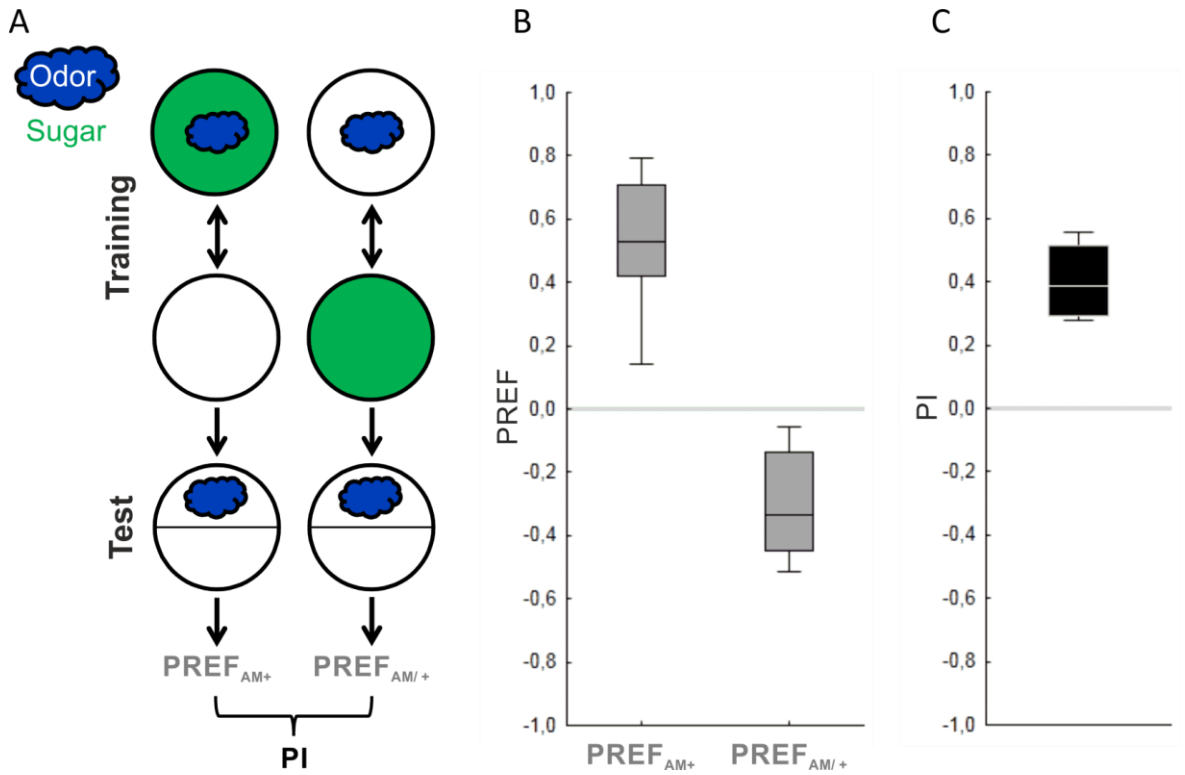

Figure 6. The associative learning paradigm. **(A)** Sketch of the learning paradigm for larval associative reward learning in its one-odor version, **(B)** the resulting odor preferences, and **(C)** associative performance indices of wild-type WT larvae. Using a Petri-dish assay plate (circles), groups of about 30 larvae were trained with either of two reciprocal training regimen, namely either with a paired or an unpaired protocol. For paired training the odor, e.g. amyl acetate (AM) (blue cloud) is presented together with the sugar reward (green fill of circle). In the subsequent test, odor preference is calculated as the number of larvae on the odor side minus the number of larvae on the other side divided by the total number of larvae ($PREF_{AM+}$). A second group of 30 larvae is trained reciprocally, that is by presenting odor and reward separately and the preference score is determined as said ($PREF_{AM/+}$). The associative performance indices (PIs) are calculated as the difference of $PREF_{AM+}$ and $PREF_{AM/+}$, divided by 2 and thus are a measure of associative memory within the boundaries of -1 to 1. Positive values indicate appetitive associative memory, zero indicates no learning effect, and negative values imply aversive associative memory. 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.

Using six experimental groups handled in parallel, we employed three different odor concentrations, in either the wild-type WT or *syn*^{97} mutant larvae (1:2000, 1:200, 1:20 dilutions of AM). The defect in odor-sugar memory of the *syn*^{97} mutant indeed was observed for the highest but not for the two lower concentrations of AM (Fig. 7A; 1:2000: $P > 0.05/3$; 1:200: $P > 0.05/3$; 1:20: $P < 0.05/3$; U= 207, 306, 213; N= 24, 24, 27, 27, 27, 27). Specifically, in the *syn*^{97} mutant associative performance indices remained at a statistically uniform low level across the range of tested concentrations ($P > 0.05/2$; H= 7.22; df= 2; sample sizes as above). In contrast, the scores of wild-type WT larvae were higher for higher concentrations of AM ($P < 0.05/2$; H= 14.16; df= 2; sample sizes as above). Strikingly, the same pattern of results was found for another odor, OCT (Fig. 7B). It thus appears that the *syn*^{97} mutant, different from the wild-type WT (Fig. 7A, B) (Mishra et al. 2013), memory strength cannot be properly adjusted to be higher for higher odor concentrations. This made us wonder whether a similar effect would be seen if stronger

memories are established on the basis of a stronger reward (Schipanski et al. 2008). In other words, is Synapsin required when a particularly strong memory needs to be established for particularly salient to-be-associated cues?

Odor-sugar memory in syn^{97} mutants is impaired only for higher sugar concentrations

We used three different concentrations of the fructose reward (FRU; 0.02 mol/l, 0.2 mol/l, 2 mol/l) (and AM as odor at the 1:20 dilution which is permissive for detecting the defect of the syn^{97} mutant). It turns out that only at the highest FRU concentration a syn^{97} mutant phenotype was detectable, while for the other concentrations memory scores of the wild-type WT and the mutant were at the approximately same level (Fig. 7C; 0.02 mol/l: $P > 0.05/3$; 0.2 mol/l: $P > 0.05/3$; 2 mol/l: $P < 0.05/3$; U= 65, 81, 26; N= 12, 12, 13, 13, 15, 15). Across sugar concentrations we observed statistically uniform scores for the syn^{97} mutant ($P > 0.05/2$; H= 3.98; df= 2; sample size as above), while associative performance indices of wild-type WT were higher for higher sugar concentrations ($P < 0.05/2$; H= 25.40; df= 2; sample size as above). Thus, the wild-type WT but not the syn^{97} mutant can adjust memory strength to be higher when higher sugar concentrations were used during training.

Taken together, in the absence of Synapsin *Drosophila* larvae can form odor-sugar memories, yet Synapsin is required in order to adjust memory strength to a higher salience of odors or of the reward for establishing stronger memories.

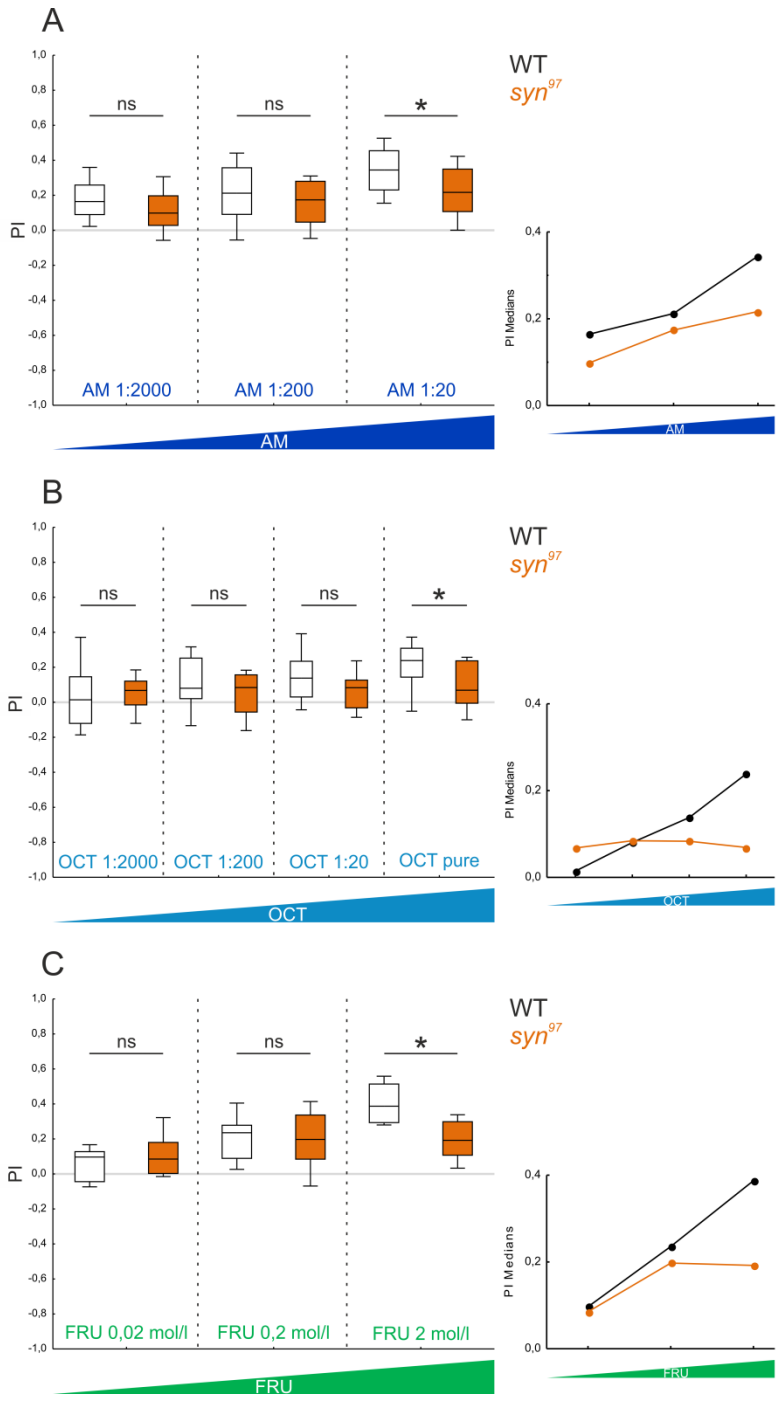

Figure 7. Odor-sugar memory in *syn*^{97} mutants is impaired selectively for high odor or sugar concentrations. **(A)** As higher concentrations of the odor AM were used (dark blue), defects in odor-sugar memory of the *syn*^{97} mutant strain become apparent (the inset shows the median PIs plotted across AM concentrations). White fill of the box plots is used for the wild-type WT strain, orange fill for the *syn*^{97} mutant strain. All displayed data were gathered in parallel. The underlying PREF scores are documented in Figure S3. ns indicates $P>$ 0.05/3, and * indicates $P<$ 0.05/3 in MWU tests. Other details as in Figure 6. **(B)** Same as in (A), for OCT as odor. The underlying PREF scores are documented in Figure S4. ns indicates $P>$ 0.05/4, and * indicates $P<$ 0.05/4 in MWU tests (from left to right: U= 312, 293, 251, 277.5; N= 27, 27, 28, 28, 27, 27, 32, 32). Comparison within a given strain and across concentrations yields $P<$ 0.05/2 at H= 16.16 for the wild-type WT strain and $P>$ 0.05/2 at H= 1.10 for the *syn*^{97} mutant strain in KW tests (df= 3 in both cases). Other details as in Figure 6. **(C)** To examine whether the odor-sugar memory scores of the *syn*^{97} mutant are also dependent of the sugar concentration, three differed fructose (FRU) concentrations were used. Only the highest fructose concentration (2 mol/l) revealed a *syn*^{97} mutant phenotype while for the other concentrations the memory scores of the wild-type WT and the mutant were at an approximately same level (the inset shows the median PIs plotted across fructose concentrations). All displayed data were gathered in parallel. The underlying PREF scores are documented in Figure S5. ns indicates $P>$ 0.05/3, and * indicates $P<$ 0.05/3 in MWU tests. Other details as in Figure 6. Regarding the wild-type WT, these results are qualitatively in line with Mishra et al. (2013) concerning odor concentration) as well as Neuser et al (2004) and Schipanski et al. (2008) concerning sugar concentration, despite some variations in wild-type genotype and paradigm.

syn^{97} *mutants are selectively impaired in short-term memory*

Memory typically is strong immediately after an event, and degrades over time. Is Synapsin required for the early 'extra' memory component that supports high levels of learned behavior shortly after training? We tested separate groups of wild-type WT and *syn*^{97} mutant larvae at either of six different time points after training: either immediately after training (0 min) or after retention intervals ranging from 5 min to 80 min (Fig. 8A). Stimuli were chosen to be conducive for detecting a phenotype (AM diluted 1:20; FRU

2 mol/l). In order to create a situation during the retention interval that was different from both the training and the test situation, the larvae were placed onto a plain plastic dish into a drop of water for the indicated time intervals.

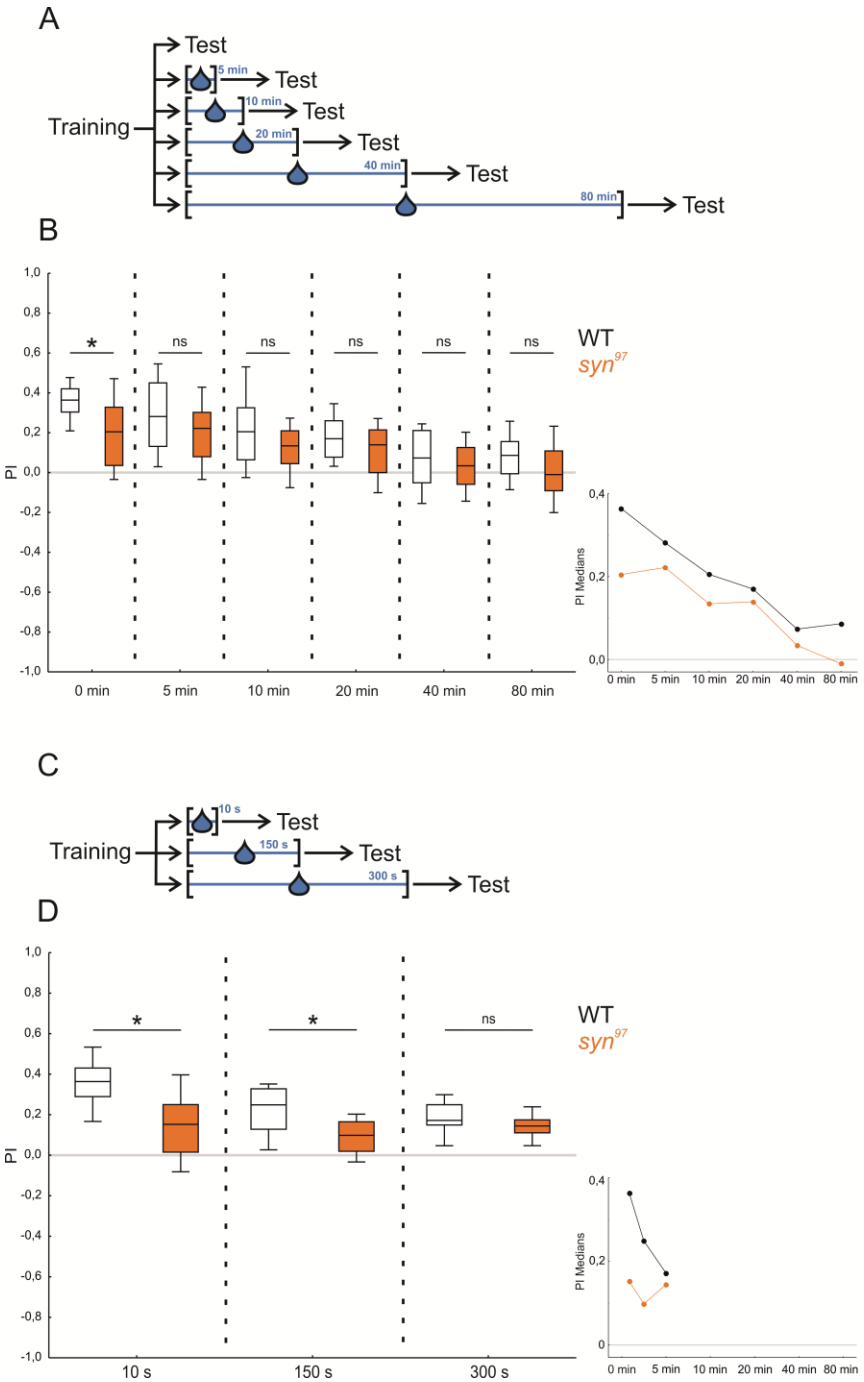

Figure 8. *syn*^{97} mutants are selectively impaired in short-term memory. To investigate memory over time, the *syn*^{97} mutant and the wild-type WT were tested at different time points after training. **(A)** The wild-type WT strain and the *syn*^{97} mutant strain were tested at either of six different time points after training. During the time interval between training and test the larvae were placed into a drop of water in all cases, except in the case when the larvae were tested immediately after training (0 min). **(B)** Only immediately after training (test at 0 min) lower associative memory scores for the *syn*^{97} mutant than the wild-type WT were found, while for all later testing time points no difference in memory scores was observed (inset, showing the median PIs plotted across time intervals). All displayed data were gathered in parallel. The underlying PREF scores are documented in Figure S6A. * indicates $P< 0.05/6$, and ns indicates $P> 0.05/6$ in MWU tests. Other details as in Figure 6. **(C)** The experiment was repeated such that all experimental groups were placed into a water droplet, and only the duration of the retention period was varied. **(D)** Both at 10 s and at 150 s after training the *syn*^{97} mutant larvae showed an impairment in memory that was gone after 300 s/ 5 min (inset, showing the median PIs plotted across time intervals). Therefore the decrease in memory early after training is related to a time-dependent process. All displayed data were gathered in parallel. The underlying PREF scores are documented in Figure S6B. * indicates $P< 0.05/3$, and ns indicates $P> 0.05/3$ in MWU tests. Other details as in Figure 6.

We found that the *syn*^{97} mutant showed a defect in memory only immediately after training (0 min) but not for any of the later time points (Fig. 8B; 0 min: $P< 0.05/6$; 5 min, 10 min, 20 min, 40 min and 80 min: $P> 0.05/6$; U= 232.5, 301, 283.5, 242.5, 275, 232.5; N= 31, 31, 28, 28, 28, 28, 25, 25, 26, 26, 26,

26). For both genotypes we observed a decay of associative performance indices over time ($P< 0.05/2$ in both cases; H= 53.71, 30.54 for wild-type WT and *syn*^{97}; df= 5 in both cases; sample sizes as above).

Given the experimental design (Fig. 8A), it remained unclear whether the requirement of Synapsin reflects a merely time-dependent process, and/or whether the placement into the water droplet is an amnesic treatment, such that Synapsin-dependent memory is erased in the wild-type WT strain (Knapek et al. 2010). We therefore repeated the experiment such that all experimental groups were placed into a water droplet and only the duration of the retention period was varied (either 10 s, 150 s, or 300 s: Fig. 8C). We found that the *syn*^{97} mutant showed an impairment in memory after both 10 s and 150 s whereas, in confirmation of the above results (Fig. 8B), after 300 s no difference in memory was detectable between the *syn*^{97} mutant and the wild-type WT (Fig. 8D; 10 s and 150 s: $P< 0.05/3$; 300 s $P> 0.05/3$; U= 34, 51, 76; N= 15 for all groups). Within this narrow time range we detected a decay of memory scores for the wild-type WT (Fig. 8D; WT: $P< 0.05/2$; H= 12.5; df= 2; sample sizes as above) while memory scores of the *syn*^{97} mutant remained effectively stable (Fig. 8D; *syn*^{97}: $P> 0.05/2$; H= 1.75; df= 2; sample sizes as above).

Thus, Synapsin is required to form memories that support high levels of learned behavior shortly after training.

The syn^{97}*/sap47*^{156} *double mutants show no additive impairment in odor-sugar memory*

Given the conspicuous residual 50 % of associative memory in the *syn*^{97} mutant, we wondered what the genetic determinants for this remaining capacity are. Specifically, we wondered whether the defect of the *syn*^{97} mutant would be additive with the likewise about 50 % decrease in associative memory scores observed in the *sap47*^{156} mutant (Saumweber et al. 2011). An additive defect in memory would result if the Synapsin and Sap47 proteins act in parallel to support memory. In contrast, a lack of additivity implies an absence of evidence for such parallel organization, and rather suggests they act in series, within the same process. We therefore probed for associative memory in the *syn*^{97} mutant, the *sap47*^{156} mutant, and a *syn*^{97}/*sap47*^{156} double mutant (DM), as well as their corresponding wild-type strains (WT, WT_2, WT_3, respectively, see Materials and Methods for nomenclature). Based on the previous data, this experiment featured AM at a 1:20 dilution, and 2 mol/l FRU as reward. All three mutants showed a significant and approximately 40 % - 60 % impairment in associative function compared to their respective wild-type (Fig. 9A; $P< 0.05/3$ in all cases; U= 104, 103.5, 117; N= 22, 22, 24, 24, 25, 25). When memory scores are normalized to the respective wild-type performance, scores of the *sap47*^{156} mutant, the one showing the stronger defect, and the DM are indistinguishable (Fig. 9A inset; $P> 0.05$; U= 261; N= 24, 25). With due caveats in mind (see Discussion), such a lack of additivity upon the lack of both Synapsin and Sap47 is suggestive of both proteins not working in parallel, but rather within the same process to confer

associative memory. Our results from the analytical chemistry of the Synapsin and Sap47 proteins are consistent with such functional interdependence (see next section).

We note that heterozygous $syn^{97}/sap47^{156}$ double mutants (DM/+: heterozygous for both the syn^{97} mutation and the $sap47^{156}$ mutation; Fig. S9) showed no impairment in memory compared to the wild-type WT_3 (Fig. 9B; $P> 0.05$; U= 568; N= 36, 36). Accordingly, both the syn^{97} and the $sap47^{156}$ mutation are not dominant in their effect on memory, meaning single functional alleles of the *synapsin* and *sap47* genes are sufficient to ensure proper associative function.

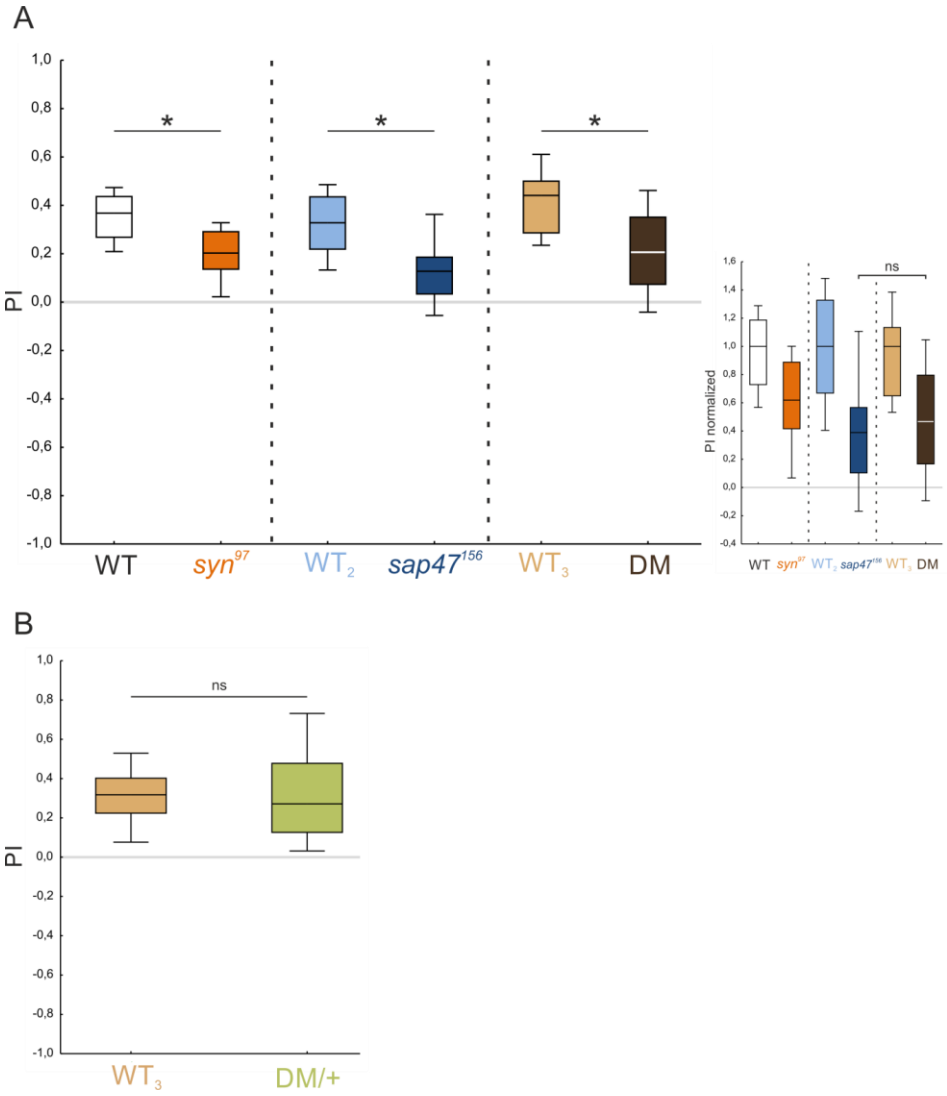

Figure 9. The memory impairments in the in syn^{97} and the $sap47^{156}$ mutants are not additive. **(A)** Memory was compared between the syn^{97} mutant, the $sap47^{156}$ mutant, and the $syn^{97}/sap47^{156}$ double mutant (DM) to investigate whether there is an additive memory defect for the double mutant. All mutants are significantly impaired in memory compared to their respective wild-type. The memory scores of the syn^{97} mutant were reduced of about 40 % compared to the wild-type WT; the $sap47^{156}$ mutant showed a reduction in memory of about 60 % compared to the wild-type WT_2. The DM revealed an impairment in memory of about 60 % in comparison to the wild-type WT_3 (inset, showing the normalized PIs, ns indicates $P> 0.05$ in a MWU test). Hence no additive effect could be detected. The color of the fill of the box plots is used to indicate genotype. All displayed data were gathered in parallel. The underlying PREF scores are documented in Figure S7A. * indicates $P< 0.05/3$ in MWU tests. Other details as in Figure 6. **(B)** Heterozygous $syn^{97}/sap47^{156}$ double mutants (DM/+) showed no impairment in memory compared to the wild-type WT_3. The underlying PREF scores are documented in Figure S6B. ns indicates $P> 0.05$ in MWU test. Other details as in Figure 6.

Synapsin phosphorylation is altered in $sap47^{156}$ mutants

Considering a possible interdependence of Synapsin and Sap47 function (see previous section), and given the additional Synapsin band in Western blots of $sap47^{156}$ mutant larvae (Fig. 5G, two rightmost lanes) as

well as the functional significance of the phosphorylation of Synapsin in general (see Introduction), we decided to compare the phosphorylation of the Synapsin protein from larval brains of *sap47*^{156} mutants to the corresponding wild-type WT_2. Using mass spectrometry (LC-MS/MS) we yielded coverage of 38 % of the Synapsin protein in WT_2 and of 47 % in the *sap47*^{156} mutant strain; within the covered regions, we could ascertain 15 different phosphorylated sites of the Synapsin protein from experimentally naïve wild-type WT_2 larvae (Fig. 10A, Table 1). Of note, Synapsin was always phosphorylated at a central motif, namely at either S480 or S482; no case was observed with a lacking phosphorylation at either site, or with phosphorylation at both these sites. The same applies in the *sap47*^{156} mutant (Fig. 10B) – with the striking difference that it is almost always only S480 that is phosphorylated (Table 1).

Particularly frequent instances of Synapsin phosphorylation were observed in *sap47*^{156} mutant larvae not only at S480, but also at a more N-terminal motif, at S128 and T138 (Table 1). Regarding this N-terminal motif, we find a higher number of differently-phosphorylated kinds of Synapsin in the *sap47*^{156} mutant than in the wild-type WT_2 (Fig. 10A, B). Lastly, we would like to mention that in a C-terminal region a double-phosphorylation at S952 and S965 was found in the *sap47*^{156} mutant, but not in the wild-type WT_2.

These alterations of Synapsin phosphorylation in the *sap47*^{156} mutant are suggestive of a functional interplay between Synapsin and Sap47.

			WT_2	*sap47*^{156}	WT_2	*sap47*^{156}
Amino acid	**Amino acid number***	**Predicted kinase (NetPhosK-Score)**	**Counts phosphorylated/ total**	**Counts phosphorylated/ total**	**P-value phosphory-lated form**	**P-value phosphory-lated form**
T	86	p38MAPK (0.53)	1/8	0/1	1.62E-03	-
T	89		1/8	1/1	1.62E-03	2.45E-03
S	107		1/2	0/7	1.62E-03	-
S	110	PKC, PKA (0.60) (0.71)	1/1	1/8	5.11E-04	2.46E-03
S	117	RSK (0.57)	1/2	0/20	5.11E-04	-
S	119	PKC (0.72)	0/2	3/20	-	2.55E-03
S	128	cdc2 (0.50)	1/17	37/75	2.95E-03	1.19E-03
S	136		2/16	8/73	1.96E-03	1.52E-03
T	138	PKC (0.76)	3/16	42/73	9.18E-04	1.19E-03
Y	149	INSR (0.50)	1/1	0/0	1.61E-03	-
S	163		1/1	0/5	1.61E-03	-
Y	204		0/0	1/1	-	3.10E-03
T	370	PKC (0.75)	1/1	0/1	2.39E-03	-
S	480	cdk5, RSK, GSK3 (0.65) (0.55) (0.51)	8/15	11/12	4.56E-07	6.07E-08
S	482	CKII (0.52)	7/15	1/12	2.11E-07	8.81E-04
T	511		0/2	1/5	-	2.93E-03
S	512		0/2	1/5	-	2.93E-03
T	612	PKC (0.51)	0/1	1/4	-	1.63E-03
S	613	CKI (0.61)	0/1	1/4	-	3.13E-03
S	623		1/1	0/1	3.06E-03	-
S	634	PKC, cdc2 (0.66) (0.58)	1/1	0/1	3.06E-03	-
S	952	CKI (0.50)	0/3	12/27	-	8.45E-04
S	965		0/3	12/27	-	8.45E-04

* Isoform D E2QCY9_DROME 1041 AS.

Table 1 Synapsin phosphorylation in wild-type WT_2 and *sap47*^{156} mutant larvae. Frequency of detected Synapsin peptides in their phosphorylated and non-phosphorylated form, compared between wild-type WT_2 and *sap47*^{156} mutant larvae. Red colouring indicates that peptides were found phosphorylated more or less often than in the respectively other genotype.

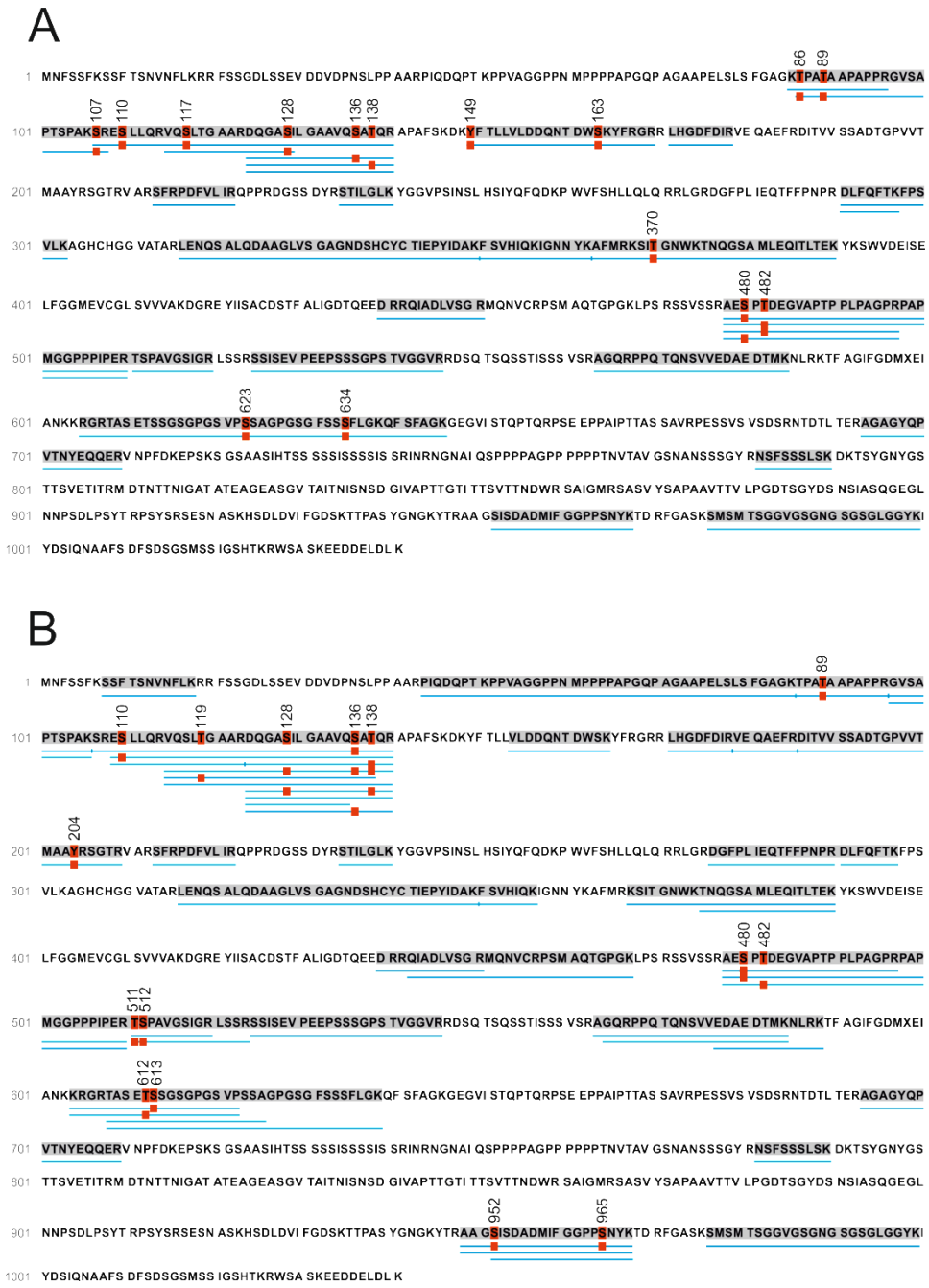

Figure 10. The pattern of Synapsin phosphorylation is altered in sap47^{156} mutants. Phosphorylation sites of Synapsin in experimentally naive wild-type WT_2 (A) and in naïve sap47^{156} mutant larvae (B). Thirteen LC-MS/MS runs were performed to analyze the phosphorylation status across the Synapsin protein in both genotypes. The number of times a phosphopeptide or its corresponding non-phosphorylated counterpart was detected is indicated as counts in Table 1. (A) We identified 15 phosphosites of Synapsin in the wild-type WT_2 and (B) 15 phosphorylated sites of Synapsin in the sap47^{156} mutant larvae. Blue bars below the sequence indicate the peptides identified as peptide-spectra matches (PSM) using the PEAKS *de novo* sequencing algorithm. The red "P" boxes indicate phosphorylation ($P< 0.005$). As an example how to read this display and Table 1, in the wild-type WT_2 all peptides covering amino acids 478-497 were found to be phosphorylated at either S480, or S482, but never were both or none of these two found to be phosphorylated. Table 1 then shows that a phosphorylated S480 site was found for 8 out of 15 peptides, while for S482 this was the case for the remaining 7 peptides.

Discussion

Drosophila larvae lacking Synapsin can form and remember odor-reward associations, but as we show Synapsin is required to profit from a high salience of odors or from a high salience of the reward for establishing strong memories (Fig. 7). Likewise, the early 'extra' memory component that supports high levels of learned behavior shortly after training, that is memory for saliently recent events, is Synapsin-dependent (Fig. 8).

Synapsin is required for short- but not longer term memory

Our observation that Synapsin is required specifically for short- but not longer term odor-reward memory in larval *Drosophila* matches what Knapek et al. (2010) found for odor-punishment memories in adult *Drosophila*. Given the requirement of Synapsin for regulating the balance between reserve-pool and releasable vesicles in a phosphorylation-dependent way (see Introduction), this seems plausible. The training-induced changes in the phosphorylation pattern of Synapsin are likely transient, such that the initial balance between reserve-pool vesicles and releasable vesicles is relatively quickly resumed. In effect, Synapsin function thus is the basis for the memory of saliently recent events.

Synapsin boosts memory strength for highly salient events

According to the working hypothesis of odor-reward learning in *Drosophila* (see Introduction and Fig. S10), it is straightforward to understand why strong rewards lead to strong odor-reward memories. A stronger reward would more strongly activate a dopaminergic reward signal, leading to a stronger activation of *inter alia* the AC-cAMP–PKA-Synapsin pathway in those mushroom body Kenyon cells that are coincidently activated by the odor. Thus, more reserve vesicles would be recruited and a stronger memory trace established. Without Synapsin, this ability to adapt memory strength to reward strength is compromised. Certainly, the eventual net effect on synaptic strength would include the effects of other activated kinases, too (see Introduction).

At first sight it seems equally straightforward that a high odor concentration will activate the mushroom body Kenyon cells more strongly and, by the same token as for the case of a strong reward, establish a stronger memory. However, according to such a scenario one would predict equal or higher memory scores if the odor concentration is increased between training and test. This is because during the test with a higher-than-trained odor concentration the mushroom body Kenyon cells would be activated more strongly, leading to at least as strong output as with the trained odor concentration. Not fitting this prediction, memory scores were found to be less when odor concentration was increased between training and test (i.e. memory is specific for the trained odor intensity: Mishra et al. 2013; also Yarali et al. 2009b). In terms of physiology, both the level of activity and the combination of activated mushroom body

Kenyon cells varies, albeit slightly, with odor concentration. It will be interesting to see whether and which parameter set of biologically plausible mushroom body models (Luo et al. 2010, Nehrkorn et al. 2015) can account for both the high memory scores found when using a high odor concentration in training and in testing (Fig. 7A, B; Mishra et al. 2013, loc. cit. Fig. 2), as well as for the decrease in memory scores when the odor concentration is increased between training and test (Mishra et al. 2013, loc. cit. Fig. 3). The circuit motif suggested by Nehrkorn et al. (2015) in principle seems to be capable to capture both these aspects.

In punishment learning of adult flies event salience has been varied by introducing temporal gaps between the to-be-associated stimuli. This revealed both Synapsin-dependent and Synapsin–independent punishment memory components for optimally-timed, highly salient, events. For suboptimally-timed, less salient cases, punishment memory is Synapsin-independent (Niewalda et al. 2015). The dataset from Niewalda et al (2015) is revealing also in another respect. That is, for optimal punishment learning the odor is presented shortly before the shock (forward conditioning), yielding punishment memory scores of PI≈ -0.6. When the sequence of odor and shock is reversed such that the odor is presented only upon the pleasantly relieving cessation of shock (backward conditioning), flies subsequently approach that odor. Such 'relief' memory typically is weaker than punishment memory, yielding scores of only PI≈ 0.2, even at an optimal backward interval (Gerber et al. 2014). Such relief memory is Synapsin-dependent. Interestingly, when a sub-optimal forward conditioning interval is used, punishment memory is just as weak as relief memory after optimal backward conditioning (PI≈ -0.2 and 0.2, respectively) - yet in the *syn*^{97} mutant no decrement in relief memory is observed! Thus, the absolute level of memory does not appear to be the sole determinant for the involvement of Synapsin. Rather, the requirement of Synapsin becomes the more obvious the closer the memory process operates at its particular upper limit.

We conclude that Synapsin is required to form memories such that they match in strength to high event salience, either in relation to odor salience, reward salience, event-recency, or event-timing. This suggests that Synapsin may be required whenever a memory process operates at its particular upper limit.

The roles of Synapsin, Sap47, and Brp for short-term memory

The present data confirm that a lack of Synapsin reduces memory scores to about half, prompting the question for the nature of the residual Synapsin-independent memory. We had found earlier, and have confirmed in this study, that a lack of the Sap47 protein likewise entails a reduction of memory scores to half (Fig. 9A; Saumweber et al. 2011). Notably, the decrements in memory upon a lack of both Synapsin and Sap47 are not additive (Fig. 9A), suggesting that the residual Synapsin-independent memory is also Sap47-independent and vice versa. Clearly, one caveat regarding this suggestion is that it is based on an absence of evidence for additivity, which must not be confused with an evidence of absence. Still, also the changes in phosphorylation of Synapsin upon a lack of Sap47 suggest an interdependence in the function

of both proteins (Table 1; Fig. 10). Whether the altered phosphorylation of Synapsin in particular at the N-terminal (S128/138), central (S480/482) and/or the C-terminal phospho motif (S952/965) are significant with respect to memory function remains to be investigated. Interestingly, the memory defect of mutants lacking Synapsin cannot be rescued by a Synapsin protein with mutated S22 and S549 sites (Michels et al. 2011; loc. cit. S6/S533); these sites were found to be phosphorylated in adult *Drosophila* (Niewalda et al. 2015), but unfortunately the present analysis, despite effort, does not yield information about their phosphorylation status in the larva. Indeed, protein mass spectrometry for larval tissue is substantially more difficult than for adult tissue, arguably because of the lower abundance of the Synapsin protein in the larva. We note that the changes in Synapsin phosphorylation in mutants lacking Sap47 are a possible cause for the additional Synapsin band seen in Western blots (Fig. 5G, two rightmost lanes) (alterations in phosphorylation of a protein can result in changes of electrophoretic mobility beyond the slight mass increases generated by the additional phospho groups themselves, i.e. 79.97 Da per phospho group).

In any event, what could be the molecular basis for the residual Synapsin- as well as Sap47-independent memory? Regarding olfactory punishment learning in adult *Drosophila* Knapek et al (2010) reported that Synapsin-independent memory is amnesia-resistant. In turn amnesia-resistant short-term memory does require the Bruchbilot protein (Brp; coding gene: *brb*, CG42344), a protein localized to the presynaptic active zones and essential for the proper formation of presynaptic dense bodies and short-term synaptic plasticity (Wagh et al. 2006; Fouquet et al. 2009, Hallermann et al 2010, Knapek et al 2011). While the role of Brp in larval memory has not yet been tested, a possible scenario thus is that short-term memory has two components, one that depends on Synapsin and on Sap47, but not on Brp, and which is amnesia-sensitive; and a second component that works without Synapsin and without Sap47, requires Brp, and is amnesia-resistant.

Materials and Methods

Flies and rearing conditions

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 12/12-h light/dark cycle. Experimenters were blind with respect to genotype and treatment condition in all cases; these were decoded only after the experiments. We used three different wild-types together with their respective null mutants:

Wild-type	CS^{2012} (WT)	CS^{NF} (WT_2)	CS^{V} (WT_3)
Mutant	$syn^{97\ CS2012}$	$sap47^{156}$	$syn^{97}/sap47^{156}$ (DM)

The wild-type CS^{2012} and the Synapsin mutant *syn*$^{97\ CS2012}$ emerged from an additional outcrossing of *syn*^{97CS} (Godenschwege et al. 2004; Michels et al. 2005) to wild-type CS for 13 generations. The *sap47*^{156} mutant strain was outcrossed to wild-type CS^{NF} for nine generations (Funk et al. 2004; Saumweber et al. 2011). Outcrossing removes marker genes introduced for mutagenesis and effectively adjusts genetic background which may otherwise distort results (de Belle and Heisenberg, 1996). The *syn*^{97}*/sap47*^{156} double mutant was generated by V. Albertova by homologous recombination and then outcrossed to wild-type CS.

For simplicity, the wild-type CS^{2012} strain is labeled WT, the wild-type CS^{NF} strain is labeled WT_2, and the wild-type CS^{V} strain is labeled WT_3. The Synapsin null mutant strain *syn*$^{97\ CS2012}$ is labeled *syn*^{97} and the *syn*^{97}*/sap47*^{156} double mutant strain DM. Animals heterozygous for *syn*^{97} as well as for *sap47*^{156} are labeled DM/+.

Single-larva PCR

To confirm the genetic status of the used strains we preformed single-larva PCR according to Gloor et al. (1993) (Fig. 5A, E shows the principle of primer design and the expected PCR products). The primer binding sites were upstream (first primer: 1= *syn* primer and I=*sap47* primer), within (second primer: 2= *syn* primer and II=*sap47* primer) or downstream (third primer: 3= *syn* primer and III=*sap47* primer) of the respective deletion. Accordingly, the first and the second primer should yield a product only if the gene is in its wild-type condition. The first and the third primer produce a product for both the wild-type and the mutant status of the gene, which can be clearly distinguished because of their size. Specifically, the following primers were used:

syn primers:	1=	5’-AGAAAATTTGGCTTGCATGG-3’;
	2=	5’- CGGGGTCTCAGTTTTGTTG-3’;
	3=	5’-CCTCTACTTTTGGCTGCCTG-3’
sap47 primers:	I=	5’- GAGAAGAGCTCGACTTTCCAG-3’;
	II=	5’- CTTCGCTCTCTTGGACTCG-3’;
	III=	5’- CCTATCCACTCAGTTTGAGGG-3’

Western blot

For homogenization and electrophoresis we used the Novex Bolt Mini Gel system (Life Technologies Carlsbad, USA). For each lane 10 larval brains were homogenized in 10 µl homogenization buffer, containing 2.5 µl LDS Sample Buffer (4X), 1 µl Reducing Agent (10X) and 6.5 µl deionized water. The sample was heated to 70 °C for 10 min and centrifuged for 30 s before electrophoresis. For gel

electrophoresis we used the Novex Bolt Mini Gel Tank. The proteins were separated in a 4-12 % Bis-Tris Plus gel at 165 V for 40 min. The proteins were transferred to nitrocellulose membrane with the iBlot Gel Transfer Device system. After the membrane was blocked with Odyssey Blocking Buffer (LI-COR, Lincoln, USA) for 1 h, it was washed three times for 10 min in 1 X PBST. For the immunoreaction we used three primary monoclonal mouse antibodies. SYNORF1 was used for Synapsin detection (Klagges et al. 1996) (diluted 1:100 in PBST); for Sap47 detection nc46 (Funk et al. 2004; Hofbauer et al. 2009) (diluted 1:100 in PBST) was used; ab49 (Zinsmaier et al. 1990, 1994) (diluted 1:100 in PBST) was used for detection of the Cysteine String Protein (CSP; Arnold et al. 2004) as loading control. As secondary antibody we used IRDye 800CW goat anti-mouse lgG (LI-COR) (diluted 1:15000 in PBST). The primary antibody incubation was performed at 4 °C over night followed by three 10-min washing steps in PBST. Secondary antibody incubation at RT for 1 h was followed by three final 10-min washing steps in PBST. Detection and visualization was performed with the ODYSSEY CLx Imaging System (LI-COR).

Immunohistochemistry

Larval brains were dissected in Ringer's solution and fixed in 3 % paraformaldehyde dissolved in PBST (0.2 % Triton X-100) for 1 h. After three 10-min washes in PBST (3 % Triton X-100), the brains were treated in blocking solution containing 3 % normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) in PBST for 1.5 h. Tissue was then incubated overnight with either SYNORF1 for Synapsin detection (diluted 1:10 in blocking solution) or nc46 for Sap47 detection (diluted 1:10 in blocking solution). Six 10-min washing steps in PBST were followed by incubation with a secondary rabbit anti-mouse antibody conjugated with Alexa 488 (diluted 1:200) (Invitrogen Molecular Probes, Eugene, USA). For orientation in the preparation we used overnight staining with Alexa Fluor 568 Phalloidin (diluted 1:200) (Invitrogen Molecular Probes), which visualizes filamentous actin. After final washing steps with PBST, samples were mounted in Vectashield (Vector Laboratories Inc., Burlingame, USA).

Analysis of Synapsin phosphorylation by LC-MS/MS

Sample preparation and LC-MS/MS analysis was performed as described earlier regarding adult *Drosophila* (Niewalda et al. 2015). In brief, brains of experimentally naïve larval *Drosophila* were dissected and lysed in 8 M urea and 1 % (w/v) RapiGest SF surfactant (Waters Corp., Milford, USA) and mechanical destruction (micro glass potter and sonification). After reduction and thiomethylation of cystein residues, proteins were digested by Trypsin (Promega, Trypsin Gold, Fitchburg, USA). Afterwards, RapiGest detergent was removed and samples were cleaned using Empore universal resin SPE-columns (3M, St. Paul, USA).

Proteome analysis was performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific, San Jose, USA) equipped with an U3000 nano-flow HPLC (Thermo Scientific, San Jose, CA). Samples were separated on a 75 µm I.D., 25 cm PepMap C18-column (Dionex, Sunnyvale, USA) applying a gradient from 2 % ACN to 35 % ACN in 0.1 % formic acid over 220 min at 300 nl/min. The LTQ Orbitrap Velos Pro MS used exclusively CID-fragmentation with wideband activation (pseudo MS3 for neutral losses of phosphate residues) when acquiring MS/MS spectra. The spectra acquisition consisted of an orbitrap full MS scan (FTMS; resolution 60,000; m/z range 400-2000) followed by up to 15 LTQ MS/MS experiments (Linear Trap; minimum signal threshold: 500; wideband isolation; dynamic exclusion time setting: 30 s; singly-charged ions were excluded from selection, normalized collision energy: 35 %; activation time: 10 ms). Raw data processing, protein identification and phosphopeptide assignment of the high resolution orbitrap data were performed by PEAKS Studio 7.0 (Bioinformatics Solutions, Waterloo, Canada). False discovery rate (FDR) was set to < 1 %. Phosphosites were accepted as confident for $P< 0.005$ (modified t-test, included in PEAKS Studio 7.0).

Petri dish preparation, odors

As assay plates for behavioral experiments we used Petri dishes (85-mm inner diameter; Sarstedt) that were filled with 1 % agarose (NEEO Ultra-Quality, Roth, Karlsruhe, Germany). We used 2 mol/l fructose (FRU; CAS: 57-48-7; Roth, Karlsruhe, Germany) as reward that was added to the agarose, unless mentioned otherwise. We used *n*-amylacetate (AM; CAS: 628-63-7; Merck, Darmstadt, Germany) or 1-octanol as odors (OCT; CAS: 111-87-5; Merck, Darmstadt, Germany). Odors were diluted in paraffin oil (AppliChem, Darmstadt, Germany, 1:20 for AM and 1:20 for OCT) unless mentioned otherwise. Custom-made odor containers made of Teflon, perforated at their lids to allow odor evaporation while preventing animals to get in direct contact with the chemicals, were filled with 10 µl of the respective odor solution.

Associative learning

Larvae were trained with either of two reciprocal training regimen and afterwards compared for their odor preference (Fig. 6A) (for a manual see Gerber et al. 2013): In one group of larvae AM was paired with the sugar reward (AM+), while a second group of larvae was trained with unpaired presentations of odor and reward. To equate both groups with respect to the total number of trials, in the paired group blank trials were interspersed. Then, animals from both groups were tested for their AM preference. Associative memory is indicated by a relatively higher preference for AM after AM+ training as compared to AM/+ training, as quantified by the performance index (PI; see below).

For example, about 30 larvae were collected from the food vial and briefly washed in tap water. Two containers loaded with AM were placed at opposing sides of an assay plate including the fructose

reward (+). Immediately before training started the larvae were gently placed onto the plate using a wet brush. The assay plate was closed with a lid. The lid featured at its middle about 15 custom-made holes (1 mm diameter) for better airflow. Then, the animals were left untreated for 5 min. Subsequently, the larvae were transferred to another assay plate, with two containers at opposing sides containing no odor (empty, EM); this time no fructose reward was included in the assay plate. This cycle of paired training (AM+) was repeated two more times, each time using fresh assay plates.

After this training, the preference of the animals for AM was recorded. Unless mentioned otherwise the larvae were immediately placed into the middle of a fresh assay plate; that fresh testing assay plate had no fructose in it. A container with AM was placed on one side, and an empty container to the other side (EM). After 3 min the number of animals on the AM side ($\#_{AM}$), on the EM side ($\#_{EM}$) and in a 1-cm wide middle stripe ($\#_{Middle}$) was counted and the preference for AM (range -1; 1; Fig. 6B) calculated as:

(1) PREF = $\#_{AM}$ - $\#_{EM}$ / $\#_{AM+EM+Middle}$

Thus, PREF values of -1 imply full avoidance, while scores of 1 would imply full attraction.

In parallel, another set of larvae was exposed to AM without fructose on a first assay plate and then to an assay plate containing fructose and an empty container, for a total of three such cycles of unpaired training (AM/ +). Then, PREF scores were determined as in equation (1). The PREF scores of all experiments are documented in Figures S2-S8.

For both paired and unpaired training, the sequence of trail types was reversed in every other repetition of the experiment (i.e. either as described AM+/EM and AM/EM+; or EM/AM+ and EM+/AM).

From these preference values the performance index (PI; range -1; 1; Fig. 6C) can be calculated. The PI describes the difference between the preference values after paired training ($PREF_{AM+}$) versus after unpaired training ($PREF_{AM/+}$) and thus indicates associative memory:

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

Positive PI scores therefore indicate appetitive associative memory, while negative scores indicate aversive associative memory.

For OCT as odor, experiments were performed likewise.

II Chapter II

Synapsin function in adult punishment- and relief-memory *

*Based on Niewalda T, Michels B, Jungnickel R, Diegelmann S, Kleber J, Kähne T, Gerber B. 2015. Synapsin determines memory strength after punishment- and relief-learning

Introduction

Painful, traumatic experiences can have a moulding influence on behavior. Current research is focused on the 'negative' memories that such experiences induce: stimuli experienced before a painful event become predictors of danger and will be avoided when encountered again. While in principle adaptive, danger-predictions can also contribute to maladaptive behavior and undesired psychological states (e.g. stress, anxiety, panic). Under such circumstances, any means to counteract these effects is welcome. We therefore extend the focus towards 'backward conditioning', that is to memories related to stimuli perceived after a painful event (Moscovitch and Lolordo, 1968; Plotkin and Oakley, 1974; Solomon and Corbit, 1974; Heath, 1976; Wagner, 1981; Wagner and Larew, 1985) (recent reviews include Gerber et al. 2014, as well as Navratilova and Porreca, 2014).

In fruit flies, odor-shock training ('forward conditioning' of the odor) leads to conditioned avoidance of the odor during subsequent tests, whereas shock-odor training ('backward conditioning' of the odor) leads to conditioned approach (Tanimoto et al. 2004). Corresponding effects are seen in humans, rabbits, rats and in the appetitive domain in bees (references in preceding paragraph as well as Hellstern et al. 1998; Andreatta et al. 2010; Andreatta et al. 2012; Felsenberg et al. 2013; Franklin, Lee et al. 2013; Franklin, Puzia et al. 2013; Mohammadi et al. 2014). Such timing-dependent valence-reversal makes sense, as after odor-shock training the odor predicts punishment, whereas shock-odor training associates the odor with relief from shock. We therefore refer to these processes as punishment-learning and relief-learning, respectively (Fig. 11A).

For punishment-learning, reasonably clear working hypotheses exist (see Discussion section), but much less is known about relief-learning, in any experimental system. In *Drosophila*, relief-memory is less strong than punishment-memory, requires six rather than just one training trial, and is strongest when using relatively mild shock intensities (Yarali et al. 2008). These parametric requirements were the likely reason why relief-learning was overlooked in classical studies (Quinn et al. 1974; Tully and Quinn, 1985). Whenever the current study compares relief- and punishment-memory, six trials and the same shock intensity are used in either case.

Given that relatively little is known about the mechanisms of relief-memory, we decided to focus on an evolutionarily conserved process of synaptic plasticity and memory. We chose the Synapsin protein as a study case (coded by the *synapsin* gene: CG3985), a ubiquitously and abundantly expressed, and evolutionarily conserved, presynaptic phosphoprotein (Klagges et al. 1996; Nuwal et al. 2011; Diegelmann, Klagges et al. 2013). It can tether reserve-pool vesicles to the cytoskeleton and, during learning, regulate their recruitment to the readily-releasable pool in a phosphorylation-dependent way. Thus, more synaptic vesicles are made eligible for release upon subsequent activation of the cell during retrieval. In this sense, Synapsin is thought to function during learning in a way that determines memory

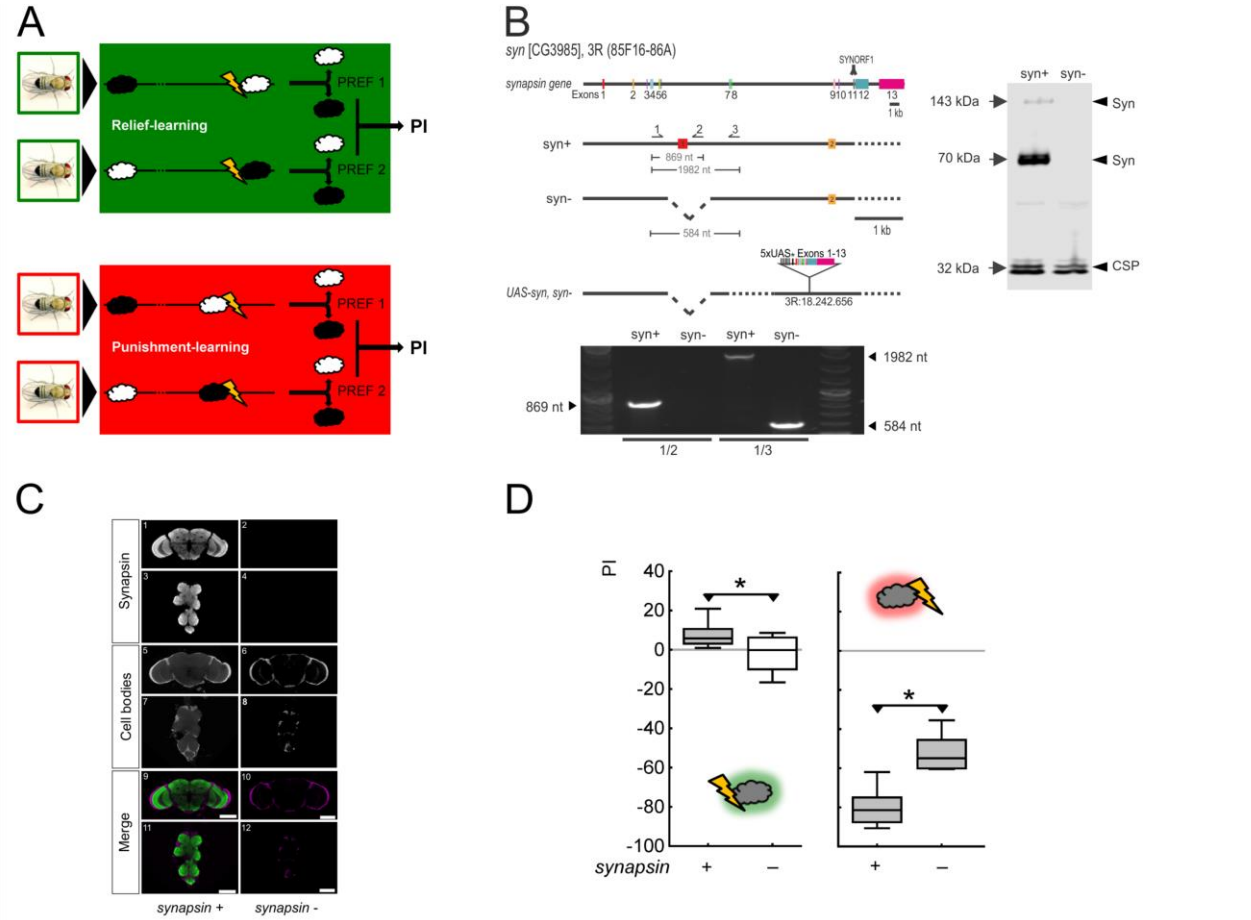

Figure 11: Mutant flies lacking Synapsin are impaired in both relief- and punishment-memory. **(A)** Schematic of the experimental paradigm for relief- and punishment-learning. For relief-learning, an odor is presented upon the cessation of shock, while for punishment-learning an odor is presented before the onset of shock. In both cases, a control odor is presented temporally far removed from shock. During the test, the flies can choose between the relief-trained *versus* the control odor, and between the punishment-trained *versus* the control odor, respectively. Swapping the chemical identity of the odors in reciprocally trained flies allows a performance index (PI) to be calculated from the difference in preference between these reciprocally trained flies. The PI thus measures associative memory and runs no risk of confounding differences in innate preference for either odor, or in non-associative memory components. For further details see Materials and Methods section. (**B)** Upper left: Genomic organization of the *Drosophila synapsin* gene. *Syn* contains 13 exons and spans a 13.7 kb genomic region in the 3R (85F16-86A) cytological interval (flybase; www.flybase.org) (the 2nd of the 14 exons previously suggested on the basis of *syn*-cDNA sequences [Klagges et al. 1996] apparently representes a splice artifact [B. Klagges, University of Leipzig, Germany; E. Buchner, University of Würzburg, Germany; personal communication]). Accordingly, the coding region for the epitope LFGGMEVCGL that is recognized by the monoclonal antibody SYNORF1 is encoded by exon 11. The *syn^{97CS}* strain (labelled *synapsin*$^{-}$ for simplicity) carries a 1.4-kb deletion spanning parts of the regulatory region and the first exon of the *synapsin* gene. The arrows indicate the binding sites for the PCR primers upstream (1), within (2), and downstream (3) of the deletion. The effector strain UAS-*syn*, *synapsin*$^{-}$ contains the *syn*-cDNA plus 156 base pairs upstream (*). Lower left: in a single-fly PCR approach, primer combination 1/2 yields an 869-nt product in *synapsin*$^{+}$ wild-type but not in *synapsin*$^{-}$ mutants, whereas primer pair 1/3 yields a 1982-nt product in *synapsin*$^{+}$ and a 584-nt product in *synapsin*$^{-}$. Right: Western blot from material obtained from three adult fly heads stained for Synapsin and for CSP as a loading control. The single band at about 143 kDa and the double band at about 70 kDa, where Synapsin isoforms are expected (Klagges et al. 1996), are found in *synapsin*$^{+}$ but not in *synapsin*$^{-}$ flies. **(C)** Synapsin immunoreactivity is absent in whole-mount preparations of *synapsin*$^{-}$ mutant flies. In the bottom row (Merge), anti-Synapsin staining is shown in green, and cell body counterstaining with propidium iodide in magenta, displayed as frontal optical sections (0.9 µm) of *synapsin*$^{+}$ (left column) and *synapsin*$^{-}$ (right column) brain and thoracic nervous system. The scale bar indicates 100 µm. **(D)** The left panel shows that relief-memory is intact in *synapsin*$^{+}$ wild-type flies, but is abolished in mutant flies lacking Synapsin (*synapsin*$^{-}$). The right panel shows that punishment-memory is impaired, but is not abolished, in *synapsin*$^{-}$ mutant flies. *: $P < 0.05$ for the between-genotype comparison within an experiment; a grey shading of the boxes indicates $P < 0.05/2$ in comparisons of either genotype to chance levels (zero) within one experiment. PI: Performance Index, indicating the difference in preference between reciprocally trained flies, and thus learned approach (positive scores) and learned avoidance (negative scores), respectively. The middle line of the box plots represents the median, the box boundaries the 25 % and 75 % quartiles, and the whiskers the 10 % and 90 % quantiles, respectively.

strength. Synapsin contributes to the regulation of synaptic output and various forms of memory throughout the animal kingdom (Silva et al. 1996; Hilfiker et al. 1999; Garcia et al. 2004; Gitler et al. 2004; Sudhof, 2004; Diegelmann, Klagges et al. 2013; Greco et al. 2013; Sadanandappa et al. 2013; Vasin et al. 2014). Regarding associative processing in *Drosophila*, Synapsin null mutants show reduced scores in 1-trial strong-shock punishment-memory, reduced spatial memory in the heat-box paradigm and, as larvae, reduced odor-sugar memory (Godenschwege et al. 2004; Knapek et al. 2010; Michels et al. 2005, 2011; Walkinshaw et al. 2015). We reasoned that Synapsin is worthy of study for its role in relief- and/or punishment-memory because if it were specifically required for either form of memory it would provide a target to tip the balance between punishment- and relief-memory by systemic manipulation. Given the conserved function of Synapsin, this may offer translation potential to e.g. selectively erase the punishment-memory component but not the relief-memory component related to a traumatic episode. On the other hand, showing a common requirement of Synapsin for both relief- and punishment memory would caution against such approaches.

Results

Synapsin is a phosphoprotein

Given that *Drosophila* Synapsin has been described as a phosphoprotein (Nuwal et al. 2011), we first sought to replicate and potentially extend the description of which sites of Synapsin are indeed phosphorylated. We found by mass spectrometry (LC-MS/MS) that in experimentally naïve, *synapsin*$^{+}$ wild-type flies the Synapsin protein features 28 phosphorylated sites and that these sites encompass consensus motifs for 11 kinases (Fig. 12, Table 2). We further note that proteins corresponding to both the edited and the non-edited version of Synapsin (Diegelmann et al. 2006) were found to be expressed (Fig. 12, Table 2).

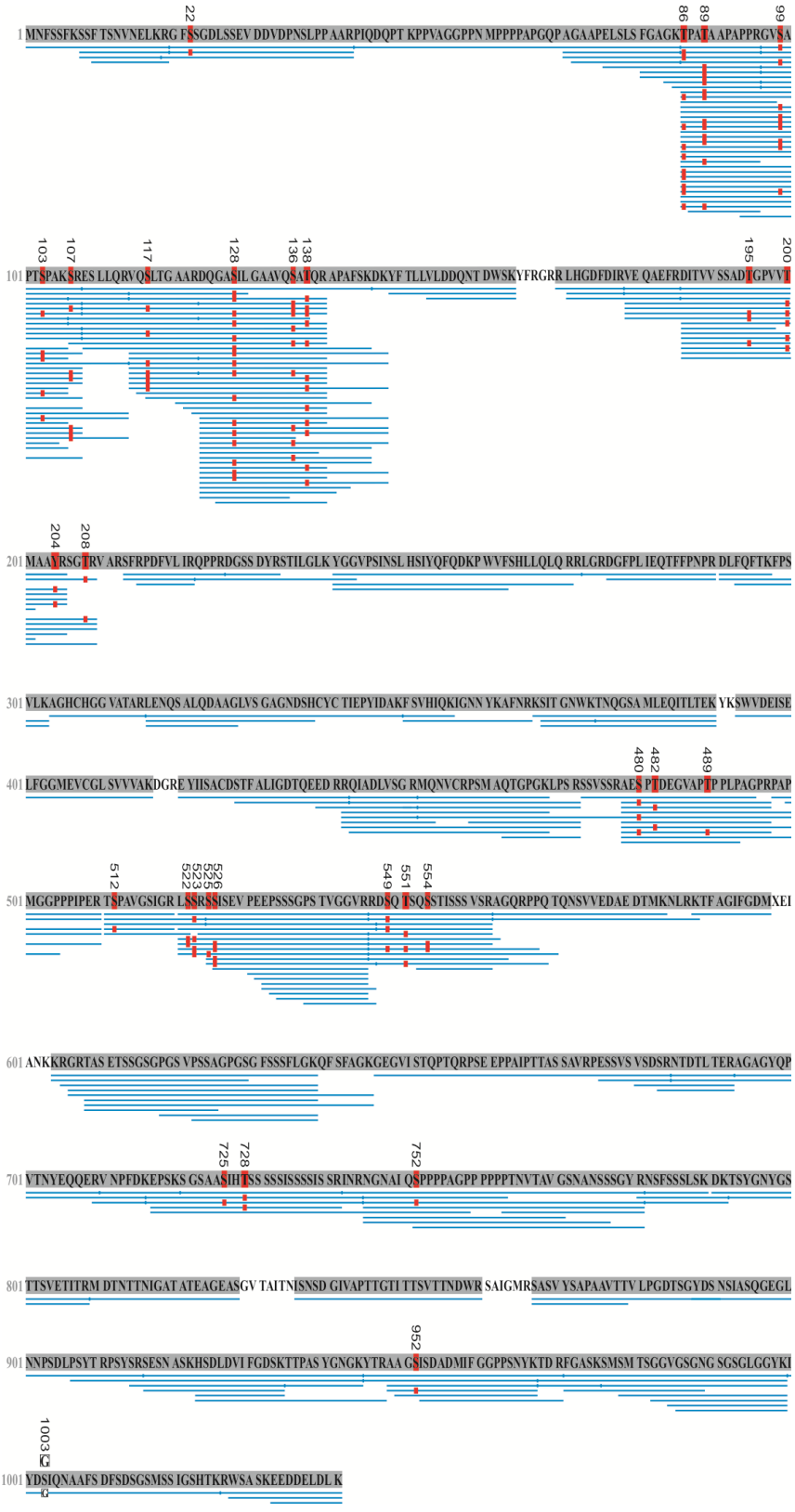

Figure 12: Phosphorylation sites of Synapsin and abundance of edited and non-edited Synapsin. LC-MS/MS analysis of experimentally naïve, wild-type fly brains to map phosphorylated sites across the Synapsin protein (see also Table 2). 85 LC-MS/MS runs were performed, consisting of a combination of 17 biological and 5 technical replicates each (coverage of the longest Synapsin protein isoform of 97 %). This identified 28 phosphorylated sites of Synapsin: 1 at tyrosine, 10 at threonine and 17 at serine. Twenty-three phosphorylated sites were identified for the first time, while 5 had been reported before (Zhai et al. 2008; Nuwal et al. 2011). Of the 7 phosphorylated sites both reported by Nuwal et al. (2011) and covered by the present data, we can confirm 3, while we found 4 of these sites to be non-phosphorylated.Both the edited and the non-edited forms of the Synapsin protein were found. That is, the pre-mRNA of the synapsin transcript is modified from the N-terminal motif RRFS (non-edited) to RGFS (edited) such that the PKA consensus motif RRFS is compromised (Diegelmann et al. 2006). In n= 85 LC-MS/MS runs the non-edited protein motif (RRFS) was found 54 times (*P*= 1.71E-07) and the edited protein motif (RGFS) was found 22 times (*P*= 9.44E-13). A phosphorylation at the motifs' serine was reliably detected only once (sic) for the edited, but not at all for the non-edited protein motif (Table 2: S22/ S6).The workflow optimized sensitivity for proteome and phosphorylation site analysis of sample amounts corresponding to only a single brain. Therefore a separation of isoforms prior to mass spectrometry was not warranted, such that discrimination between isoforms is not possible. Given a 97 % coverage, however, it is possible to ascertain the longest isoform (isoform D, E2QCY9_DROME: www.uniprot.org); this D isoform emerges from transcription starting at the first start codon and read through at the first stop codon (Klagges et al. 1996; Jungreis et al. 2011). A shorter isoform based on transcription from the second start codon and thus lacking 16 amino acids at the N-terminus (Q24546_DROME: www.uniprot.org) was confirmed in Nuwal et al. (2011). Blue bars below the sequence indicate peptide-spectra matches (PSM) identified by LC-MS/MS and the PEAKS *de novo* sequencing algorithm. The red "P" boxes indicate phosphorylation (*P*< 0.005). Note the S1003 → G mutation (white "G" box).

Table 2. Phosphosites of the Synapsin protein determined by LC-MS/MS

Amino acid	Phosphosite in Synapsin isoform D E2QCY9_DROME 1041 AS	Phosphosite in Synapsin Q24546_DROME 1025 AS[C]	Frequency of detection (n= 85)	*P*-value	Predicted kinases (NetPhosK-Score)	Lit.
S*	22	6	1	6.31E-07	PKA, 0.85 RSK, 0.65	
T	86	70	40	7.94E-04	p38MAPK, 0.53	
T	89	73	43	1.26E-03		
S	99	83	6	2.00E-03	PKA, 0.66 PKG, 0.59	
T	102	86	0	c.a.n.p*	PKC, 0.79 cdc2, 0.50	Nuwal et al. 2011
S	103	87	4	2.51E-03	cdk5, 0.75 p38MAPK, 0.58 GSK3, 0.53	Nuwal et al., 2011
S	107	91	38	2.00E-03		
S	117	101	4	2.00E-06	RSK, 0.57	
S	128	112	25	1.58E-03	cdc2, 0.50	
S	136	120	11	2.00E-03		
T	138	122	37	1.58E-03	PKC, 0.76	
T	195	179	7	1.58E-03		
T	200	184	11	1.26E-03		
Y	204	188	8	1.26E-03		
T	208	192	4	1.58E-03	PKC, 0.71	
S	480	464	81	5.01E-10	cdk5, 0.65 RSK, 0.55 GSK3, 0.51	Nuwal et al., 2011
T	482	466	26	2.51E-09	CKII, 0.52	Nuwal et al., 2011
T	489	473	1	1.58E-04	cdk5, 0.62 p38MAPK, 0.53 GSK3, 0.51	
S	512	496	24	1.00E-03		
S	522	506	2	1.26E-08	PKC, 0.79 PKA, 0.52	
S	523	507	4	5.01E-10	RSK, 0.59	
S	525	509	2	1.26E-08		Zhai et al., 2008
S	526	510	18	7.94E-12	PKA, 0.67 CKI, 0.60	Zhai et al., 2008
S	549	533	29	1.00E-11	PKA, 0.81 DNAPK, 0.61 RSK 0.60	
T	551	535	56	7.94E-14	PKC, 0.78	
S	554	538	0	c.a.n.p*		Nuwal et al., 2011
S	725	709	26	1.58E-03		
T	728	712	17	3.16E-04	PKC, 0.51	
S	752	736	5	5.01E-04		
S	952	936	3	3.98E-07	CKI, 0.50	
S	977	961	0	c.a.n.p*	cdc2, 0.50	Nuwal et al., 2011
Y	998	982	0	c.a.n.p*		Nuwal et al., 2011

S*- only in the edited protein phosphorylation at this site was detected. c.a.n.p*- covered as non-phosphorylated (peptides containing this motive were found as non-phosphorylated only). [C]For convenience we added amino acid numbering as reported previously (Zhai et al., 2008; Nuwal et al., 2011) using the matrix (Q24546_DROME; http://www.uniprot.org)

Synapsin⁻ mutant flies have defects in both relief- and punishment-memory

Mutant flies lacking Synapsin (*synapsin⁻*; Fig. 11B, C) are deficient in both relief- and punishment-memory scores (Fig. 11D). That is, after shock-odor training, the associative performance indices for the *synapsin⁺* wild-type strain are higher than for the *synapsin⁻* mutant, indicating reduced relief-memory in the *synapsin⁻* mutant (U= 72.0, $P< 0.05$; N= 16, 16). The *synapsin⁺* wild-type flies show the expectedly small, yet significant relief-memory scores ($P< 0.05/2$) while the performance indices for *synapsin⁻* mutant flies are not different from zero ($P> 0.05/2$). We conclude that relief-memory is abolished in Synapsin null mutant flies.

After odor-shock training, *synapsin⁻* mutant flies show less negative performance indices, and thus less punishment-memory, than *synapsin⁺* wild-type flies (U= 3.0, $P< 0.05$; N= 12, 12). As both genotypes show significantly negative performance indices ($P< 0.05/2$ in both cases), we conclude that punishment-memory is impaired, but not abolished, in Synapsin null mutant flies.

Is relief- and punishment-memory strength indeed lessened?

Given the known dependence of conditioned valence on the timing between odor and shock (the inter-stimulus-interval, ISI) (Gerber et al. 2014), there are actually five scenarios as to how a lack of Synapsin could lead to defects in both relief- and punishment-memory. That is, suppose only one ISI each were chosen for relief- and punishment-training (as indicated by the vertical arrows in Fig. 13). Under such conditions all scenarios but the one in Fig. 13A1 predict that the respective maximum of the relief- and punishment-memory scores is actually unchanged. In other words, a reduction in the associative memory score using a single given ISI in any given mutant is not sufficient to argue for a lessening in strength of the underlying associative processes – which would show by a consistent lessening of associative memory scores and thus a dampening of scores along the Y-axis of the ISI function (Fig. 13A1). We therefore decided to compare the full ISI function between *synapsin⁺* wild-type and *synapsin⁻* mutant flies.

As shown in Fig. 13B and C, we find that after punishment-learning the performance indices are consistently less negative in *synapsin⁻* mutant flies than in the *synapsin⁺* wild-type (U= 63, 43, 30, 31, 26; $P> 0.05$ for the ISIs of -150, -45 s, and $P< 0.05$ for the ISIs of -30, -15, 0 s; N= 10-12). Likewise, after relief-learning the performance indices are consistently less positive in *synapsin⁻* mutant than *synapsin⁺* wild-type flies (U= 42, 316, 59, 182; $P> 0.05$ for the ISIs of 25, 125, 200 s and $P< 0.05$ for the ISI of 50 s; N= 12-30). In other words, after both punishment- and relief-learning memory strength is lessened, corresponding to the scenario in Fig. 13A1. This argues that a lack of Synapsin entails a lessening of associative memory rather than an alteration in the temporal properties of coincidence detection (Fig.s 13A2-5).

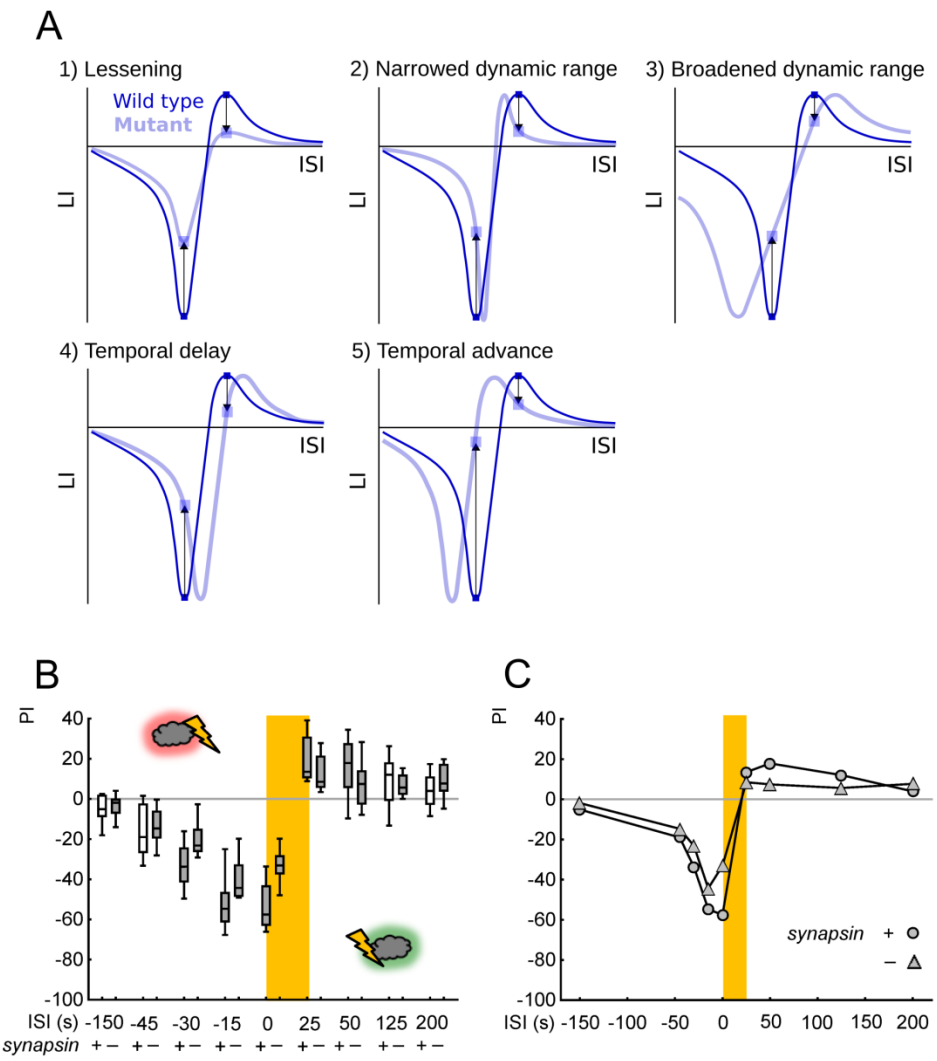

Figure 13: Memory strength is lessened, rather than features of coincidence detection modulated, in mutant flies lacking Synapsin. **(A)** When using only one inter-stimulus-interval (ISI) for relief- and one ISI for punishment-learning, less strong scores in both relief- and punishment-memory could be a result either of a lessening in strength of the associative memory (A1), a narrowed dynamic range of associative coincidence detection (A2), a broadened dynamic range (A3), a temporal delay (A4) or a temporal advance (A5), if the ISIs happened to be chosen as indicated by the arrows. We therefore decided to compare the full ISI function between *synapsin*$^{+}$ and *synapsin*$^{-}$ mutant flies. **(B)** Associative performance indices of wild-type *synapsin*$^{+}$ (respective left plots) and the mutant *synapsin*$^{-}$ flies (respective right plots) for the indicated ISIs. *: $P<$ 0.05/5 for the between-genotype comparison after punishment-learning, and $P<$ 0.05/4 after relief-learning. A grey shading of the boxes indicates $P<$ 0.05/10 and $P<$ 0.05/8, respectively, in comparisons to chance levels (zero). Other details as in Figure 11. **(C)** The median performance indices (PIs) from (B) are plotted across the ISIs. The consistent lessening of scores throughout the ISI function resembles scenario (A1), suggesting a lessening of associative memory in the mutant *synapsin*$^{-}$ flies.

Task-relevant sensory and motor faculties are intact in synapsin^{-} *mutant flies*

To ascertain whether the lessening in strength of both relief- and punishment-memory in the *synapsin*$^{-}$ mutant is actually secondary to some sort of sensory or motor impairment, we test whether behavior towards the to-be-associated stimuli is impaired. This is found not to be the case. There is no between-genotype difference in shock avoidance (Fig. 14A; U= 104, $P>$ 0.05; N= 16, 16). Likewise, *synapsin*$^{+}$ wild-type and *synapsin*$^{-}$ mutant flies do not differ in their behavior towards benzaldehyde (BA) or limonene (LM) (Fig. 14B, C; U-tests: U= 116, 158, $P>$ 0.05 in both cases; N= 19, 16, 20, 16). We conclude that avoidance of the shock as well as of both the odors in question is indistinguishable between genotypes, in experimentally naïve animals.

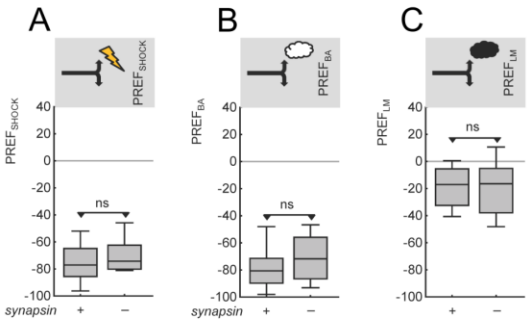

Figure 14: Behavior towards the to-be-associated stimuli is normal in experimentally naive mutant flies lacking Synapsin. Avoidance of the shock **(A)** and of the odors (**B**: BA; **C**: LM) is not different between experimentally naive flies of the two genotypes. ns: $P > 0.05$. Other details as in Figure 11.

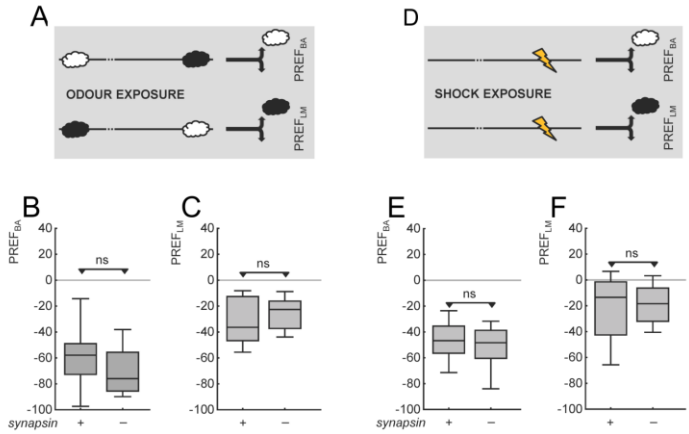

Figure 15: Olfactory behavior is normal in mutant flies lacking Synapsin also after training-like stimulus exposure. Genotypes do not differ in olfactory behavior after either odor exposure **(A)** (**B**: BA, **C**: LM) or shock exposure **(D)** (**E**: BA, **F**: LM). Other details as in Figure 14.

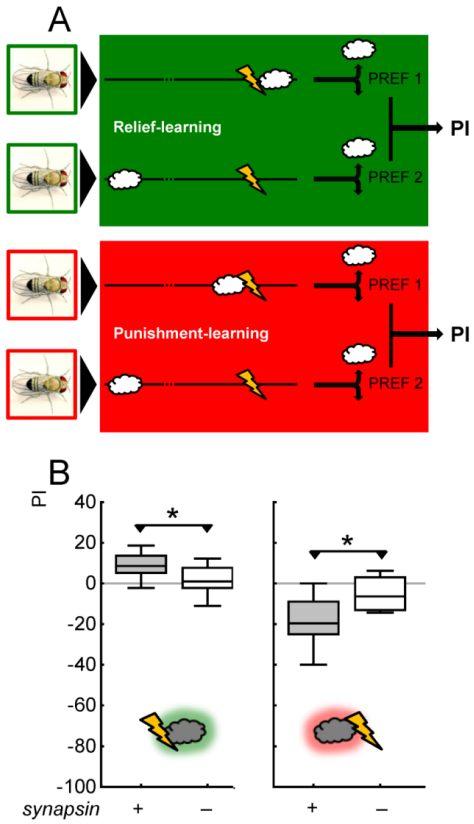

Figure 16: Mutants lacking Synapsin are defective also in non-discriminatory relief- and punishment-learning tasks. **(A)** Schematic of the one-odor versions of the relief- and punishment-learning tasks. The procedure is as in the two-odor version of the paradigm (Figure 11A; Materials and Methods section), except that one odor is omitted. That is, for one-odor relief-learning, the odor (benzaldehyde: BA) is presented upon the cessation of shock, while for punishment-learning the odor is presented before the onset of shock. In both cases, a second experimental group receives unpaired presentations of odor and punishment. The difference in odor preference between paired- and unpaired-trained groups indicates associative memory, and is quantified as performance index (PI). **(B)** The left and right panels show that also in non-discriminatory, one-odor versions of the paradigm, relief-memory and punishment-memory are strongly impaired in mutant flies lacking Synapsin (*synapsin*$^{-}$). Other details as in Figure 11D.

These kinds of control procedure have been state of the art since the first studies of associative odor learning in flies (Quinn et al. 1974; Dudai et al. 1976; Tully and Quinn, 1985). However, testing olfactory behavior in experimentally naïve animals only shows that at the beginning of the learning experiment the mutants are normal in sensory-motor ability – whether these faculties are still intact at the

moment of the test remains unclear (Préat, 1998; Michels et al. 2005; Knapek et al. 2010; see discussion in Gerber and Stocker, 2007). For example, the handling during the experiment, exposure to odors, and/or exposure to the shocks during training can non-associatively alter olfactory behavior (Préat, 1998; Boyle and Cobb, 2005; Colomb et al. 2007; Knapek et al. 2010; Sadanandappa et al. 2013). If the mutant differed from the wild-type in its susceptibility to these effects, the mutant but not the wild-type could be distorted in its olfactory behavior at the moment of test - and this could be mistaken as an associative memory phenotype. Indeed, for isoamylacetate and 1-butanol, *synapsin*$^{+}$ wild-type and *synapsin*$^{-}$ mutant flies differ in olfactory behavior after such exposure (H. Tanimoto, Tohoku University, Sendai, Japan, personal communication). We therefore run two kinds of 'sham training' control: animals are handled just as in normal training, but either the shock is omitted (odor exposure, Fig. 15A) or the odors are omitted (shock exposure, Fig. 15D). After this kind of treatment, we test whether *synapsin*$^{+}$ wild-type and *synapsin*$^{-}$ mutant flies differ in their behavior towards either odor. We do not find any between-genotype differences in these tests (Fig. 15B, C, E, F: U=146, 163, 113, 120, $P > 0.05$ in all cases; N= 16-21). We conclude that also at the moment of test, those sensory and motor faculties that are required to show learned odor behavior are not defective in the *synapsin*$^{-}$ mutant.

Do the synapsin^{-} *mutant defects reflect defects in odor discrimination?*

Both the control procedures for olfactory behavior, the testing of experimentally naïve animals (Fig. 14B, C) and the testing of animals after sham training (Fig. 15), feature a test situation where the flies chose between an odor-arm versus a blank-arm of the T-maze. However, both relief- and punishment-memory are assessed in a choice situation between the trained odor and the control odor (Fig. 11A). The rationale, throughout the literature, for not running the control procedures with two odors as well is that if both odors were (roughly) equally salient, one might obtain choice scores of about zero in both wild-type and mutant flies, but for different reasons: the wild-type flies may be effectively indifferent between the two odors, while the mutants may be anosmic. However, intact performance in one-odor control procedures still allows one to argue that the *synapsin*$^{-}$ mutant defect in two-odor memory tests may not actually be due to defects in memory, but to an inability of the *synapsin*$^{-}$ mutant flies to tell the two odors apart. If this is the case, deficits should not be observed in non-discriminatory tasks, i.e. if one-odor versions of the task are used (Fig. 16A). However, also in these non-discriminatory tasks, deficits in relief- and punishment-memory are found (Fig. 16B; U-tests: U= 67, 21, $P > 0.05$ in both cases; N= 16, 16, 16, 16).

We conclude that the defects of the *synapsin*$^{-}$ mutant flies after relief- and punishment-learning reflect defects in associative memory and are unrelated to odor discrimination ability.

RNAi mediated knock-down phenocopies the relief- as well as punishment-memory defect

We next test whether the defect of the *synapsin*$^{-}$ mutant flies in relief- and punishment-memory is indeed due to the lack of the Synapsin protein, or, despite our outcrossing efforts, to residual differences in genetic background or to side effects of the deletion. To this end, we use an RNAi approach, combining a UAS-RNAi-*syn* effector strain with the broad neuronal driver *elav*-Gal4 (knock down).

A reduction in Synapsin levels (Fig. 17A) leads to reduced relief-memory in the knock-down flies compared to both genetic controls (Fig. 17B; U= 19, 38, $P< 0.05/2$ in both cases; N= 12, 15, 14) (a test across all genotypes yields: $P< 0.05$, H= 12, df= 2). Both genetic controls show small yet significant relief-memory scores ($P< 0.05/3$ in both cases), while the scores of knock-down flies are not significantly different from zero ($P> 0.05/3$). We conclude that relief-memory is intact in control flies but abolished in knock-down flies.

Knock-down flies also show reduced punishment-memory as compared to both genetic control flies (Fig. 17C; U= 47, 48, $P< 0.05/2$ in both cases; N= 13, 19, 15) (a test across all genotypes yields: $P< 0.05$, H= 9, df= 2). All three genotypes show significant levels of punishment-memory ($P< 0.05/3$ in all three cases). We conclude that punishment-memory is reduced, but is not abolished, in flies with reduced levels of Synapsin.

Given that two independent means of reducing Synapsin levels (i.e. the deletion mutant and the RNAi-mediated knock-down) yield concordant memory defects, we conclude that it is the effect upon Synapsin levels, rather than their respectively different potential genetic background or off-target effects, which is responsible for the defects in relief- and punishment-memory.

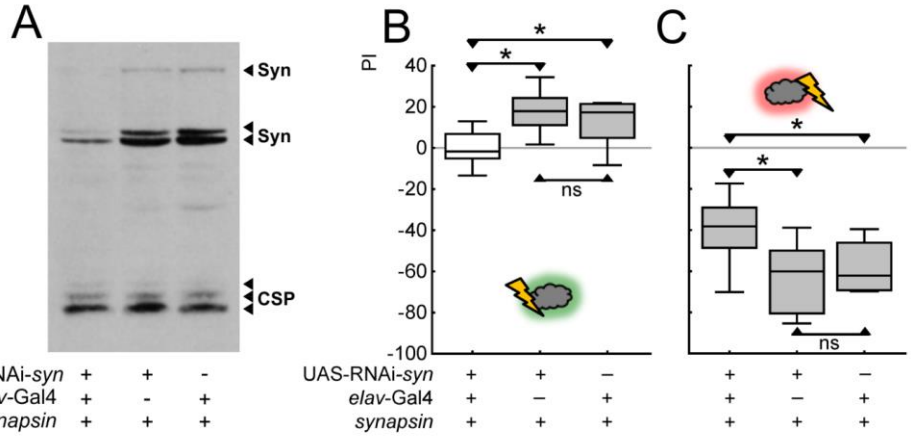

Figure 17. RNAi-mediated knock-down of Synapsin impairs both relief- and punishment-memory. **(A)** Western blot of material obtained from three heads stained for Synapsin, and for CSP as a loading control. The blot is loaded with double heterozygous *elav*-Gal4;; UAS-RNAi-*syn* flies to the left (knock-down), UAS-RNAi-*syn* heterozygous flies in the middle (effector control), and *elav*-Gal4 heterozygote flies to the right (driver control). In the knock-down flies, a reduction of all Synapsin isoforms is apparent. **(B)** Relief-memory is abolished in knock-down flies as compared to controls. **(C)** Punishment-memory is reduced in knock-down flies as compared to controls. *: $P< 0.05/3$ and ns: $P> 0.05/3$ are used for pair-wise comparisons. Grey shading of the boxes indicates significance from chance (zero) at $P< 0.05/3$.

Locally restoring Synapsin restores relief- and punishment-memory

To further scrutinize the role of the Synapsin protein, we test whether restoring Synapsin expression locally in the mushroom bodies, using *mb247*-Gal4 as a driver and UAS-*syn* as an effector in the *synapsin*$^-$ mutant background, can rescue the mutant defect in relief- and/or punishment-memory.

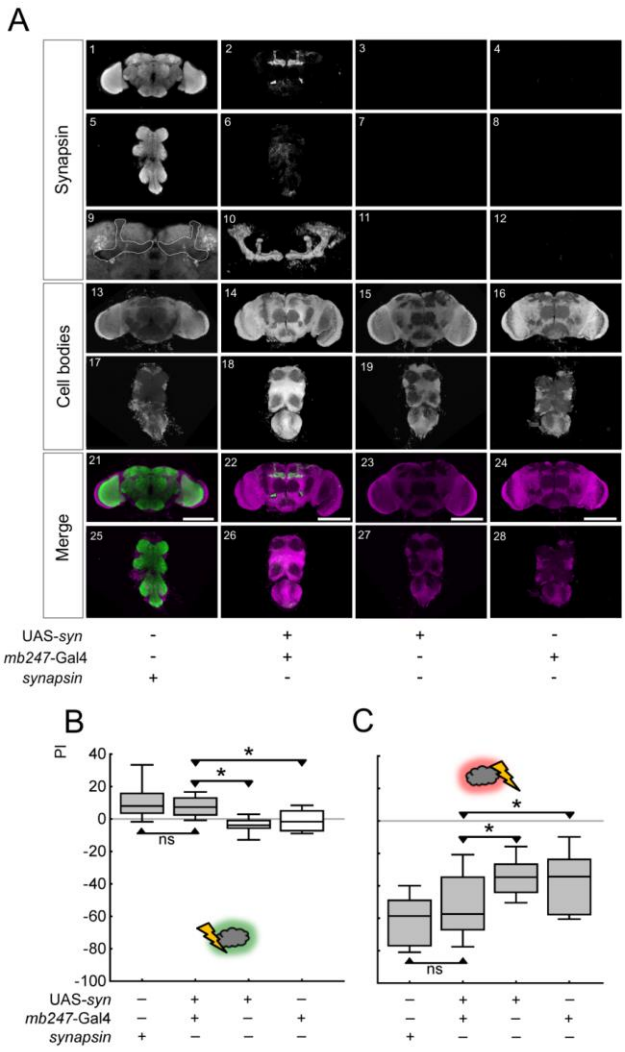

Figure 18. Locally restoring Synapsin restores relief- and punishment-memory. **(A)** Expression pattern of Synapsin in flies of the indicated genotypes. In the bottom row (Merge), anti-Synapsin staining in brains and thoracic nervous systems is shown in green, and cell body counterstaining with propidium iodide is shown in magenta, from 0.9 µm frontal optical sections of the indicated genotypes. In (9-12), the mushroom body regions from (1-4) are shown at higher magnification. In (9) the mushroom bodies are indicated by the stippled line. In (10) the expression of Synapsin using the *mb247*-Gal4 driver is shown as a three-dimensional display. Scale bars: 100 µm. **(B)** Relief-memory of *synapsin*$^-$ mutant flies is fully restored upon rescue expression of Synapsin using the *mb247*-Gal4 driver. **(C)** Punishment-memory, too, is fully restored upon locally expressing Synapsin. *: $P<$ 0.05/3 and ns: $P>$ 0.05/3 are used for pair-wise comparisons. Grey shading of the boxes indicates significance from chance (zero) at $P<$ 0.05/4.

The expression of Synapsin in the rescue flies is indeed restricted to the mushroom bodies (Fig. 18A). Upon such local expression, we find that both relief- and punishment-memory are fully rescued: after relief-learning, rescue flies perform better than both control genotypes (Fig. 18B; U= 26, 48, $P<$ 0.05/3 in both cases; N= 17, 15, 15, 15), and actually do as well as *synapsin*$^+$ wild-type flies (U= 115, $P>$ 0.05/3; N=15, 17) (a test across all genotypes yields: $P<$ 0.05, H= 22, df= 3). We conclude that restoring Synapsin locally in the mushroom body fully restores the *synapsin*$^-$ mutant defect in relief-memory.

Also after punishment-learning, rescue flies perform better than either kind of control flies (Fig. 18C; U= 150, 185, $P<$ 0.05/3 in both cases; N= 26, 26, 26, 26). Indeed, their punishment-memory scores

are as high as those of *synapsin*$^{+}$ wild-type flies (U= 290, P> 0.05/3; N= 26, 26) (a test across all genotypes yields: P< 0.05, H= 30, df= 3). We conclude that the *synapsin*$^{-}$ mutant defect in punishment-memory is also fully restored upon restoring Synapsin locally in the mushroom body.

Taken together, we conclude that restoring Synapsin in the set of cells covered by *mb247*-Gal4, covering the mushroom body alpha, beta and gamma lobes and with faint if any background expression (Aso et al. 2009) (Fig. 18A), is sufficient to restore the defects in both relief- and punishment-memory which ensue upon a lack of Synapsin.

Acutely and locally restoring Synapsin restores relief- and punishment-memory

We next wanted to see whether Synapsin functions during embryonic, larval and pupal development, or rather acutely during adulthood. Using *tub*-Gal80^{ts} we combined the expression of Gal80^{ts}, a temperature-dependent inhibitor of Gal4, together with *mb247*-Gal4 as a driver and UAS-*syn* as an effector. Thus, the induction of Synapsin in the mushroom bodies can be achieved by raising the temperature acutely only during adulthood, which inactivates Gal80^{ts}, releases the inhibition of Gal4, and allows Synapsin expression in the *mb247*-Gal4 pattern (compare Fig. 19A10 to Fig. 20A10). As compared to constitutive expression of Synapsin, such induced expression is notably faint (compare Fig. 18A22 to Fig. 20A22).

In control conditions without induction, relief-memory scores in the experimental genotype are less than in the *synapsin*$^{+}$ wild-type flies (Fig. 19B: U= 46, P< 0.05/3, N= 21, 21), and not different from effector- and driver-control (U= 116, 68, P> 0.05/3, N= 21, 21). Acute induction of Synapsin expression restores relief-memory to levels indistinguishable from those observed in *synapsin*$^{+}$ wild-type flies (Fig. 20B: U= 151, P> 0.05/3, N= 16, 16). Indeed, relief-memory scores upon acute Synapsin expression are higher than in effector- and in driver-control flies (U= 99, 116, P< 0.05/3; N= 16, 16).

Likewise, after punishment-learning acute Synapsin expression supports a full rescue in comparison to *synapsin*$^{+}$ wild-type flies (Fig. 20C: U= 231, P> 0.05/3, N= 25, 22); these flies indeed show stronger punishment-memory scores than both the effector- and driver-control (U= 135, 117, P< 0.05/3 in both cases, N= 22, 19). Without induction, punishment-memory remains at levels less than in *synapsin*$^{+}$ wild-type flies (Fig. 19C: U= 38; P< 0.05/3, N= 15, 12) and indistinguishable from effector- and driver-control flies (U= 69, 51, P> 0.05/3 in both cases, N= 13, 12).

We conclude that restoring Synapsin at fairly low levels, acutely during adulthood, and locally in the mushroom bodies, restores the *synapsin*$^{-}$ mutant defects in both relief- and punishment-memory.

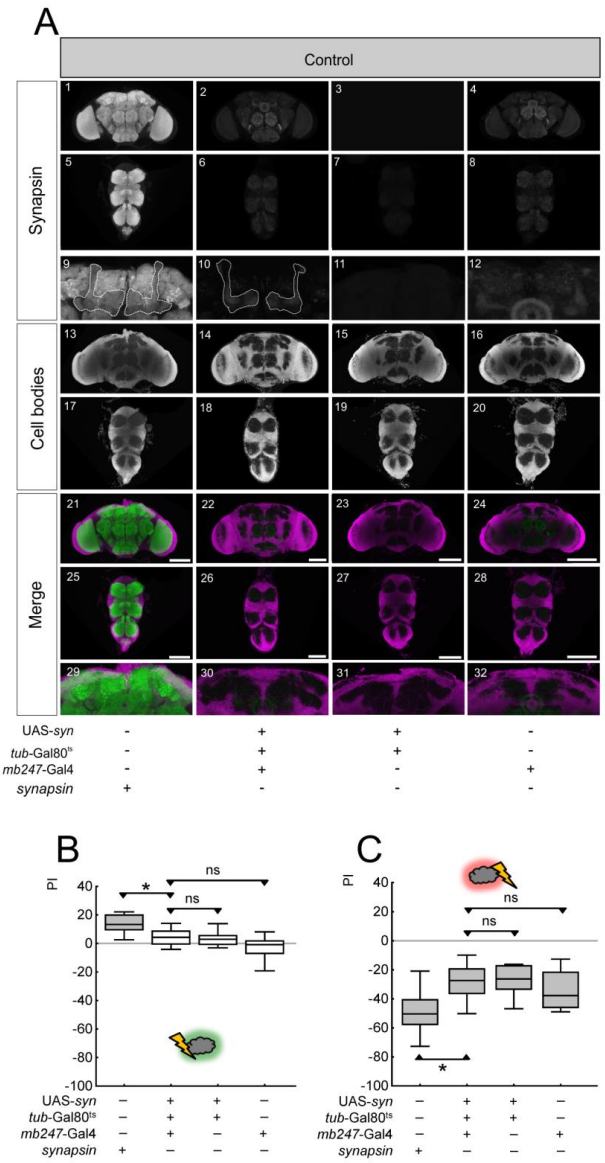

Figure 19. Relief- and punishment memory remain impaired in control conditions without acute and local restoration of Synapsin. **(A)** Expression pattern of Synapsin in uninduced control flies of the indicated genotypes. In the bottom rows (Merge), anti-Synapsin staining in brains and thoracic nervous systems is shown in green, while cell body counterstaining with propidium iodide is shown in magenta. The mushroom body region of (A1-4) is shown at higher magnification in (A9-12). The stippled line in (A9, 10) indicates the mushroom body neuropil. The mushroom body region of (A21-24) is shown at higher magnification in (A29-32). Note the absence of anti-Synapsin staining in (A10, 30). Scale bars: 100 µm.
(B-C) In un-induced control conditions, relief- (B) and punishment-memory (C) remain abolished in the experimental genotype. *: $P < 0.05/3$ and ns: $P > 0.05/3$ are used for pair-wise comparisons. Grey shading of the boxes indicates significance from chance (zero) at $P < 0.05/4$.

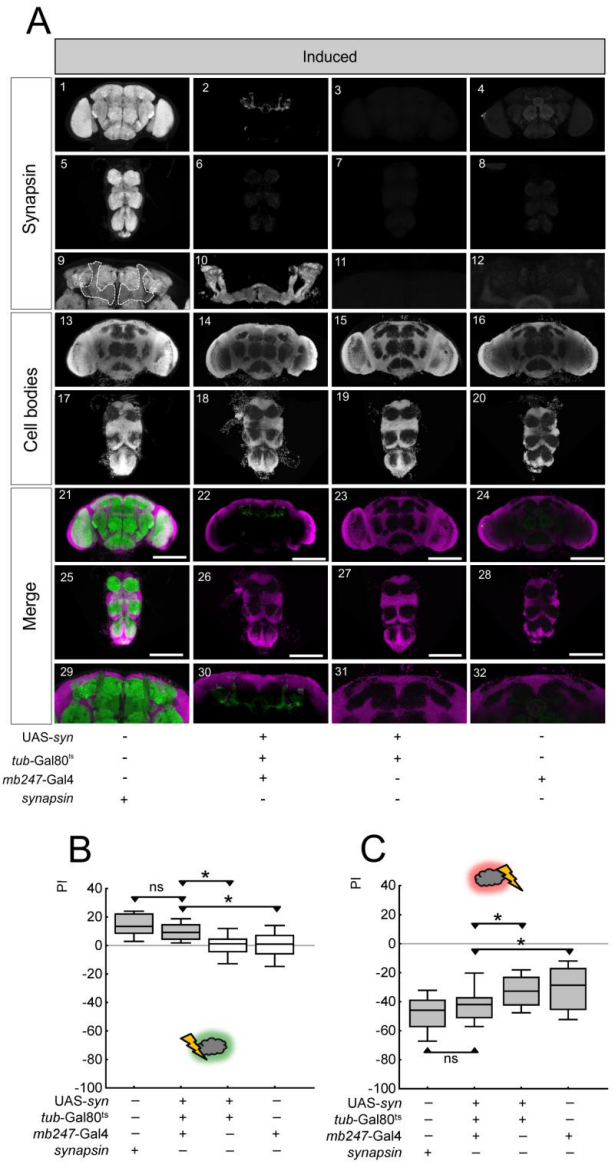

Figure 20. Acutely and locally inducing Synapsin expression restores relief- and punishment-memory. **(A)** Expression pattern of Synapsin in induced rescue flies of the indicated genotypes. In the bottom row (Merge), anti-Synapsin staining in brains and thoracic nervous systems is shown in green, while cell body counterstaining with propidium iodide is shown in magenta. The mushroom body region of (A1-4) is shown at higher magnification in (A9-12). The stippled line in (A9) indicates the mushroom body neuropil. In (A10) the expression of Synapsin induced by the *mb247*-Gal4 driver is shown as a three-dimensional display. The mushroom body region of (A21-24) is shown at higher magnification in (A29-32). Scale bars: 100 µm. **(B)** Relief-memory of *synapsin*$^{-}$ mutant flies is restored upon acutely induced local expression of Synapsin using the *mb247*-Gal4 driver in combination with Gal80^{ts}.
(C) Punishment-memory, too, is fully restored upon acutely and locally expressing Synapsin. *: $P < 0.05/3$ and ns: $P > 0.05/3$ are used for pair-wise comparisons. Grey shading of the boxes indicates significance from chance (zero) at $P < 0.05/4$.

Discussion

We report Synapsin as a determinant for memory strength in a behavioral assay of timing-dependent associative plasticity (Fig.s 11, 13, 16-19). The defects upon a lack of Synapsin do not reflect any task-relevant sensory-motor impairment, as manifest either in naïve responsiveness to odors or shock (Fig. 14) or in responsiveness to the odors after sham-training procedures (Fig. 15). Also, the outcrossing regimen for the mutant, the RNAi-phenocopy of the mutant phenotype, and the spatially as well as temporally-specific rescue make it highly unlikely that effects of genetic background or off-target effects of our genetic manipulations impact these results.

The 'fingerprint' of Synapsin dysfunction is distinct from what has been found for the w^{1118} mutation, which shifts the overall balance between relief- and punishment-memory towards generally more negative valence (Yarali and Krischke et al. 2009). That is, in the w^{1118} mutants any event including an electric shock establishes a more negative net effect in memory. Fittingly, Nakamura et al. (1999) reported a relation of genetic variance in the human ABCG1 gene, the homolog of the *white* gene, to the susceptibility for panic and mood disorders.

We find that punishment-memory is partially but not completely abolished both in Synapsin null-mutant flies and upon an RNAi-mediated knock-down of Synapsin. The observation that this defect is partial is in agreement with what Godenschwege et al. (2004) and Knapek et al. (2010) reported for the Synapsin null-mutant, namely a 25 - 30 % decrement in punishment-memory scores. A somewhat stronger yet still partial defect (50 %) was also seen after larval odor-sugar learning (Michels et al. 2005, 2011). Interestingly, in wild-type flies an application of cold-shock between training and test induces a partial amnesia of punishment-memory, and the residual amnesia-resistant memory does not require Synapsin (Knapek et al. 2010). Given that relief-memory is fully abolished upon loss-of-function of Synapsin, one would thus reason that cold-shock should fully abolish relief-memory – and intriguingly this is what Diegelmann, Preuschoff et al. (2013) have found. The emerging scenario is thus that punishment-learning establishes two short-term memory components: one that is Synapsin-dependent and amnesia-sensitive, and one that is Synapsin-independent and amnesia-resistant. In contrast, relief-learning establishes only Synapsin-dependent and amnesia-sensitive short-term memory. This implies that if in a natural succession of events a traumatic experience were to induce both relief- and punishment-memory for the stimuli experienced respectively after and before the trauma, an amnesic cold-shock treatment would leave only punishment-memory intact.

Our observation that restoring Synapsin in the Synapsin null-mutant fully restores both relief- and punishment-memory scores (Fig. 18) demonstrates that it is the absence of the Synapsin protein that causes the defects in associative memory, rather than other effects of the deletion or of differences in

genetic background that remained despite our extensive outcrossing regimen. The current study is thus the first actually to prove a role of the Synapsin protein in the associative memory of adult *Drosophila*.

The rescue of associative memory was obtained by restoring Synapsin both in a temporally specific way, acutely only during adulthood (Fig. 20), and in a spatially specific way, in the mushroom body (Fig.s 18 and 20). Notably, this was the case for both relief- and punishment-memory. This prompts the question how the fly brain is organized on the cellular and/or molecular level so as to establish memories that support opposite behaviors (i.e. conditioned avoidance *versus* approach). For such a discussion, a brief sketch of the current working hypothesis on punishment-learning is warranted (reviews include Heisenberg, 2003; Gerber et al. 2004; Davis, 2005; Margulies et al. 2005; Keene and Waddell, 2007; Kahsai and Zars, 2011; Gerber et al. 2014; see also the seminal recent work of Aso, Hattori et al. 2014, and Aso, Sitaraman et al. 2014):

- A given odor activates a specific combination of olfactory sensory neurons, according to the ligand profile of the expressed receptor protein. Sensory neurons expressing a given receptor converge at typically a single glomerulus in the antennal lobe; within these glomeruli they provide output to olfactory projection neurons. The pattern of activated projection neurons is additionally shaped by lateral connections between glomeruli. The olfactory projection neurons further connect to two target areas, the mushroom body and the lateral horn. Thus, dependent on receptor expression, ligand profile and connectivity within this system, odors can be coded combinatorially across these ascending pathways. Both mushroom body and lateral horn then connect to premotor circuitry.
- Processing of the electric shock is less well understood. What is clear is that shock activates a subset of dopaminergic neurons mediating an internal punishment signal. These neurons provide input (*mb-input* neurons) to a sufficient number of mushroom body neurons to cover the full olfactory stimulus space (Ito et al. 1998; Schwaerzel et al. 2003; Riemensperger et al. 2005; Kim et al. 2007; Aso et al. 2009; Claridge-Chang et al. 2009; Mao and Davis, 2009; Aso et al. 2010, 2012; Burke et al. 2012; Pech et al. 2013; Das et al. 2014; Galili et al. 2014; larval *Drosophila*: Schroll et al. 2006; Selcho et al. 2009).
- Thus, only in an odor-specific set of mushroom body neurons does a coincidence of odor- and shock-induced activity take place, resulting in a modulation of connection between these mushroom body neurons and their postsynaptic partners, the mushroom body output neurons (*mb-output* neurons). The AC-cAMP-PKA signalling cascade is one of the necessary processes for detecting and enacting this coincidence (Zars et al. 2000; Thum et al. 2007; Blum et al. 2009; Tomchik and Davis, 2009; Boto et al. 2014; see Gervasi et al. 2010 for appetitive learning). Arguably Synapsin is one of the relevant targets of this cascade in the context of associative learning (Michels et al. 2011). If after training the learned odor is encountered again, activity in

the *mb-output* neurons – because of their modified input from the mushroom body neurons – is altered such that learned odor avoidance can take place (Séjourné et al. 2011; Menzel, 2012). Non-trained odors do not support conditioned avoidance – unless sufficiently similar in quality and/or intensity to the trained one (Yarali and Ehser et al. 2009; Niewalda et al. 2011; Campbell et al. 2013; Barth et al. 2014).

The mushroom body is under intense study with respect to the organization of punishment-learning *versus* reward-learning. The emerging picture is that punishment- and reward-learning may engage different sets of mushroom body neurons as well as different input and output neurons – as if the mushroom body were internally multiplexed according to valence (*mb*: Perisse et al. 2013; Boto et al. 2014; *mb-input*: Liu et al. 2012; *mb-output*: Séjourné et al. 2011; Plaçais et al. 2013; Aso, Hattori et al. 2014; Aso, Sitaraman et al. 2014). With respect to the *mb-input* neurons, those dopaminergic neurons that are required for punishment-learning, defined for example by the *TH*-Gal4 driver, are dispensable for relief-memory (Yarali and Gerber, 2010). It is not known which *TH*-Gal4 negative *mb-input* neurons and which *mb-output* neurons are participating in relief-learning. Whether different subsets of the mushroom body neurons harbour the memory trace after relief- and punishment-learning likewise remains to be determined.

Alternatively, relief- and punishment-memory may be dissociated at the level not of the Synapsin protein as such, but at the level of its phosphorylation pattern. Given the 28 phosphorylated sites of Synapsin, targeted by up to 11 different kinases (Fig. 12, Table 2) (see also Michels et al. 2011; Nuwal et al. 2011; Diegelmann, Klagges et al. 2013; Sadanandappa et al. 2013), it is conceivable that different kinases and/or phosphorylation sites of the Synapsin protein could be employed during relief- and punishment-learning. Likewise, the mRNA-editing observed for one of the phosphorylation sites of Synapsin (Diegelmann et al. 2006) could be selectively involved in relief- and punishment-learning, in particular as the proteins corresponding to both the edited and the non-edited version are indeed expressed (Fig. 12, Table 2).

To summarize, the current results regarding Synapsin point to shared genetic and molecular determinants for relief- and punishment-memory in *Drosophila*. Given our shared evolutionary heritage in general and the conserved role of Synapsin in mammalian associative memory in particular, this may caution against systemic pharmacological approaches for reducing excessively strong punishment-memory in humans, for example after traumatic experiences. This is because such traumatic experiences may induce both relief- and punishment-memory. If the target of the pharmacological treatment affects both memories, a systemic drug treatment will unwittingly reduce relief-memory as well and may thus have a net detrimental effect (for a more detailed discussion see Gerber et al. 2014). For example in the case of Synapsin, a pharmacological erasure of Synapsin-dependent memory would abolish relief-memory but would leave

punishment-memory partially intact (see also the discussion of cold-shock amnesia above). In this sense, and with due caveats in mind, the current study can help in avoiding adverse effects of medical treatment after traumatic experiences.

Materials and Methods

Genotypes and rearing of flies

To compare flies with *versus* without Synapsin, we compared the deletion mutant *syn*^{97CS} (Fig. 11) to a Canton-S wild-type strain. The *syn*^{97CS} strain had undergone 13 outcrossing steps to this very CS wild-type strain to yield effectively identical genetic backgrounds (described and used in Godenschwege et al. 2004; Michels et al. 2005, 2011). To ensure a stably identical genetic background over the course of this project, 13 further outcrossing steps were undertaken. For simplicity, these strains are referred to as *synapsin*$^{-}$ for the deletion mutant and *synapsin*$^{+}$ for the wild-type throughout this study.

We used the following parental driver and effector strains (in addition to the mentioned status, all strains are homozygous *w*^{1118}):

- *elav*-Gal4 [X] (strain c155 of Lin and Goodman, 1994);
- *mb247*-Gal4 [III], *synapsin*$^{-}$, which was generated by using *mb247*-Gal4 (Schulz et al. 1996), recombined into the *synapsin*$^{-}$ mutant background;
- UAS-*syn* [III], *synapsin*$^{-}$, which was generated on the basis of Löhr et al. (2002);
- *tub*-Gal80^{ts} [II]; UAS-*syn* [III], *synapsin*$^{-}$ (Michels et al. 2011);
- UAS-RNAi-*syn* [III] (Michels et al. 2011).

For experiments we used the F1 progeny of the following crosses:

- RNAi: To knock down Synapsin, *elav*-Gal4 [X] females were crossed to UAS-RNAi-*syn* [III] males, yielding double-heterozygous *elav*-Gal4/+;; UAS-RNAi-*syn*/+ flies. As an effector control, females without any transgene were crossed to males carrying UAS-RNAi-*syn*, yielding single-heterozygous UAS-RNAi-*syn*/+. As a driver control, female *elav*-Gal4 were crossed to males without any transgene, yielding single-heterozygous *elav*-Gal4/+.
- Rescue: To rescue Synapsin, female *mb247*-Gal4 [III], *synapsin*$^{-}$ flies were crossed to male UAS-*syn* [III], *synapsin*$^{-}$, yielding double-heterozygous *mb247*-Gal4/UAS-*syn* flies in the homozygous *synapsin*$^{-}$ mutant background. As a driver control, female *mb247*-Gal4, *synapsin*$^{-}$ were crossed to male *synapsin*$^{-}$, yielding single-heterozygous *mb247*-Gal4/+ in the homozygous *synapsin*$^{-}$ mutant background. As an effector control, *synapsin*$^{-}$ females were crossed to male UAS-*syn*, *synapsin*$^{-}$ to yield single-heterozygous UAS-*syn*/+ in the homozygous *synapsin*$^{-}$ mutant background.
- Induced rescue: To restrict expression of Synapsin to the adult stage we made use of the temperature-inducible transgene *tub*-Gal80^{ts}. We crossed female *mb247*-Gal4 [III], *synapsin*$^{-}$ to

male *tub*-Gal80^{ts} [II]; UAS-*syn* [III], *synapsin*$^{-}$, yielding triple-heterozygous *tub*-Gal80^{ts}/+; *mb247*-Gal4/UAS-*syn* in the homozygous *synapsin*$^{-}$ mutant background. As an effector control we used the offspring of female *synapsin*$^{-}$ and male *tub*-Gal80^{ts}; UAS-*syn*, *synapsin*$^{-}$ to yield double-heterozygous *tub*-Gal80^{ts}/+; UAS-*syn*/+, in the homozygous *synapsin*$^{-}$ mutant background. As a driver control, *mb247*-Gal4, *synapsin*$^{-}$ females were crossed to male *synapsin*$^{-}$ to yield heterozygous *mb247*-Gal4/+ in the homozygous *synapsin*$^{-}$ mutant background.

We note that preliminary experiments using *elav*-Gal4 as a driver strain did not result in a rescue of associative memory (not shown); this is in accordance with the lack of rescue observed when using this driver to restore *rutabaga* function (Zars et al. 2000).

All flies were kept in mass culture at 25 °C, 60-70 % humidity and a 16/8 hour light/ dark cycle. Unless mentioned otherwise, one- to five day-old flies were collected and kept at 18 °C until the following experimental day. Experiments were performed at 22-25 °C and 75-85 % relative humidity. For the induced rescue experiment, flies were raised at 18 °C and, after collection, either shifted to 30 °C for 4 days to allow inactivation of Gal80^{ts} and thus expression of Synapsin (Induced), or retained at 18 °C for 4 days (Control).

Throughout, we used flies in groups of about 100, handled in a tube system based on Tully and Quinn (1985) modified to allow the handling of four groups of flies in parallel. Training was performed in dim red light to allow sight for the experimenter (but not for the flies); tests were run in darkness. The electric shock was applied via an electrifiable grid, covering the inner side of the training tubes. A vacuum pump ensured removal of odor-saturated air. As odorants, 80 µl benzaldehyde (BA; CAS number 100-52-7; Fluka, Steinheim, Germany) or 110 µl limonene (LM; CAS number 5989-27-5; Sigma-Aldrich, Steinheim, Germany) were applied in Teflon containers of 5 mm or 7 mm diameter, respectively.

Learning experiments and behavioral controls

For punishment- as well as for relief-learning, flies received 6 training trials. In the following example, BA features as the to-be-learned odor and LM as the control odor. At time 0:00 (min:s), flies were loaded into the experimental set-up, which took approx. 1 min. After an additional accommodation period of 3 min, LM was presented for 15 s. Then, for punishment-learning, BA was presented from 7:15 to 7:30. At 7:30, the electric shock was delivered. Thus, for punishment-learning, the interstimulus interval (ISI) between the onset of the shock and the onset of BA was –15 s. The shock consisted 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, the flies were transferred back to food vials for 16 min until the next trial started.

For relief-learning, all parameters were identical, except that BA was presented from 8:20 to 8:35, leading to an shock-offset to odor-onset interval of 25 s, which corresponds to the optimal delay in this paradigm (Tanimoto et al. 2004; Yarali et al. 2008; Yarali and Krischke et al. 2009).

In half of the cases, BA served as the to-be-learned odor and LM as the control, while these assignments were swapped in the other half.

For the experiment describing the inter-stimulus-interval functions in *synapsin*$^{+}$ wild-type and *synapsin*$^{-}$ mutant flies, the timing of odor and shock was as indicated in the Results section.

Once training was completed, a 16 min break was given before the animals were again loaded into the set-up for the memory test. After an accommodation period of 5 min, the animals were transferred to the choice point of a T-maze, where they could choose between BA and LM. After 2 min, the arms of the maze were closed and the number of animals (denoted # in the following) within each arm was counted. The relative preference between BA and LM (PREF) was then calculated as:

(1) $PREF = (\#_{BA} - \#_{LM}) \times 100 / \#_{Total}$

The difference in PREF scores between the two reciprocally trained sets of flies was then calculated to obtain an index of associative memory performance (PI) that ranges between -100 and 100, and indicates conditioned approach by positive PIs and conditioned avoidance by negative PIs (the papers by Tully and Quinn (1985) as well as Gerber and Stocker (2007) feature discussions of why these PIs yield measures of associative memory, cleared of non-associative effects):

(2) $PI = (PREF_{1:BA\ to\text{-}be\text{-}learned} - PREF_{2:LM\ to\text{-}be\text{-}learned}) / 2$

All procedures were the same for the one-odor version of the tasks, except that LM was omitted.

Behavioral controls

To test for shock avoidance, the flies were loaded into the experimental set-up. After an accommodation period of 2 min, the red light was switched off and flies were transferred to the choice point where they could enter either arm of the maze; ten seconds later, shock was applied in one arm of the maze as specified above. Ten seconds after the onset of the last shock pulse, the maze was closed and the flies were collected and counted. A preference index for the shock ($PREF_{SHOCK}$) was calculated to provide negative values for avoidance of the electrified arm:

(3) $PREF_{SHOCK} = (\#_{Electrified\ arm} - \#_{Non\text{-}electrified\ arm}) \times 100 / \#_{Total}$

To assess olfactory behavior, the flies were loaded into the experimental set-up. After an accommodation period of 4 min, the red light was switched off and the flies were brought to the choice point of the T-maze and allowed to choose between a blank arm with air only and the other arm scented with odor (either

BA or LM); after 2 min, the maze was closed and the flies were collected and counted. A preference index ($PREF_{ODOR}$) was calculated as:

(4a) $PREF_{BA} = (\#_{BA} - \#_{Air}) \times 100 / \#_{Total}$

(4b) $PREF_{LM} = (\#_{LM} - \#_{Air}) \times 100 / \#_{Total}$

Please note that, ever since their introduction as a control procedure in *Drosophila*, these tests for olfactory behavior do not involve a choice between the two odors; this is because otherwise a failure in the ability to detect the odors in the mutant could not be distinguished from an indifference between the two odors in the wild-type.

For the odor-exposure and shock-exposure controls, the flies were handled as in normal training, except that we omitted either the shock (in the case of the odor-exposure control) or the odors (in the shock-exposure control). Then, the preference towards BA and towards LM was measured as described in the preceding paragraph (equations 4a and 4b).

Statistical analyses of behavioral data

Non-parametric statistics were used throughout. Kruskal-Wallis or Mann-Whitney U-tests (KW or MWU) were used to compare multiple or two groups of flies, respectively. To test for differences from zero, we used one-sample-sign-tests (OSS). The significance level was $P < 0.05$. For multiple comparisons within a dataset, *P*-levels were adjusted by a Bonferroni correction ($P < 0.05$ divided by the number of comparisons), a conservative approach to maintain the experiment-wide error-rate below 5 %. Data were plotted as box plots, representing the median as the middle line, the 25 % and 75 % quantiles as boundaries of the box and the 10 and 90 % quantiles as whiskers.

Single fly PCR

PCRs were carried out according to Gloor et al. (1993), using material from individual flies. 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. 11).

Immunohistochemistry and Western blotting

For whole-mount immunohistochemistry, brains were dissected in Ringer's solution and fixed for 2 h in 4 % formaldehyde with PBST as the solvent (phosphate-buffered saline containing 0.3 % Triton X-100). Samples were blocked in 3 % normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and subsequently incubated overnight with the mouse monoclonal anti-Synapsin

antibody SYNORF1 3C11 (DSHB, Iowa, USA; diluted 1:20 in PBST) (Klagges et al. 1996; Godenschwege et al. 2004). The sample was then incubated overnight with an Alexa488-coupled goat anti-mouse Ig (diluted 1:200 in PBST) (Invitrogen Molecular Probes, Eugene, OR, USA) to detect the primary antibody. All incubation steps were followed by multiple PBST washes. Incubations with antibodies were done at 4 °C; all other steps were performed at room temperature. The brains were mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA), containing propidium iodide for counterstaining of DNA and hence of cell nuclei. Preparations were examined under a confocal microscope.

For Western blots, three adult heads per lane were homogenized in 10 µl Laemmli-buffer. The sample was heated to 70 °C for 5 min and centrifuged for 2 min before electrophoresis. Proteins were separated by 12.5 % SDS-PAGE in a Multigel chamber (100 mA, 2 h; Peqlab, Erlangen, Germany) and transferred to a nitrocellulose membrane (Kyhse-Andersen, 1984). The membrane was blocked overnight (5 % milk powder in 1 x TBST). Immunoreactions were successively performed with two mouse monoclonal antibodies: SYNORF1 for Synapsin detection (Klagges et al. 1996) (dilution 1:100) and ab49 (Zinsmaier et al. 1990, 1994) (DSHB, Iowa, USA; dilution 1:133) for detection of the Cysteine String Protein (CSP; Arnold et al. 2004) as a loading control (incubation time: 1.5 hours). The membrane was then incubated with the secondary antibody (goat anti-mouse IgG-HRP coupled, 1:3700; 1 h). The incubation steps were followed by multiple washing procedures (1 x TBST). Visualization was achieved with the ECL Western blot detection reagents (Amersham Bioscience, Freiburg, Germany).

Analysis of Synapsin phosphorylation by LC-MS/MS

Brains of experimentally naïve adult *Drosophila* were dissected in Ringer's solution containing halt protease and phosphatase inhibitor cocktail, EDTA-free (1:100; Thermo Fisher Scientific, Waltham, USA) to prevent enzymatic protein degradation. Samples were then stored at -80 °C in a liquid-free manner. Five adult brains were resolubilized in 50 µl water containing 8 M freshly deionized urea. Tissue and cell destruction was achieved by means of a micro glass potter and pulsed sonification on ice for 1 h. Biological membranes were destroyed and membrane proteins solubilized by the addition of 200 µl of 50 mM NH_4HCO_3 buffer (pH 8.0) containing 2 mM dithiothreitol and 1 % (w/v) RapiGest SF surfactant (Waters Corp., Milford, USA) to obtain a final concentration of 2 M urea and 0.75 % RapiGest. After gently shaking at room temperature for 60 min, 10 mM methyl methane thiosulfonic acid was added for an additional 1 h for thiomethylation of previously reduced cysteins. Limited proteolysis was started by adding 1 µg trypsin (Promega, Trypsin Gold, Fitchburg, USA) followed by incubation at room temperature for 12 h. After complete digestion, RapiGest was hydrolysed by adding trifluoric acetic acid (TFA) to a final concentration of 1 % and incubation for 1 h, at room temperature. Precipitated RapiGest

fragments were spun down at 10 000 × g for 10 min and the supernatant was applied to an empore universal resin SPE-column (3M, St. Paul, USA), equilibrated with 2 ml methanol and subsequently washed with 0.1 % TFA. Resin-bound peptides were washed with 5 ml of 0.1 % TFA and eluted twice with 0.5 ml of 70 % acetonitrile (ACN), 0.1 % TFA. Eluates were pooled and dried in a vacuum evaporator centrifuge (Savant, Thermo Fisher Scientific, Waltham, USA). Proteome analysis was performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Scientific, San Jose, USA) equipped with an EASY-nLC ultra HPLC (Thermo Scientific, San Jose, CA). Samples were resolubilized in 50 µl of 0.1 % TFA, 2 % ACN and divided in five 10 µl aliquots as technical replicates, corresponding to a sample amount of one adult brain per injection, on a 75 µm I.D., 25 cm PepMap C18-column, packed with 2 µm resin (Dionex, Sunnyvale, USA). Separation was achieved by applying a gradient from 2 % ACN to 35 % ACN in 0.1 % formic acid over a 220 min gradient at a flow rate of 300 nl/min. The LTQ Orbitrap Velos Pro MS used exclusively CID-fragmentation with wideband activation (pseudo MS3 for neutral losses of phosphate residues) when acquiring MS/MS spectra. The spectra acquisition consisted of an orbitrap full MS scan followed by up to 15 LTQ MS/MS experiments (TOP15) on the most abundant ions detected in the full MS scan. Essential MS settings were as follows: full MS (FTMS; resolution 60,000; m/z range 400-2000); MS/MS (Linear Trap; minimum signal threshold 500; wideband isolation; dynamic exclusion time setting 30 s; singly-charged ions were excluded from selection). Normalized collision energy was set to 35 %, and activation time to 10 ms.

Raw data processing, protein identification and phosphopeptide assignment of the high resolution orbitrap data were performed by PEAKS Studio 7.0 (Bioinformatics Solutions, Waterloo, Canada). False discovery rate (FDR) was set to < 1 %. Phosphosites were accepted as confident for $P < 0.005$ (modified t-test, included in PEAKS Studio 7.0). The prediction of putative kinases responsible for the motif-specific phosphorylations was performed using the NetPhosK 1.0 Server (Blom et al. 2004).

General discussion

How does the brain work? This fundamental question runs like a red thread through the work of nearly every neurobiologist. The brain is probably one of the most complex organs that developed during evolution and is capable to fulfill many complex tasks. A muscle for example has basically only one task, to contract and to relax. In contrast, the brain accomplishes many fascinating tasks, many of those we are not even aware of. For example, it ensures that visual and auditory stimuli are properly processed and thereby allowing us to see and to hear. It makes sure that we don't fall when walking or standing by, usually subconsciously, coordinating our movement, holding the balance and keeping us thus literally on our feet. Another fascinating feature of the brain is its plasticity enabling memory formation, which allows us to gain experience and to remember it. The present thesis addresses this topic dealing with the interesting question of how a memory is established and how memories and experiences are translated into learned behavior. Here the focus is on the presynaptic protein Synapsin and how it contributes to different types of learning and memory processes.

Chapter I investigate the role of Synapsin in associative odor-sugar reward learning in *Drosophila* larvae. Larvae were trained to associate a certain odor with a sugar reward. Therefore the larvae were presented an odor together with a sugar reward and in a subsequent test animals will approach the previously rewarded odor in expectation of sugar. It was reported before that Synapsin is necessary for reward learning and for the establishment of a proper reward memory (Michels et al. 2005). Synapsin deletion mutants (syn^{97}) that cannot express Synapsin showed a significant impairment in reward memory compared to wild-type larvae. This fact was taken up in Chapter I and further investigated. For this purpose, in an associative odor-sugar learning experiment, either the odor or the sugar concentration were varied or the time interval between training and test. Interestingly, it turned out that only for the highest odor concentration a difference in learning performance between the wild-type and the syn^{97} mutant can be observed while for lower concentrations no difference in memory scores between genotypes were detectable (Fig. 7A-B). Similarly, for different sugar concentrations it could be shown that wild-type larvae learn better only if higher sugar concentrations are used as reward (Fig. 7C). The same logic applies to the time interval between training and test (Fig. 8). Immediately after training wild-type larvae show higher memory scores compared to the syn^{97} mutant but already after a five-minute interval differences in memory performance are below limit of detection. Accordingly, the role of Synapsin depends on the nature of the to-be-learned task. The results suggest that Synapsin is only required for tasks that are "easy" to learn (high odor or sugar concentration, short time interval between training and test). For such salient events the animals in principal can learn and remember without Synapsin but with Synapsin they seem to learn better and establish stronger memories. For highly salient events Synapsin seems to function as a

natural cognitive enhancer that raises the upper limits of mnemonic capacity. Hence it seems to be required to learn more efficiently. In this context this work contributes to a better understanding of how memory strength is tailored to task salience. The key question in this regard is whether there is a protein dependent basis for the difficulty of a task? This is also relevant from the medical point of view because it could help to find a treatment for patients suffering from learning and memory deficits.

Chapter II deals with the role of Synapsin in two further kinds of associative learning paradigms, namely punishment learning (Tully and Quinn 1985; Godenschwege et al. 2004; Knapek et al. 2010; Walkinshaw et al. 2015) and pain-relief learning (Tanimoto et al. 2004; Yarali et al. 2008; Gerber et al. 2014) in the adult fly. Punishment learning is similar to reward learning but instead of a reward a punishment is associated with an odor. Flies were presented an odor followed by an electric shock, in a subsequent test flies will avoid this previously punished odor (Tully and Quinn 1985). For pain-relief learning the animals are also supposed to associate a certain odor with an electric shock but here the odor is presented after the electric shock instead of before the electric shock like for punishment learning (Tanimoto et al. 2004). Hence the odor is presented at the offset of the electric shock, at a moment of pain-relief. Therefore the odor is associated with something "good" (end of pain) and in a subsequent test animals will approach this odor. Please note that the only difference between punishment learning and pain-relief learning is the sequence of odor and shock presentation. Interestingly this change in sequence of odor and electric shock shifts the behavior from avoidance to approach. Accordingly timing matters, a topic that is discussed in detail later. As mentioned above chapter II addressed the requirement for Synapsin in these two different types of learning paradigms and it could be shown that Synapsin is required for both: proper punishment learning and proper pain-relief learning (Fig.s 11, 13, 16-19). Animals lacking Synapsin showed a significant reduction in memory performance compared to wild-type after both learning procedures. Relief from pain can be seen as relief from a bad situation. Therefore understanding pain-relief learning may contribute to identify possibilities to counteract undesired avoidance or undesired approach behavior. This is particularly relevant with regard to psychopathologies like drug addiction or anxiety disorders as well as traumatic experiences as they massively influence our behavior and can lead to avoidance behavior and/ or undesired psychological states such as stress, anxiety or even panic. Concerning those psychopathologies, it is easy to imagine that the offset of e.g. a panic attack or withdrawal symptoms have a reward-like effect on coincidentally presented stimuli, which then will be approached, which may lead to a maintenance of the disorder (Andreatta et al. 2010). The potential impact of Synapsin for pathologies is further discussed with two examples, namely self-cutting and arachnophobia in the later discussion. It is also very interesting to investigate whether punishment and pain-relief learning are established via the same pathway and molecules since any intervention in one of these learning types can have severe influences on the other one as well.

This work contributes to the general understanding of the function of the brain by showing that Synapsin plays an important role in learning and memory processes especially with regard to the question why and how important things can be learned better. Therefore, this work can be used as basis for future studies to investigate the natural enhancement of learning and memory processes by Synapsin. This is gaining importance due to the fact that the man himself also has Synapsin, probably with similar function (Garcia et al. 2004; Fassio et al. 2011; Greco et al. 2013; Kharlamova et al. 2015). Furthermore this work contributes to better understanding of pain-relief learning and of the establishment of pain-relief memories what could be of great significance to understand pathological diseases and for its therapy.

Processing of stimulus concentration and quality of odors and taste rewards

Memories resulting from associative odor-reward learning include information about the reward concentration (the 'how much?' of a reward; larva: Schleyer et al. 2011, adult: Shiraiwa 2008). They are also specific with respect to the reward quality (the 'what kind?' of a reward; larva: Schleyer et al. 2015; adult: first hints in Lin et al. 2014) as well as for odor concentration (the 'how much?' of an odor; larva: Mishra et al. 2013; adult: Yarali et al. 2009) and odor quality (the 'which kind?' of an odor; larva: Chen et al. 2011; adult Niewalda et al. 2011; Campbell et al. 2013; Barth et al. 2014). How are these four different kinds of information processed within the *Drosophila* brain?

At least for the odor quality there is a widely accepted working hypothesis as to how the information is processed via the olfactory pathway such as the glomerular one of larval *Drosophila* (Fig. 3; and reviewed in Gerber et al. 2009): The larva houses 21 olfactory sensory neurons (OSNs) on each body side that usually express a single type of olfactory receptor gene (*Or*); the olfactory receptor molecules (ORs) are located at the dendrites of the OSNs in the dome of the dorsal organ. A given OR type is expressed in but one OSN and can bind several different ligands. Likewise, a given ligand can bind to different ORs. Accordingly, a given odor activates a specific combination of the 21 OSNs (Kreher et al. 2005 and 2008). Each OSN projects to one glomerulus in the larval antennal lobe (LAL). The LAL consists of about 21 glomeruli and each glomerulus receives input from one of these OSNs. The OSNs also target local interneurons that link many or even all glomeruli and thereby are able to shape signaling (Thum et al. 2007; Wilson 2013). From the glomeruli the signal is carried forward by projection neurons (PNs) that typically receive input in one glomerulus of the LAL. The PNs project the signal to two higher-order processing centers: the mushroom bodies (MBs) and the lateral horn (LH) (Masse et al. 2009). Consequently, odor quality can be encoded along the olfactory pathway as odor-specific combinatorial activity patterns of the OSNs, PNs and MB KCs. Such combinatorial activation enables the larva to differentiate between many different odors despite a relatively low number of OSNs. For the adult fly, odor quality is processed in a comparable way yet with increased cell numbers (see also general Introduction).

In contrast, there is no established working hypothesis how information about sugar reward quality is processed. That could be linked to the fact that the gustatory pathway and the connectivity of the gustatory system are less well understood (Apostolopoulou et al. 2015). The larva has about 80-90 gustatory sensory neurons (GSNs) on each body side that are located in three internal organs, the ventral, dorsal and posterior pharyngeal sense organs and in three external organs, the terminal organ, the ventral organ and the bulge of the dorsal organ (reviewed in Gerber & Stocker 2007). From here the taste information bypasses the brain and is directly projected to the subesophageal ganglion (SEG). The SEG is a primary gustatory center and has a hub function because here all GSN input information is collected and further distributed to the downstream targets. Two of these targets are modulatory octopaminergic/tyraminergic and dopaminergic neurons which relay the information towards the Kenyon cells of the mushroom body (Rohwedder et al. 2016). Please note that the precise connectivity between the SEG and the modulatory neurons is not known. Besides the KCs a distinct set of interneurons also relay information to the ventral nerve cord, as well as to (pre-)motor system. Similar to the olfactory system, the connection towards the motor system is supposed to mediate innate gustatory behavior.

The MB can be divided into several domains defined by the innervation of specific dopaminergic MB input neurons (DANs) and mushroom body output neurons (MBONs) (Selcho et al. 2009, Pauls et al. 2010; Rohwedder et al. 2016; for adults see also Aso et al. 2014a). It is possible that information about reward quality is mediated combinatorially by a reward specific activation pattern of DANs, similar to odor specific activation of PNs. Alternatively, a certain reward could activate a for exactly this reward specific subset of DANs that is directly connected to the MB (see discussions in Schleyer et al. 2015, Rohwedder et al. 2016). Hence a different reward would activate a different specific subset of DANs and therefore no overlapping or combinatorial activation of DANs would occur (a so-called ‘labeled line coding’). These two possibilities are currently under investigation, but at the moment no clear answer can be given yet.

In any event, via a yet unknown number of synaptic steps, the sugar reward activates DANs (Fig. 3B, causing an internal appetitive reinforcement signal that is relayed to most if not all Kenyon cells (KCs) of the MB (for larva: Pauls et al. 2010; Rohwedder et al. 2016; for adults: Schwaerzel et al. 2003; Aso et al. 2010; Aso et al. 2014a). Upon odor stimulation, the OSNs are activated and the signal is carried forward by PNs to the MB, Thus, upon joint odor and sugar stimulation odor-evoked activity and the internal appetitive reinforcement signal converge at the MB. Notably, only in those KCs that receive both the reinforcement signal (as most KCs do) *and also* the odor signal, appetitive memory will occur (Heisenberg 2003; Tomchik and Davis, 2009; Gervasi et al. 2010, Diegelmann et al. 2013 and many more). In other words associative odor-sugar memory emerges when the odor signal and the reward signal converge to the same KCs.

How does event salience fit into this scenario? In a simplistic scenario, stronger rewards may activate their DANs more strongly; likewise, higher-concentration odors would activate their KCs at least slightly more than low concentrations. If the strength of memory would scale with these activations, the strength of the MB-output synapse would code for an integrated "event strength", that is for a combined value of odor and sugar concentration. This appears to be in contrast to what Mishra et al. (2013) found. In an associative odor-sugar experiment larvae were trained at a medium odor concentration and tested at the same medium concentration, at a higher concentration and at a lower concentration respectively. Only for the medium odor concentration they observed strong memory scores while for both the higher and the lower odor concentrations memory strength was significantly weaker compared to the medium concentration. Accordingly, memory is specific for the odor concentration, i.e. the odor intensity is coded within the brain. This immediately raises the interesting question how memories can be stronger for higher concentrations of odor on the one hand (Kleber et al. 2016), and specific for the previously-rewarded odor concentration on the other hand (Mishra et al. 2013). This appears to be possible if, in addition to the scenario detailed above, the combination of activated KCs would slightly vary with odor concentration (Mishra et al. 2013). According to such a combined scenario, a given odor quality would be coded by slightly different sets of KCs if presented at different concentrations, and in addition the respective KCs would be activated slightly more strongly for higher odor concentrations. If a higher-than-trained odor concentration is presented, it activates only a subset of the KCs that house the memory trace, and therefore retrieves only a partial memory. It would be fascinating to find a set of parameters of a mushroom body simulation and a connectivity scheme that would capture both the specificity of odor memory for the previously rewarded odor, and the observation that memories get stronger when using higher concentrations of odor.

Synapsin phosphorylation

The working model for Synapsin predicts that Synapsin can tether reserve-pool vesicles to the cytoskeleton and, during learning, regulate their recruitment to the readily releasable pool in a phosphorylation-dependent way (see general introduction, Fig. 4; Klagges et al. 1996; Michels et al. 2011). Thus, phosphorylation represents a key step towards the regulation of Synapsin function. Till today little was known how strong Synapsin is phosphorylated in naive animals, at which sites it is phosphorylated and whether this is modulated by learning. We were able to identify several phosphorylation sites that harbor consensus motifs for several kinases for both the adult fly (Fig. 12, table 2) as well as for larva (Fig. 10, table 1). Thus, we could confirm that Synapsin is a phosphor-protein and could describe a detailed phospho-map. Given that Synapsin is related to learning and memory processes, an immediate question arises: Is Synapsin phosphorylation associated with learning and memory processes? In 2011 Michels and colleagues could show that transgenic expression of Synapsin containing

two dysfunctional kinase-consensus sites cannot rescue the defect of the *syn*^{97} null mutant larvae in associative function (Fig. 12, table 2: mutated at S22 and S549; Michels et al. 2011; loc. cit. S6/S533). This interesting observation leaves some essential questions unanswered. Given that the Synapsin protein harbors more than two phosphorylation sites (table 1 and 2) an important issue is to figure out all those phosphorylation sites that are relevant for learning and memory processes. An experimental approach to mutate all kinase-consensus sites one by one and validate them by learning experiments would be very laborious. A potentially more efficient way to address this problem is to use a LC-MS/MS approach as described in Chapter 1 (Materials and methods section). An ideal experiment would allow comparing the phosphorylation pattern of Synapsin before and after associative conditioning. The focus here would be on the Kenyon cells (KCs) of the mushroom body because they harbor the olfactory memory trace (Schwaerzel et al. 2003; see general introduction). The first intuitive idea that comes to our mind was to train animals and compare the Synapsin phosphorylation pattern with untreated, naive animals via LC-MS/MS. But given that only about 5% of the KCs are activated by odors (Turner et al. 2008; Honegger et al. 2011) and therefore are involved in the plastic mechanism of associative learning, it is very unlikely that one can detect a difference in phosphorylation in such a small fraction of cells (even if in all of the 5% odor activated KCs a memory trace is build up) compared to 95% of KCs that are not involved in forming an associative memory. With other words, the signal-to-noise ratio between the 5% of KCs that are directly involved in associative memory formation and the 95% of KCs that are not is far too low. According to Vasmer et al. (2014) a way to tackle this problem is to express both *d*TRPA1 and Synapsin in the same random set of Kenyon cells (using GAL4-UAS system, see general introduction) in animals of Synapsin null mutant background. *d*TRPA1 is a thermo-inducible cation-channel and therefore cells expressing *d*TRPA1 can be activated by raising temperature to 30°C or higher (Hamada et al. 2008). Accordingly, in all of these, and in only these, random Kenyon cells both Synapsin and *d*TRPA1 are expressed and memory can be established. In an associative learning experiment the odor-activation of KCs can be substituted by heat-activation. Thus, for paired training the heat is presented together with the sugar-reward and for unpaired training the heat and the sugar-reward are presented separately (Honda et al. 2014). After such training the phosphorylation pattern of Synapsin can be investigated with LC-MS/MS analysis and can be compared with the phosphorylation pattern of untrained animals with the same genetic background. In a test situation a temperature gradient can be applied and animals potentially accumulate at the temperature that was previously used for odor substitution. This method would increase significantly the signal-to-noise ratio because all cells, and only these cells, expressing Synapsin get activated, and therefore would allow to investigate where and how strong Synapsin gets phosphorylated during learning and memory processes. One may wonder why not expressing Synapsin and *d*TRPA1 in all Kenyon cells of the mushroom body. It was reported that learning, induced through artificial activation of Kenyon cells, coincident with an electric shock, requires a minimum and a maximum number of Kenyon

cells (Vasmer et al. 2014) and therefore a mushroom body wide expression could probably fail. Additionally, given the odor specific combinatorial activation of KCs an artificial activation of nearly all KCs would prevent odor-coding within the KCs.

Synapsin and its role in high and no high salient events

How could Synapsin be involved in memories for high salient events? Considering the working hypothesis of Synapsin, it regulates a reserve pool of synaptic vesicles (SVs) by tethering them to the cytoskeleton and allows their recruitment to the readily-releasable pool upon associative odor-reward coincidence (Greengard et al. 1993; Hosaka et al. 1999; Südhof, 2004; Hilfiker et al. 2005). It is plausible that the memory strength is limited by the number of ready releasable vesicles. Therefore Synapsin could fine tune synaptic strength by regulating the SVs available for release (see above and Fig. 4). In other words proper Synapsin function leads to a recruitment of more SVs from the reserve pool which may result in higher memory strength and performance. But this scenario seems only to fit for salient events and/or easy to learn tasks. Here the release of neurotransmitters can be easier accomplished and therefore Synapsin functions as a natural cognitive enhancer. But for events that are not highly salient or more difficult to learn (low odor- and/or sugar concentration, long time interval between training and test etc.; for more details see chapter I) it does not matter whether Synapsin is expressed or not. Wild-type and syn^{97} mutant did not show a difference in memory performance (Fig.s 7-8). Hence and accordingly to the working model of Synapsin (Fig. 4) during training no synaptic vesicles are recruited from the reserve pool. Otherwise the memory strength of the wild-type would increase compared to the mutant which is not the case. In turn, this leads to the assumption that for non-highly salient events the AC–cAMP–PKA cascade is not active or its action is massively reduced and therefore Synapsin is not or less phosphorylated. Accordingly, the synaptic strength and hence the memory strength can only be regulated by the readily releasable pool. In this context it would be thrilling to see whether an artificial increase of cAMP levels during training of non-salient tasks would result in higher memory scores in the wild-type but not in the Synapsin mutant and further whether one could detect a difference in the phosphorylation pattern of Synapsin between animals with and without artificial increase of cAMP levels using LC-MS/MS.

Mushroom body output potentiation or depression

Based on electrophysiology as well as calcium imaging it was recently reported that the response of particular mushroom body output neurons (MBONs) to a conditioned odor was reduced compared to a control odor and the KC-MBON synapse was depressed (Owald et al. 2015; Hige et al. 2015; Cohn et al. 2015). This seems to be in contrast to the working model of Synapsin, predicting an enlargement of the readily releasable pool of synaptic vesicles during training, ultimately resulting in enhanced

neurotransmitter release if the conditioned odor is encountered again (Fig. 4). A depression and not a potentiation of the KC-MBON synapse would be in accordance with Synapsin function, however, if the KC comprises (maybe next to others) inhibitory neurotransmitters. Accordingly, a strong release of inhibitory neurotransmitters would result in reduced activity, if not inhibition, of the MBONs. Additionally and as mentioned above Synapsin houses *several* kinase consensus motifs (Nuwal et al. 2011; Sadanandappa et al. 2013; Niewalda et al. 2015) and therefore could serve not only as substrate for PKA but also for other kinases that may have an opposite effect on the balance between reserve-pool and readily releasable pool, e.g. by stronger tethering synaptic vesicles to Synapsin and actin filaments instead of loosening the association. In turn PKA may have other targets than Synapsin that may lead to a decrease of the readily releasable pool and/or increase of the reserve pool. Therefore the net effect of associative learning on the balance between reserve-pool and releasable vesicles and on synaptic transmission is difficult to predict. In any event, the level of activity of the MBONs is thought to be the basis for learned olfactory behavior (Séjourné et al. 2011; Plaçais et al. 2013; Aso et al. 2014a,b; Menzel, 2014)

Different types of learning and memories

Punishment learning and pain relief-learning are two types of learning. Interestingly the only difference between these two is the sequence of US (electric shock) and CS (odor) presentation. This sequential change in the order of odor-shock presentation for punishment learning to shock-odor presentation for pain-relief learning turns avoidance behavior into approach behavior (Tanimoto et al. 2004; Fig's. 2, 21A). Changing the inter stimulus interval (ISI), which is defined by the time between two stimuli (e.g. odor and shock or odor and reward) can result in a further kind of memory. If an odor and electric shock are presented in an explicitly unpaired way (very long ISI), the odor becomes a predictor for punishment-absence leading to a safety memory and learned approach (Gerber et al. 2014; Schleyer et al. 2015; Fig. 21 A).

Accordingly, the variation of the ISI in combination with a reward also results in three distinct types of memory: A paired presentation of odor and reward establishes a reward memory and learned approach. Presentation of an odor after the reward, in the moment of reward offset, results in a reward-loss memory and leads to learned avoidance. A long ISI and therefore an unpaired presentation of odor and reward generates a reward absence memory (Fig. 21B).

Please note that the nature of the US (reward or punishment) can also determine which type of memory is established. Substituting shock with sugar shifts punishment to reward learning, safety to reward-absence learning and pain-relief to reward-loss learning. Taken together, negative events can possibly establish three kinds of memory (punishment, relief and safety memory), and analogously to

positive events (reward, reward-loss and reward absence memory). Of these six types of memory, three establish learned approach, and three learned avoidance (Fig. 21C-D).

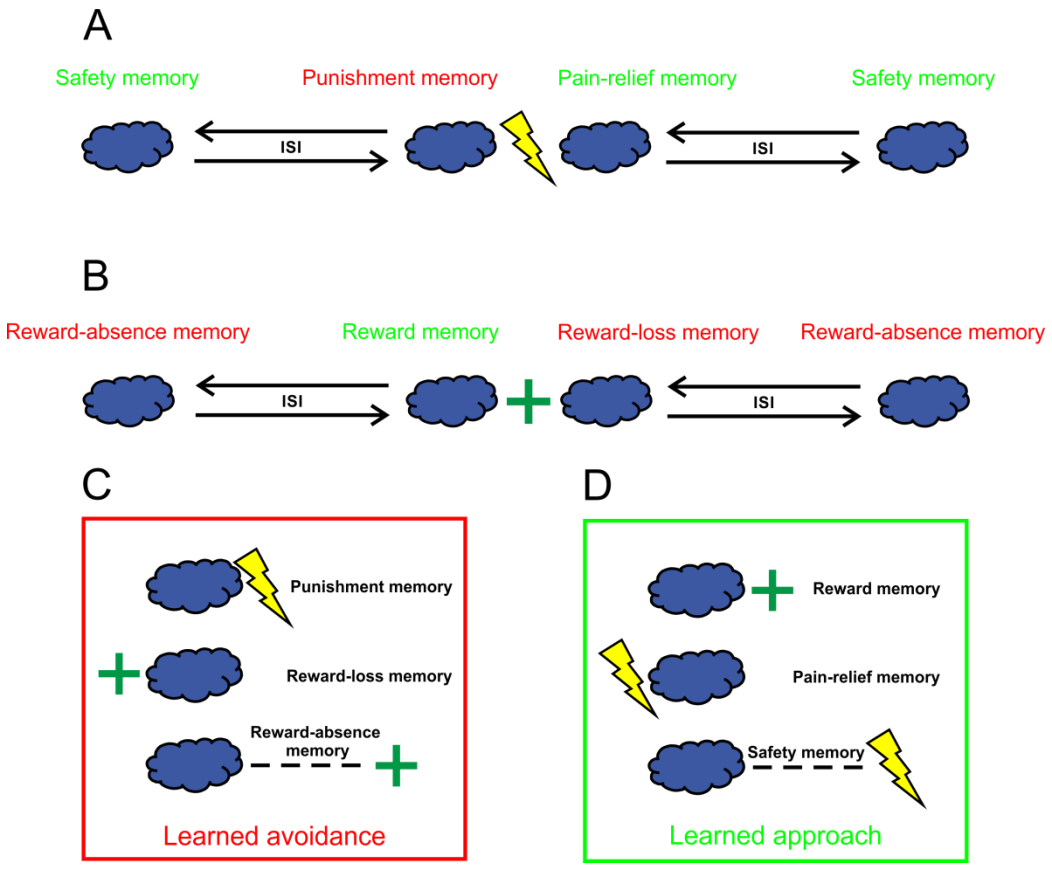

Figure 21. Different kinds of memory are established with different inter stimulus intervals (ISIs). **(A)** Three different kinds of associative odor-shock memories. The variation of the ISI in a way that the sequence of CS-US is changed to US-CS leads from punishment learning, resulting in punishment memory to pain-relief learning and the establishment of a pain-relief memory and vice versa. Increasing of the ISI leads from both punishment learning and pain-relief learning to safety-learning resulting in safety memory. Decreasing the ISI results either in punishment learning or pain relief learning, depending on the order of CS and US. Memories leading to learned avoidance are colored in red and memories leading to learned approach are colored in green. **(B)** Three different kinds of associative odor-reward memories. According to A the switch in the order of CS and US leads from reward learning and reward memory to reward-loss learning and reward-loss memory and vice versa. Extension of the ISI results in reward absence memory and reduction of the ISI yields reward memory or reward-loss memory. Of these six types of memory, three **(C)** establish learned approach, and **(D)** three learned avoidance.

To disentangle the varieties of these different learning mechanisms on the molecular and cellular level will be challenging but possible in respect for Synapsin. It is still not a trivial question whether to use a two-odor or a one-odor paradigm towards this end. In a two-odor paradigm, e.g. in reward learning two types of memory could be formed: reward memory for the odor presented along with the reward (paired odor), and a reward-absence memory for the odor presented separately from the reward (unpaired odor) (Fig. 22). Therefore the one-odor paradigm may be the better choice because here no learning about a second odor can take place and thus it could be easier to investigate the different types of memories

supporting learned approach or avoidance (Saumweber et al 2011a). The one-odor paradigm is discussed more in detail in the next section.

Using the one-odor paradigm enables to address some very interesting questions: How is Synapsin involved in the formation of the different kinds of memories? Is Synapsin required not only for salient events for reward memory (as shown in chapter I) but also for other types of memories? Are different phosphorylation sites of Synapsin involved for different memories? Are these memories located in different sets of mushroom body Kenyon cells and do they differ in content specificity?

Figure 22. A two-odor paradigm for associative reward learning can lead to the establishment of two different kinds of memories: a reward memory and a reward-absence memory. In a paired odor-reward trial (blue cloud and green plus) reward memory can be build up resulting in learned approach towards this odor. In a subsequent odor-only trial a different odor (white cloud) is present but no reward; thus a reward-absence memory could be formed resulting in learned avoidance of this (white) odor. For more information about how these kinds of memory could be established see general discussion.

What can be learned in a one-odor paradigm?

Similar to the two odor version also the one odor paradigm consists of two reciprocal training regimens (see Materials and Methods part and Fig. 6). These are two distinct situations where either the odor is presented together with the reward (paired group) or the odor is presented separately from the reward (unpaired group). After paired training larva search for sugar during the test situation, resulting in positive preference scores, because the odor becomes a signal to indicate reward (Schleyer et al. 2011). For the unpaired case larvae search for the sugar where the odor is not present, resulting in negative preference scores, because the odor becomes a signal for the absence of reward. This means, that the animals do not only learn about the presence but also about the absence of a reward (Saumweber et al. 2011a). This raises the interesting question whether learning about the presence or the absence of a reward has the same fundamental principles and how such learning can come about. In 1972, Rescorla and Wagner claimed that three factors are essential for associative conditioning: contiguity, contingency, and prediction error (Rescorla and Wagner 1972). They developed a still widely established model to predict associative strength, which also comprises that the presence as well as the absence of the unconditioned stimulus has an effect on the reinforcement. In case of paired odor-sugar training (as well as for the two odor paradigm, because one of the two odor is always paired with a US) the Rescorla and Wagner model seems to be straightforward. The fact that for paired training the odor and reward are always presented together ensures contiguity, because the odor can be associated with the reward. Contingency is a measure for the probability that the US and the CS occur together. With regard to this fact please note that the CS can occur either in the presence or the absence of the US during paired or unpaired training, respectively, (see below) and therefore contingency is given to a large extent.

Before training the animals cannot expect anything, especially no reward. Also the odor is not yet predictive for the reward. But when training starts, suddenly and unexpectedly a reward is presented, resulting in a positive prediction error (animals receive more reward than predicted). This positive prediction error is associated with the presence of the odor, and the associative strength increases rapidly. As training continues, animals learn to associate the odor with the sugar reward. In other words, the odor gets predictive for the reward. Hence, during training the positive prediction error becomes smaller until the animals expect exactly the same amount of sugar as they receive. At this point the learning process ceases and the associative strength reaches its maximum.

What happens during unpaired odor-sugar training? Here, no temporal coincidence of the odor and the reward is obvious. However, the animals possibly learn to associate the reward with a given context. At the beginning of an unpaired reward-only training trial the animals do not expect sugar. If they receive sugar they may form an associative memory between the experimental context e.g. quality of the agarose surface (Apostolopoulou et al. 2014), light conditions (von Essen et al. 2011) and the reward. In the subsequent odor-only trial the experimental context is the very same and due to the previously formed context memory this context predicts for a reward. But in this odor-only situation no sugar is present, resulting in a negative prediction error, (less reward is received than predicted). This moment could be experienced as a moment of frustration and therefore in this situation the odor can be associated with this frustration caused by the absence of the sugar reward. With other words in that situation the odor becomes a signal for no reward, and animals subsequently avoid it in their search for food (Schleyer et al. 2011).

These different situations could involve different types of learning and memory processes on the cellular and molecular level, and therefore could differ in their requirement of Synapsin. The memory strength or the performance index (PI) is defined by the difference of odor preferences (PREF) between the paired and unpaired group (see Materials and Methods Chapter 1 and Fig. 6). To reveal a difference of how the PI's of the wild-type and of the syn^{97} mutant come about it is necessary to compare the odor preferences of the paired and unpaired groups of both genotypes. Therefore, we pooled all PREFs for the paired and unpaired groups of all experiments where we used 2 mol/l FRU as reward and a 1:20 dilution of AM (Fig. S8). It turned out that both the PREFs of the paired group and the unpaired group of the syn^{97} mutant are significantly different from the corresponding PREFs of the wild-type. Hence, Synapsin seems to be required for both, for memory that emerges from paired training and for memory that emerges from unpaired training. Interestingly, if one compares the PREF scores of all single experiments this may be not an overall rule. Although all experiments were performed under the same conditions we do not find always a significant difference in both reciprocally trained groups (Fig. S8A).

Maybe a simple but reasonable way of looking at such a topic is to put oneself in the shoes of a larva. From the larva perspective the only interesting question is: where shall I go? After paired training the larva expects that where the odor is also sugar will be present. Thus the larva can use the odor for

orientation that guides it the way to the reward. After unpaired training the larva may not know where the reward is but it knows the reward is for sure not where the odor is, therefore it avoids the odor.

Pain-relief memory and traumatic experience

As mentioned above it is very plausible that pain-relief learning and the resulting pain-relief memories play an important role in pathology. In the following this will be explained for two examples.

Some people suffer from a disorder where they harm themselves e.g. by self-cutting. This disorder often goes along with other disorders like depression or anxiety. It is assumed that patients, who are e.g. sad, depressed or anxious, are searching for an escape or a relief from these bad feelings. In such a situation they hurt themselves, not because they want to feel the pain but for the sake of relief from pain. This pain relief creates at least for a short moment of positive feelings. Therefore self-harm is associated with something good, with the positive feeling of pain-relief and the suppression of bad feelings. Hence the self-harming action can be seen as an instrumental action to bring about relief. Accordingly, pain-relief learning and pain-relief memory could contribute to the maintenance of the symptoms of such disorder.

Another example where pain-relief learning might play an important role is in phobias, e.g. arachnophobia, the fear of spiders. People who suffer from this disease are not able to touch them or even to come close to spiders without getting panic or anxious. Hence the close proximity to spiders brings these people in a very bad situation. But if a patient faces his fear and e.g. let a spider crawl over his hand it may happen that he experiences that nothing bad happens and that his fear was unfounded. This moment in which he realizes that no harm occurs is a relief from panic and anxiety states. Therefore it could be that here relief learning may contribute to the cure of this disease. Indeed, exposing patients to stimuli they fear is a common therapy for phobias.

Please note that pain-relief learning has a different kind of significance in the chosen two examples. In the first example relief learning could contribute to the maintenance of the disorder while for the second example it may contribute to the healing of the disease.

Interaction of Synapsin and another presynaptic protein, Sap47

An important aspect in understanding the molecular function of a given protein is to identify functional partners, e.g. other proteins that share the same functional pathway. In this work we find evidence that Synapsin interacts directly or indirectly with the presynaptic protein Sap47 (Synapse associated protein of 47 kDa; Reichmuth et al. 1995; Saumweber et al. 2011b). We were able to show an additional Synapsin band in animals lacking the Sap47 protein on Western blots (Fig. 5G, right most lane). Larvae missing Sap47 show a reduction in odor-sugar memory of about 50 – 60%, as well as a distortion in short-term plasticity (Saumweber et al. 2011b). In wild-type animals three Synapsin bands are detectable, two at about 72 kDa and one at 143 kDa (Fig. 5F-H). In larvae lacking Sap47, however, an additional Synapsin

band is detectable on Western blots slightly above the 72 kDa-band. This prompted us to ask whether regarding establishing memory Synapsin and Sap47 work in the same or in different, parallel molecular pathways. We therefore tested whether the impairment in memory formation of the Synapsin and the Sap47 mutants are additive in Synapsin/Sap47 double mutants lacking both proteins. If Synapsin and Sap47 were operating in series, no such additivity should be observed. Indeed, we could show the deficit in memory did not add up in the Synapsin/Sap47 double mutants (Fig. 9A), suggesting that Synapsin and Sap47 work in the same molecular pathway. Furthermore the observation of the additional Synapsin band leads to the suggestion that this band represents a more strongly phosphorylated Synapsin.

Thus we gave priority to investigate the phosphorylation pattern of Synapsin in the Sap47 null mutant compared with those from wild-type larvae. Indeed it turned out that in animals lacking Sap47 the Synapsin phosphorylation pattern is altered (Fig. 10, table 1). Whether the altered phosphorylation status of Synapsin is significant with respect to memory function remains to be investigated e.g. by mutating the respective kinase consensus motifs in order to prevent phosphorylation or alternatively to train animals and do LC-MS/MS like described above.

A different way to find possible partners of Synapsin in the molecular network is to do a quantitative proteomic comparison between mutants and wild-type animals. In a proteomic study it would be very interesting to know the expression pattern of other presynaptic proteins (at best all proteins) in the *synapsin* mutant. Which proteins are up regulated or down regulated due to the lack of Synapsin? Possible candidates can be further verified by creating the respective mutants and test them for their associative memory performance. In this context and given that the lack of Sap47 alters the phosphorylation status of Synapsin it would be interesting to know whether also the expression pattern of Synapsin is altered in the $sap47^{156}$ mutant.

Synapsin-independent memory

Notably the learning scores for the syn^{97} mutants do not become zero (Fig's. 7-9). This indicates that these animals still have learned and that a Synapsin-independent memory trace was formed. How does this Synapsin independent memory come about? Interestingly, in 2010 Knapek et al. reported that Synapsin contributes to anesthesia-sensitive (ASM) but not to anesthesia resistant memory (ARM) in adult flies. Accordingly proteins other than Synapsin must be involved in ARM formation. Indeed, further experiments of Knapek et al. (2011) revealed that the protein Bruchpilot (Brp) is required for olfactory memory and in particular for the formation of ARM. Brp is localized at the presynaptic active zones, which are essential sites for proper formation of the presynaptic dense bodies (T-bars; Wagh et al. 2006; Fouquet et al. 2009). Interestingly a Brp knockdown barley impaired ASM for which Synpasin is required (see above). Thus Knapek et al. concluded that the two components of olfactory memory, ASM and ARM can be distinguished by the function of different presynaptic proteins. Already in 2010 Hallermann et al.

reported that Brp has an effect on short-term plasticity at neuromuscular active zones of *Drosophila* (reviewed by Hallermann in 2010). A meaningful experiment to test for interdependence between Synapsin and Brp is to examine a Synapsin/Brp double mutant in a similar way as we validated the Synapsin/Sap47 double mutant interaction (see above; Fig. 9).

Another possible candidate that could be relevant for ASM formation is the protein Tomosyn (Fujita et al. 1998). Tomosyn was shown as a negative regulator of secretion by directly competing with Synaptobrevin, a SNARE-protein involved in the formation of SNARE-complexes, to form nonfusogenic Tomosyn SNARE-complexes, which are involved in the vesicle fusing machinery (Fujita et al. 1998; Hatsuzawa et al. 2003; Pobbati et al. 2004). In 2011 Chen K et al. could show that Tomosyn adjusts synaptic transmission at the neuromuscular junction (NMJ) by regulating SNARE-complex assembly and thereby controlling the size of the readily releasable pool of synaptic vesicles. The authors reported that Tomosyn is required for late ASM, but not for ARM and that Tomosyn is necessary in the Kenyon cells of the mushroom body to form late ASM. Interestingly they claim that Tomosyn may be an important downstream target of cAMP dependent PKA phosphorylation. Accordingly and similar to Synapsin the function of Tomosyn could be regulated by phosphorylation and thus both Synapsin and Tomosyn could be required to establish cAMP/PKA dependent ASM. Mass spectrometry can be used to reveal phosphorylation status of Tomosyn and whether phosphorylation is involved in learning and memory processes (see above). Please note that Tomosyn is only required for the late phase of short-term memory (test after 3 hours) but not for the early phase of short-term memory (test after 3 min; Chen et al. 2011b). Strikingly this is in contrast to what we have observed for Synapsin: Synapsin is only required for early short-term memory (up to 5 min after training; Fig. 8), but not for later short-term memory. A fascinating aspect could be that during short-term memory formation the requirement for Synapsin for the early phase may change to the requirement for Tomosyn for the late phase. To address this exciting topic one could use Synapsin/Tomosyn double mutants and test them for learning and memory impairments. If these two proteins supersede by each other during memory formation one would expect no additive effect in memory impairment, but would observe a decrement in both early and late short-term memory. Subsequently it would be fascinating to see if an expression of Synapsin in the Synapsin/Tomosyn double mutants could rescue early short-term memory, but not late short-term memory and accordingly an expression of Tomosyn would rescue late short-term memory but not early short-term memory.

In 2011b Saumweber et al. showed that Sap47 is required for proper associative function, but it remains unknown to which kind of memory, i.e. ARM or ASM. Notably, we could show that the lack of both, Synapsin and Sap47, in the Synapsin/Sap47 double mutant did not lead to an additive impairment in memory (Fig. 9A), suggesting that Synapsin and Sap47 are acting in the same molecular cascade rather than in parallel. Therefore we speculate that Sap47 may not contribute to Synapsin-independent memory and vice versa.

In summary a possible scenario is that early short-term memory has at least two components. The first is amnesia-sensitive and depends on Synapsin and maybe Sap47. The other one is amnesia-resistant and depends on Brp. A subsequent late short-term memory could also consist of an amnesia-sensitive and an amnesia-resistant component and the protein Tomosyn contributes to the amnesia-sensitive component. Please note that the requirement for Sap47 for ASM and/or ARM has to be clarified. Our observations lead to the suspicion that Sap47 does not contribute to the residual memory of the *syn97* mutants while the role of Tomosyn and Brp in learning and memory function need to be further investigated.

Synapsin editing

Twenty years ago, a process was discovered in the kinetoplastid of trypanosomes called RNA editing that altered pre-mRNA (Benne et al. 1996). Similar processes have been observed for a large number of genes from different species, including *Drosophila melanogaster (*Simpsopn et al. 1996; Gott et al.2000*)*. RNA editing modifies the information encoded by the genomic DNA post-transcriptionally at the RNA level (Simpson et al. 1996; Gott et al. 2000; Diegelmann et al.2006). This pre-translational modification is catalyzed by the adenosine deaminase acting on RNA (ADAR) enzyme. In 2006 Diegelmann et al. reported that the N-terminal phosphorylation consensus motif RRxS that is conserved in all Synapsins investigated so far, is modified in *Drosophila* by pre-mRNA editing. The *synapsin* DNA codes for the amino acid sequence RRFS. RNA editing replaces adenosine by guanosine resulting in a modification of the sequence from RRFS to RGFS (Fig. 23). Interestingly and as mentioned above the ADAR target sequence RRFS also represents the target site of protein kinase A (PKA) and calcium/calmodulin dependent protein kinase I/IV (CaMKII) (Diegelamm et al. 2006; Niewalda et al. 2015).

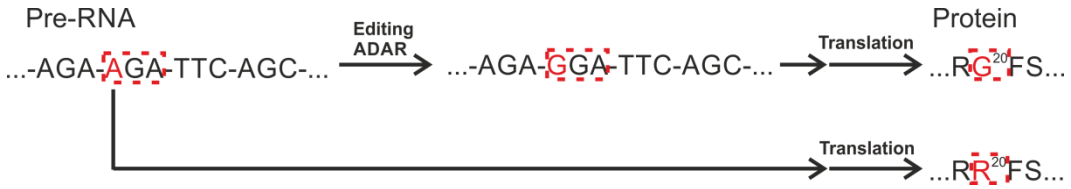

Figure 23. Synapsin pre-mRNA can undergo a process called RNA editing. This process modifies the information encoded by the genomic DNA post-transcriptionally at the pre-mRNA level (Simpson et al. 1996; Gott et al. 2000; Diegelmann et al.2006). This pre-translational modification is catalyzed by the enzyme adenosine deaminase acting on RNA (ADAR). The *synapsin* DNA codes for RRFS on protein level. However, by RNA editing adenosine is replaced by guanosine resulting in a modification of RRFS to RGFS (stippled red box). Please note that the ADAR target sequence RRFS also represents the target site of protein kinase A (PKA) and calcium/calmodulin dependent protein kinase I/IV (CaMKII).

These observations give rise to some fascinating questions. First, is every pre-RNA edited and therefore only the edited protein version of Synapsin is translated? Second, given that editing changes a phosphorylation consensus motif does this influence the phosphorylation of target proteins (first hints based on in-vitro phosphorylation by bovine PKA: Diegelman et al. 2006)? Third, given the fact that Synapsin is required for proper associative function in a phosphorylation-dependent way, has editing an

effect on learning and memory processes? Lastly, what could be the physiological reason for this alteration in nucleotide sequence?

In 2006 Diegelmann et al. detected cDNA of the edited Synapsin version in both the adult fly and the larva, but no non-edited cDNA. Please note that this is an indirect evidence for the existence of the edited Synapsin version. No edited or non-edited Synapsin protein had been successfully sequenced so far. We were able to proove with LC-MS/MS for the first time that both protein versions, the edited and the non-edited, coexist in the adult fly (Fig. 12, table 2) in the ratio of 40/60 (edited/non-edited). Unfortunately, for the larval case the coverage of the LC-MS/MS does not include the editing sequence (Fig. 10A) and therefore no conclusions about the presence of the edited or non-edited Synapsin version can be drawn. Has editing an impact on phosphorylation? As already pointed out, the target sequence for editing is also a consensus sequence for two kinases: The PKA has a recognition motif of RRxS and the CaMKII has a recognition motif of RxxS. Accordingly, editing (RGFS) eliminates the recognition motif of the PKA but leaves the target sequence for the CaMKII intact, whereas non-edited Synapsin (RRFS) comprises the consensus motifs for both kinases. Thus it is very plausible that non-edited Synapsin can be phosphorylated more efficiently (Diegelmann et al. 2006) and the likelihood for phosphorylation is higher. According to the working model for Synapsin these possible differences may have an effect on learning and memory processes. An elegant way to experimentally address this question is to express edited and non-edited Synapsin separately in Synapsin null mutant animals and test them together with a wild-type control for associative memory formation. In a follow up experiment it would be further very interesting to investigate, if one can detect a difference in the phosphorylation pattern of edited Synapsin and non-edited Synapsin, especially between trained and untrained animals using LC-MS/MS. Interestingly for naïve flies we found only once a phosphorylation at this site and only for the edited version (Table 2). Maybe this site gets stronger phosphorylated during associative memory formation.

What could be the reason for the existence of a mechanism that alters the sequence of pre-RNA? Editing could modulate the function of ubiquitously expressed Synapsin in a cell-specific manner during development and adulthood. Accordingly, expressing the edited or non-edited version of Synapsin could fine-tune learning and memory by controlling the possible degree of phosphorylation.

Outlook

The brain with its network of synapses and connections is comparable, on a simple base, with an electric circuit. Signals are transmitted and distributed, enhanced or depressed, and finally result in a certain reaction, e.g. a light turns on or a muscle contracts. A major common goal, not only in neurobiology but also in medicine and related fields is to know the complete connectome of the entire brain. One would like to be able to trace a signal from its origin through all steps to its final destination (if there is one). Another important step towards the better understanding of the functioning of the brain is to know all genes and

gene products that are required and play a role in certain processes like learning and memory processes. It would be fascinating if, when something is not working properly e.g. the muscle is not contracting anymore, one could look at the circuit, find and fix the problem. Currently an intensive effort is invested to get closer to these goals. In this context nearly the whole chemosensory circuit, important for associative learning and memory formation (see above), with all cells and synapses is already known in the larva on the light-microscopy level (Diegelmann et al. 2013; Schleyer et al. 2013). The clarification and the reconstruction of the entire connectome of the larval brain is in the focus of intensive current research, and the progressive development in EM technologies allows great strides in this field (Cardona et al. 2010; Saalfeld et al. 2012).

Furthermore it is already possible to investigate how the proteome is composed in different subsets of cells (Dieterich et al. 2006, 2010; Erdmann et al. 2015) and the rapidly progressing development in this field makes it very likely that this can be achieved on single cell level in the near future. A recently developed technique allows in vivo cell specific labeling and measuring of newly synthesized proteins in *Drosophila* (Dieterich et al. 2006, 2010; Erdmann et al. 2015). This method is based on a mutated tRNA synthetase which is using the non-canonical amino acid azidonorleucine (ANL) instead of methionine to synthesize new proteins (Link et al. 2006; Ngo et al. 2009). ANL can be tagged with e.g. a fluorophore-tag (FUNCAT method; Dieterich et al. 2010) or with a Biotin-tag (BONCAT method; Dieterich et al. 2006, 2010; Hodas et al. 2005; Erdmann et al. 2015). The fluorophore-tag allows visualization and therefore localization of newly synthesized proteins, whereas the Biotin-tag can be used for affinity purification followed by immunoprecipitation or MS analyses, allowing access to the qualification of newly translated proteins. Cell specificity can be achieved by transgenically expressing the mutated tRNA via the GAL4-UAS system and its improved versions (see general introduction). Temporal control can be accomplished by feeding ANL to the animals. Another important step would be to examine the entire transcriptome of each single cell in the entire brain.

These possibilities and the technical progress allow playing with the idea that it will be possible to reconstruct a complete and functional brain in the near future. But even if the whole connectome and all genes and gen products are known that are required for any processes, it is still a long way to go because still much will be missing. Such as the knowledge about the spatial arrangement of molecules and proteins: where in the cell are which proteins located and how are they arranged? Information is lacking about the functional partners of proteins and which isoforms and isomers of which proteins are present. Which proteins are modified in which way, e.g. phosphorylated? What is about molecules and substances that are not transcribed or translated such as gases like oxygen and carbon dioxide or elements like phosphor and iron, and how does the geometry of the brain factor in with all this?

Therefore much more effort is required until one is able to reconstruct an entire functional brain but *Drosophila melanogaster* and especially the larval stages are a suitable model organism to pursue

these goals. It is more complex than e.g. nematodes with respect to anatomy as well as behavior, but much simpler than vertebrates. *Drosophila* gives access to many genetically manipulation tools, which are in this extend unique for the fruit fly (for more details see general introduction). *Drosophila* has a central nervous system that functions according to the same principles as for higher organisms but the architecture of the brain is much simpler, especially in terms of cell numbers and connections. This makes *Drosophila* and especially its larval stages a powerful model organism to clarify many fundamental mechanisms and to reveal general principals in brain function.

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Supplementary Material

A

Source:	Tanimoto et al. 2004. Fig. 1B									
ISI (seconds)	-83	-53	-23	-3	7	22	32	42	67	187
N										
1	-4,05	-7,11	-47,02	-42,86	-30,7	18,07	21,13	-4,04	-3,62	6,41
2	-1,65	-10,16	-46,66	-69,88	-36,41	9	18,4	17,93	6,89	-2,68
3	-10,91	-38,66	-60,83	-27,86	0,09	-7,99	10,03	20,62	5,66	-1,9
4	-7,01	-35,74	-45,38	7,25	-10,45	-2,54	15,6	11,01	7,3	1,33
5	7,9	-10,74	-59,78	-59,87	-21	1,44	11,11	17,81	24,74	9,14
6	3,79	-7,69	-64,66	-74,32	-22,3	22,75	-0,31	17,81	12,9	8,33
7	22,73	-3,76	-62,67	-57,26	-6,33	24,58	14,18	8,89	4,55	-0,64
8	0,44		-58,6	-26,14	-7,03		-1,11	12,58	-0,61	20,67
9	-11,38		-33,53				10,85	3,66		1,11
10	-0,01		-26,98				14,98	4,34		8,81
11	-16,13		-56,91				7,49	8,38		6,98
12			-55,27				14,82			4,5
MEDIAN	**-1,65**	**-10,16**	**-56,09**	**-50,06**	**-15,725**	**9**	**12,645**	**11,01**	**6,275**	**5,455**

B

Source:	Tanimoto, unpublished			
ISI (seconds)	-150	-25	25	300
N				
1	9,3163944	-47,40841	18,115942	-7,064502
2	14,375	-35,82979	6,523562	-0,106686
3	7,3529412	-89,95098	22,764228	-9,669811
4	-12,63158	-87,09677	4,7853624	3,4782609
5	-42,79379	-64,72091	-18,71698	0,9178744
6	9,3223658	-71,33838	34,452511	-15,59829
7	-2,226721	-94,11303	3,6315789	1,5243902
8	-34,58824	-81,80091	-0,370959	-10,54545
9			24,074074	14,305556
10			40,726934	-4,561404
11				-9,157509
MEDIAN	**2,5631103**	**-76,56965**	**12,319752**	**-4,561404**

C

Source:	Yarali et al. 2008b. Fig. 1D			
ISI (seconds)	-150	-15	40	250
N				
1	10,392157	-89,9435	3,3741259	-8,227848
2	-14,16317	-74,27536	12,690058	-2,108486
3	5,1233397	-88,53276	3,9677976	-4,525862
4	5,0877193	-70,32967	9,1754975	-10,8229
5	-9,090909	-82,34536	13,651138	-1,082072
6	-10,2465	-80,8207	4,4313689	-0,811688
7	-12,00717	-70,60678	7,42	-4,088151
8	2,4889603	-74,88889	1,5022138	4,4093944
9			8,7421945	-13,72414
10				11,833333
MEDIAN	**-3,300974**	**-77,85479**	**7,42**	**-3,098319**

D

Source:	Yarali et al. 2008b.Fig. 3A. (ISI= 15s unpublished)	
ISI (seconds)	-15	40
N		
1	-77,45704	6,742592219
2	-77,69015	15,71319603
3	-81,09218	19,01781416
4	-70,41328	6,103286385
5	-66,70807	23,62240289
6	-81,92059	13,15789474
7	-66,39911	2,997972901
8	-63,79552	-2,244609403
9	-81,22016	15,65246788
10	-92,60753	8,118466899
11		-6,888327722
12		14,04320988
MEDIAN	**-77,57359**	**10,63818082**

E

Source:	Yarali et al. 2009. Fig. 1B							
ISI (seconds)	-150	-45	-15	0	20	40	70	200
N								
1	5,6218714	-17,47685	-72,08696	-62,82051	6,043956	10,560038	11,024531	0,7936508
2	-0,779327	-15,05376	-91,0101	-80,78335	12,733447	7,5405215	13,717949	17,642467
3	-12,96992	-24,35897	-73,00557	-54,72727	-5,519481	9,4965675	-5,200433	5,3178729
4	-8,569182	-34,39153	-84,86842	-18,87872	-3,841463	18,671946	5,3216374	3,1609195
5	8	-38,82698	-80,91168	-71,55298	7,1818182	-1,058201	7,5757576	12,380952
6	5,2565181	-45,02342	-90,32508	-68,45238	-8,114478	-4,351174	-1,003135	-6,396588
7	-5,277778	-15,26151	-89,063	-63,60887	1,048218	5,5039139	9,8474946	16,630482
8	-5,263158	-30,95238	-86,90418	-65	7,567368	7,1618037	11,62876	-6,230848
9		-15,76355	-79,48718	-76,41196	15,17094	5,1820728	-17,30927	2,6190476
10		-39,21376	-90,42033	-77,21165	6,5462163	20,574713	-2,582728	-12,15633
11		-43,24324	-62,31003	-39,70588	16,393443	2,9892142	21,22449	-11,11111
12		-35,80943	-66,13667	-61,90476	9,6355913	-1,68691	-13,04348	3,8383838
13		-37,83422	-91,48607	-58,00416	13,636364	5,5882353		
14		-35,47745	-93,92015	-52,75128	11,590909	11,020408		
15		-17,45164	-84,95475	-39,59184	33,129904	14,893617		
16		-50,55995	-79,24528	-57,36325	6,6869301	-6,13354		
17		-26,71371	-84,3465	-56,57476	25,649351	6,0172872		
18		-28,75732	-92,30769	-58,97616	9,0909091	23,138958		
19		-35,20449	-88,15789	-38,03245	10,083333	18,683429		
20		-51,12179	-98,27586	-41,09195	-10,79365	3,5097814		
21		-21,46543	-74,08907	-46,15385	27,067669	-9,285714		
22		-29,84127	-88,125	-89,375	22,37037	7,1428571		
23		-29,71602	-83,88889	-51,55844	7,1428571	20,294599		
24		-35,2381	-89,51094	-65	1,2820513	6,5637066		
25			-62,22222	-57,3701		2		
26			-84,29487	-62,46334		2,7777778		
27			-84,07258	-68,32298		18,351648		
28			-91,66667	-83,33333		15,721925		
29			-71,81818	-45,06579		-1,724138		
30			-65,04638	-70,83333		1,4006342		
31			-82,57919	-44,54545		13,565993		
32			-93,75	-71,05263		13,809715		
33				-61,90476		33,926585		
34				-67,0778		12,538462		
35				-31,38402		8,2321188		
36				-89,58333				
37				-50,21645				
38				-68,52395				
39				-49,04762				
40				-69,96337				
41				-50,8125				
42				-59,34343				
43				-45,88003				
44				-69,35976				
45				-50,60606				
46				-59,07928				
47				-38,09524				
MEDIAN	**-3,021243**	**-32,67196**	**-84,60746**	**-59,07928**	**8,3291386**	**7,1618037**	**6,4486975**	**2,8899836**

F

Source:	Diegelmann et al. 2013. Fig. 1B				
ISI (seconds)	-15	40	ISI (seconds)	-15	40
N			N		
1	-58,33333	12,7567568	26		11,475084
2	-65,81197	-3,52941176	27		4,0784314
3	-73,21429	40,9677419	28		5,2114552
4	-67,30506	26,6666667	29		13,126566
5	-46,9697	37,84461153	30		-1,605191
6	-60,18519	-0,11627907	31		8,5481682
7	-58,01282	12,80788177	32		-8,4375
8	-22,76888	21,52690864	33		16,515837
9	-67,77751	6,643356643	34		31,25
10	-42,15418	3,009259259	35		9,7662338
11	-56,06061	19,90424077	36		24,431583
12	-56,66415	-3,052503053	37		27,666667
13	-70,63492	8,840275697	38		27,295285
14	-70,12579	90,03496503	39		14,926661
15	-57,05128	17,53246753	40		32,594417
16	-67,13555	29,49308756	41		11,791383
17	-89,56229	1,666666667	42		4,2191142
18	-81,5	-3,921568627	43		17,082087
19	-66,57469	-1,454545455	44		10,769231
20	-63,97849	38,17204301	45		1,0942761
21		15,75268817	46		12,956811
22		19,29775281	47		-20,12579
23		9,919137466	48		14,761905
24		6,872635561	49		8,5365854
25		0	50		1,233672
			51		13,888889
			MEDIAN	**-64,89523**	**11,791383**

H

Source:	Niewalda et al. 2015. Fig. 3B (CS wild type only)								
ISI (seconds)	-150	-45	-30	-15	0	25	50	125	200
N									
1	-7,709059	-2,751167	-49,68553	-46,10123	-59,04762	7,8806907	29,761905	17,727273	17,333333
2	-7,924985	1,9638649	-42,54992	0	-42,85714	11,836735	-19,96047	12,414467	12,854031
3	-28,26389	-5,147426	-34,5021	-54,44763	-61,29032	26,277651	12,857143	-17,2619	7,3232323
4	-8,388158	-16	-39,71861	-60,93117	-44,16667	13,285949	33,333333	17,714286	0,8333333
5	-8,768873	-21,95341	-31,60987	-67,72727	-66,17647	11,458333	38,00905	26,259947	2,0430108
6	-18,09524	1,1904762	-56,48148	-61,11111	-64,13255	14,274258	-7,407407	2,1008403	31,025641
7	1,2195122	-28,19876	-32,95455	-47,55072	-44,45813	10,042965	-12	-13,30049	-1,111111
8	2,4444444	-38,33333	-16,07818	-55,67376	-56,07843	13,761194	19,230769	-3,056027	9,6327096
9	-2,164502	-26,42857	-23,67347	-72,81489	-21,43834	39,111111	19,37799	12,698413	-4,72561
10	5,2873563	-24,39394	-13,68421	-24,98509	-33,63787	34,848485	20,238095	11,726079	7,3667712
11	2		-25,54244	-54,90196	-77,15054	41,98783	5,8823529	27,033108	-2,495746
12	-2,464646		-37,73585	-53,47368	-60,0726	8,8319088	8,8948787	9,4164456	3,9583333
13							19,047619		10,6676
14							15,869219		4,1291291
15							17,918314		5,6666667
16							17,846154		-8,611111
17							-4,032258		-10,4123
18							-2,923977		-7,407407
19							5,0709939		-21,72414
20							20,23988		3,3160991
21							-16,71088		1,1111111
22							8,6580087		11,478261
23							27,303523		39,242424
24							35,565476		
25							32,467532		
26							7,8947368		
27							28,368794		
28							40,201465		
29							17,481481		
30							26,814516		
MEDIAN	**-5,086853**	**-18,9767**	**-33,72832**	**-54,6748**	**-57,56303**	**13,523571**	**17,882234**	**12,070273**	**3,9583333**

G

Source:	Yarali et al. 2008. Fig. 7				
ISI (seconds)	-15	40	ISI (seconds)	-15	40
N			N		
1	-90,70175	-3,880952	23		8,3928571
2	-92,2619	-8,727273	24		-10,9206
3	-88,16723	3,1198686	25		6,3268608
4	-98,09524	2,4056604	26		13,874113
5	-75,21902	10,813492	27		-0,058754
6	-93,92998	-7,52394	28		0
7	-89,07157	46,186441	29		-4,312668
8	-78,30504	10,275264	30		9,8179812
9	-26,41355	21,760155	31		-5,674157
10	-55,75	2,9411765	32		2,464948
11	-71,61765	10,744467	33		-9,52381
12	-59,79798	-1,654048	34		3,0092593
13	-76,82343	-0,730994	35		11,500701
14	-86,14973	27,852085	36		16,730605
15	-98,33333	4,2514345	37		18,129572
16	-73,57724	25	38		31,234964
17		-1,801802	39		9,7940311
18		26,644737	40		21,134974
19		-2,666667	41		21,054519
20		8,2115755	42		-10,94017
21		2,194617	43		16
22		8,8057445			
			MEDIAN	**-82,22739**	**6,3268608**

I

Source:	Appel, Yarali, unpublished							
ISI (seconds)	-15	40	ISI (seconds)	-15	40	ISI (seconds)	-15	40
N								
1	-84,25926	6,9221968	56	-56,91057	8,1730769	111		63,333333
2	-38,29787	9,8214286	57	-81,48148	-22,22222	112		-2,252907
3	-83,26014	16,231884	58	-42	20	113		-24,82482
4	-94,73684	5,6958309	59	-89,65517	14,705882	114		13,675214
5	-98,13574	-4,002193	60	-47,61905	-9,288538	115		2,1235521
6	-83,20965	15,425532	61	-53,33333	0	116		-1,403509
7	-65,40616	24,973262	62	-100	-1,754386	117		24,761905
8	-55,26898	-6,158358	63	-64,28571	15,079365	118		16
9	-82,91631	28,174603	64	-32,85714	-12,22222	119		19,908467
10	-50,05708	15,598291	65	-89,35574	2,3809524	120		21,187584
11	-90,95607	2,2360248	66	-73,33333	1,5873016	121		46,947674
12	-86,59371	9,906139	67	-75,65217	-9,230769	122		5,3488372
13	-73,52657	51,011482	68	-37,87879	40,421053	123		-2,352941
14	-72,4359	5,9808612	69	-75,37879	-12,38095	124		6,1722488
15	-72,02112	23,003953	70	-56,25	-12,30769	125		0,7864489
16	-66,04979	20,625	71	-67,76786	-21,66667	126		-0,534759
17	-44,99109	5	72	-46,50538	6,0577819	127		15
18	-66,26263	14,705882	73	-90,625	-11,51961	128		2,678818
19	-40,6237	46,25	74	-89,89899	17,663818	129		13,821138
20	-70,37037	8,9285714	75	-39,90683	48,214286	130		7,1428571
21	-72,50646	45,261438	76	-62,82051	-10,41667	131		29,852858
22	-50,22399	17,106843	77	-76,03486	14,285714	132		19,955654
23	-47,61905	27,666667	78	-41,66667	-24,74916	133		0,588697
24	-92,30769	-26,36364	79	-21,31579	20,125	134		44,308943
25	-83,33333	-5,902778	80	-56,90058	2,006689	135		-17,65481
26	-33,06452	20,512821	81	-61,1336	50	136		6,25
27	-20	7,9365079	82	-30	-4,166667	137		37,142857
28	-85,96059	13,888889	83	-65	33,653846	138		22,826087
29	-71,18644	43,333333	84	-78,59238	48,888889	139		34,242424
30	-69,44444	16,287879	85	-28,23529	38,450292	140		-8,064516
31	-51,61943	-12,5	86	-84	38,333333	141		37,237027
32	-64,02597	13,235294	87	-50,11765	9,0704225	142		20
33	-10,16949	-8,333333	88	-75	20,952381	143		24,545455
34	-80,18648	9,6969697	89	-50	17,16129	144		37,248677
35	-53,33333	-1,666667	90	-33,25062	-25,90909	145		-9,31677
36	-29,16667	22,222222	91	-69,23077	81,25	146		9,3085106
37	-37,03704	-1,904762	92		16,666667	147		14,210526
38	-61,90476	-4,444444	93		23,076923	148		7,8333333
39	-24,77273	-36,36364	94		31,812865	149		-15
40	-71,42857	2,7777778	95		-3,964912	150		-7,407407
41	-86,36364	25	96		10,833333	151		11,050157
42	-28,57143	-1,428571	97		16,666667	152		-19,63134
43	-86,90909	27,083333	98		-1,655307	153		16,648649
44	-64,28571	6,7307692	99		-5,67297	154		16,692035
45	-45,45455	17,559524	100		16,666667	155		10,784314
46	-81,81818	-2,412281	101		0	156		24,444444
47	-81,74603	33,517241	102		10,01642	157		14,012097
48	-53,97727	-0,404858	103		-4,347826	158		4,8245614
49	-90,47619	24,637681	104		34,155598	159		42,10091
50	-61,18012	0,3809524	105		8,3591331	160		-29,87013
51	-66,66667	10,964912	106		31,516021	161		17,960089
52	-57,89474	-3,076923	107		3,5714286	162		0,7024266
53	-77,77778	7,9365079	108		17,037037	163		-7,539683
54	-100	-14,90385	109		-7,168459	164		0,8333333
55	-79,16667	56,25	110		-15,31746			
						MEDIAN	**-66,04979**	**9,7591991**

Figure S1. (A-I) Original data used for Figure 2 A,C

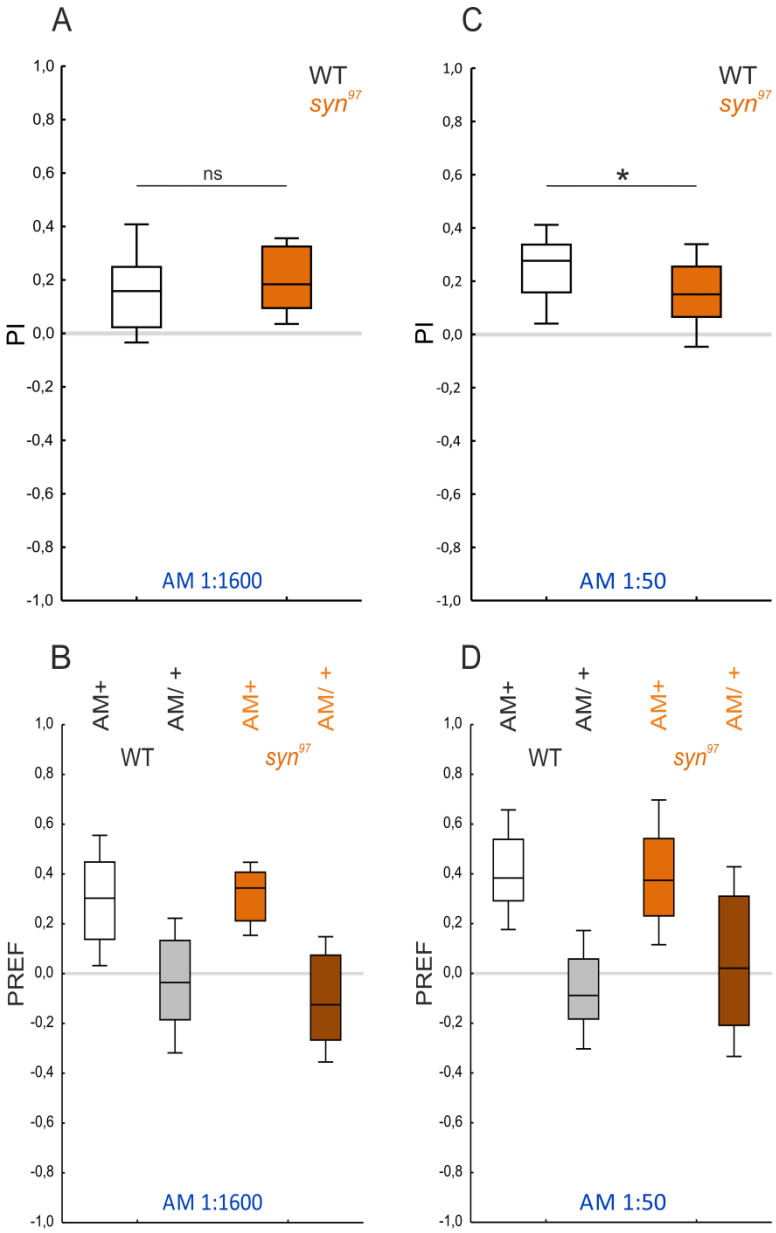

Figure S2. Odor-sugar memory in *syn*^{97} mutants is intact at low but is impaired at high odor concentration. Using the odor-sugar learning paradigm depicted in Figure 2, no memory impairment was detectable in the *syn*^{97} mutant strain with a dilution of AM as odor of 1:1600 **(A) (***P*> 0.05; U= 184; N= 21, 21**),** whereas when using a higher AM concentration in a separate set of experiments (1:50 dilution) reduced associative performance indices were observed **(C) (***P*< 0.05; U= 190; N= 21, 21**)**. White fill of the box plots is used for the wild-type WT strain, orange fill for the *syn*^{97} mutant strain. **(B, D)** Corresponding PREF scores. ns indicates *P*> 0.05, and * indicates *P*< 0.05 in MWU tests (see body text for details). Other details as in Figure 6.

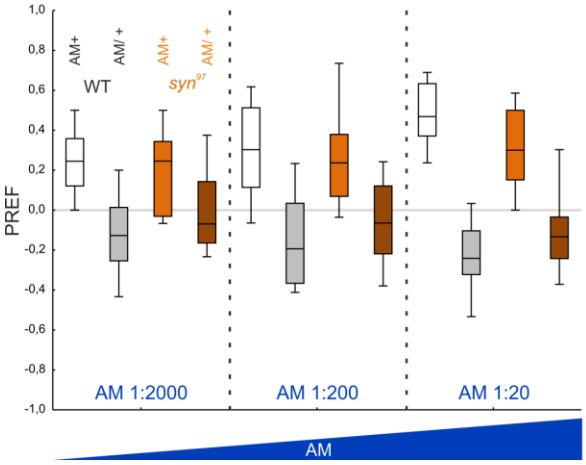

Figure S3. Plotted are the preference scores of reciprocally trained groups of larvae from the experiment displayed in Figure 7A.

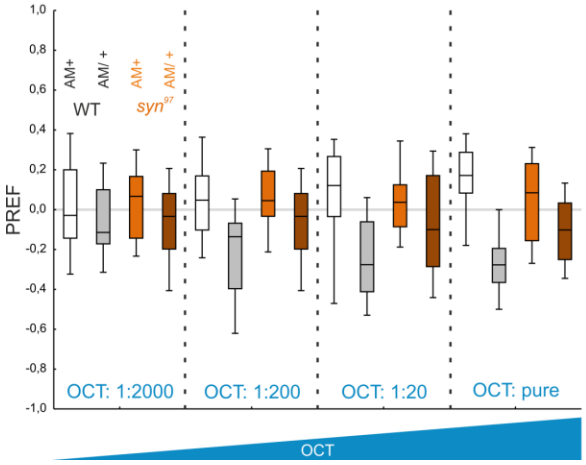

Figure S4. Plotted are the preference scores of reciprocally trained groups of larvae from the experiment displayed in Figure 7B.

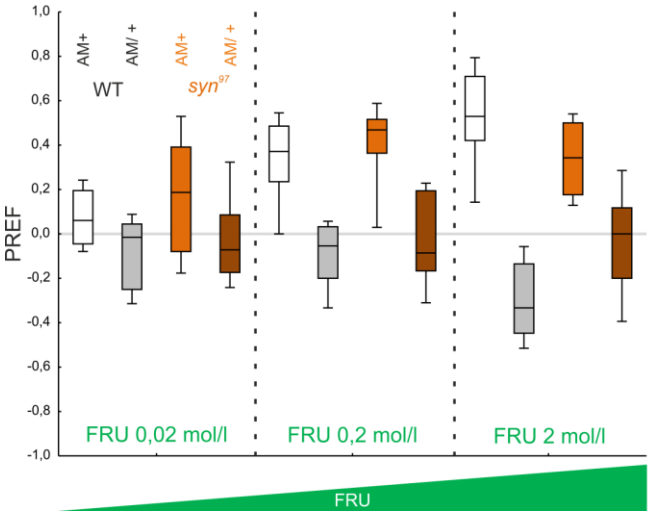

Figure S5. Plotted are the preference scores of reciprocally trained groups of larvae from the experiment displayed in Figure 7C.

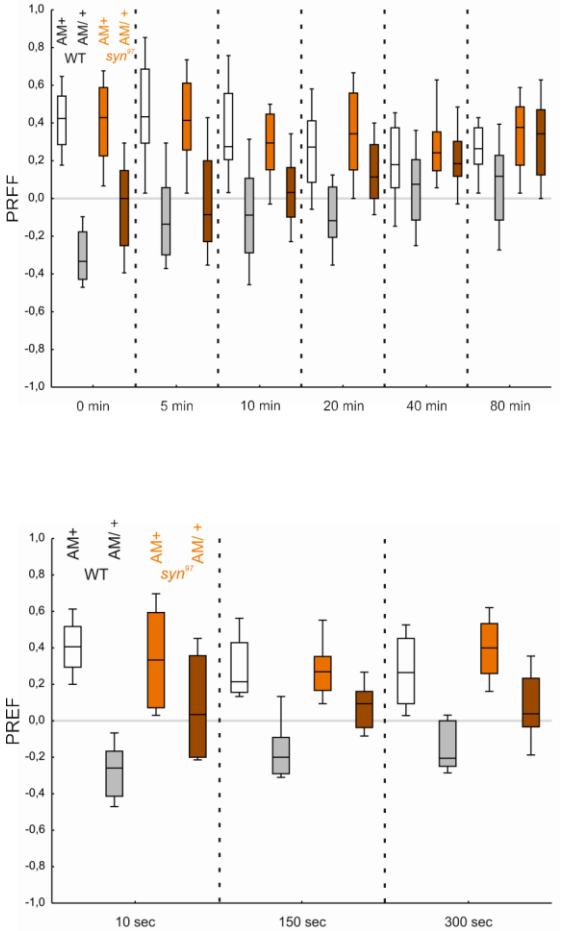

Figure S6. Plotted are the preference scores of reciprocally trained groups of larvae from the experiment displayed in Figure 8B, D.

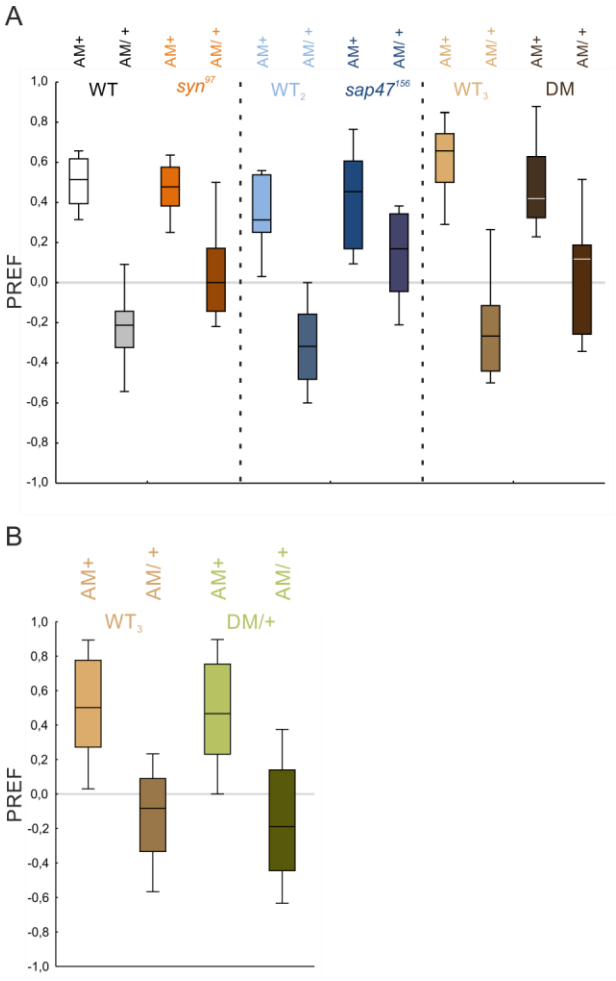

Figure S7. Plotted are the preference scores of reciprocally trained groups of larvae from the experiment displayed in Figure 9A, B.

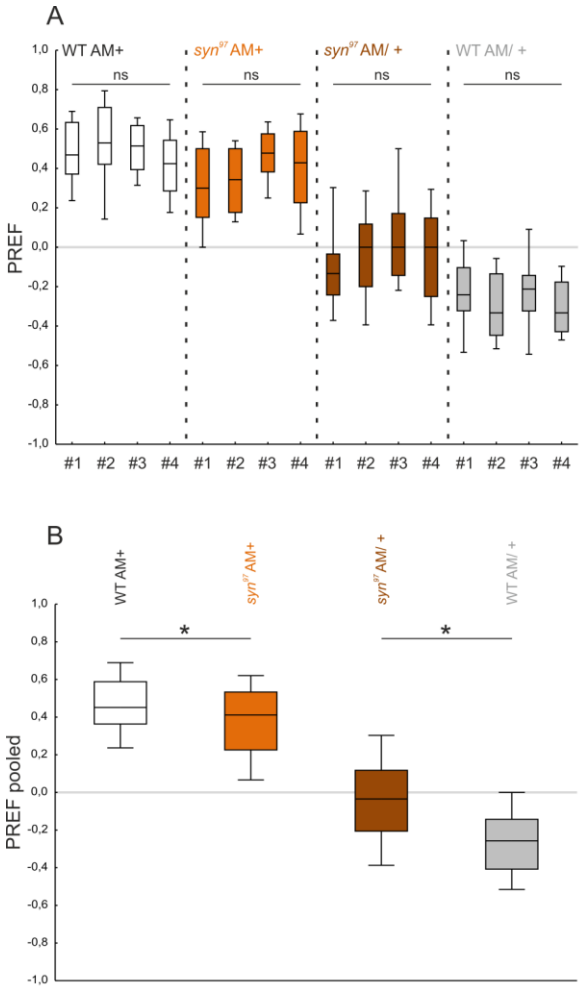

Figure S8. (A) For all experiments that were performed under the same conditions, namely with a FRU concentration of 2 mol/l and a dilution of AM of 1:20, we plotted are the preference (PREF) scores of the wild-type WT after paired training (white fill) and after unpaired training (grey fill) and the PREF of the *syn*^{97} mutant after paired training (orange fill) and after unpaired training (brown fill). The corresponding experiments are displayed in Figures 9A and S3 (#1, sample size for the respective genotype and training condition N= 27, 27, 27, 27), Figures 3C and S4 (#2, N= 15 in all cases), Figures 10B and S6A (#3, N= 31 in all cases), as well as in Figures 11A and S7 (#4, N= 22 in all cases). In no case do PREF scores differ across experiments (P> 0.05 and df= 3 in all cases; from left to right H= 3.79; H= 4.45; H= 5.03; H= 2.86 in KKW tests, indicated by ns). Pooling across experiments **(B)** reveals statistically significant differences between the wild-type WT and the *syn*^{97} mutant after both, paired training (P< 0.05/2; U= 3549.5; N= 95, 95) and after unpaired training (P< 0.05/2; U= 2219.5; N= 95, 95) (* indicates P< 0.05/2 in MWU tests).

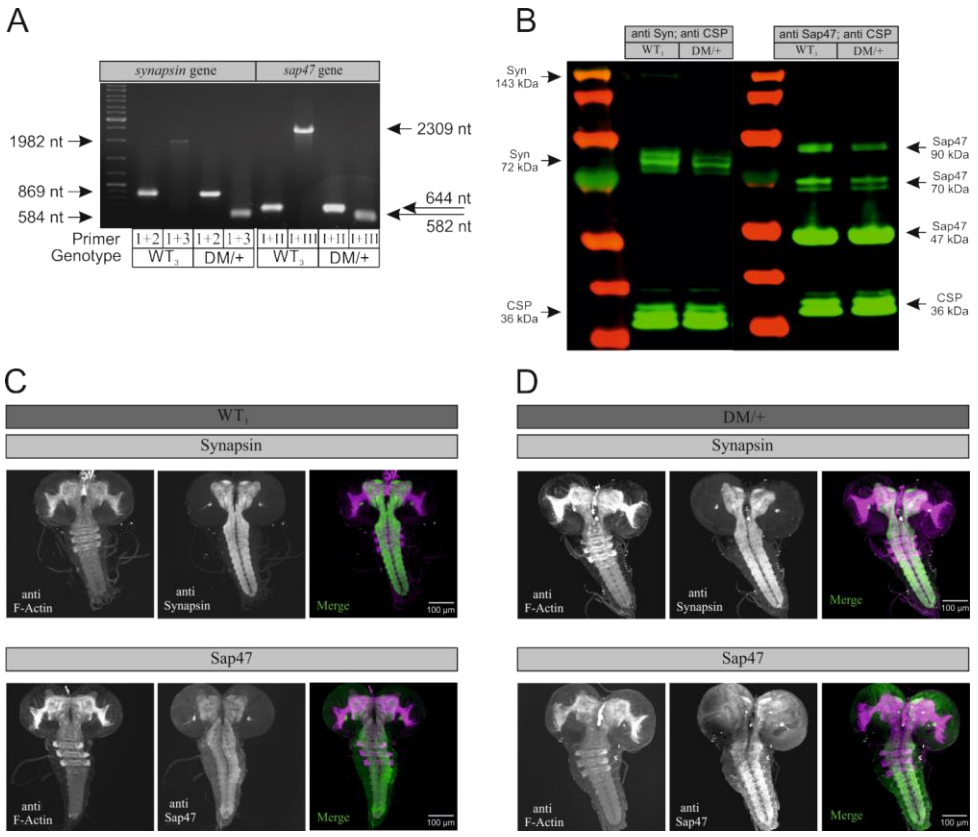

Figure S9. (A) Validation of the genetic status of the double heterozygous *syn*^{97}*/sap47*^{156} mutant (DM/+) via PCR (for details see Methods section and Figure 5A, E). We note that for primer pair 1+3 as well as for primer pair I+III only the energetically favorable short fragment and not the long fragment is detected in the DM/+. **(B)** Western blots of larval brains probed for Synapsin and Sap47 of the indicated genotypes (for details see Methods section). Expected Synapsin bands at 72 kDa are present, but the Synapsin band at 142 kDa, which has been reported before to be occasionally weak or even missing (Godenschwege et al. 2004; Michels et al. 2011), is hardly detectable in the WT_3 and is missing in the DM/+. **(C-D)** Whole mount larval brains probed for Synapsin and Sap47 of the indicated genotypes (for details see Methods section). We note that Synapsin levels appear slightly reduced in the double heterozygous *syn*^{97}*/sap47*^{156} mutant, both as judged from the Western blot (B), and the whole mounts (C, D).

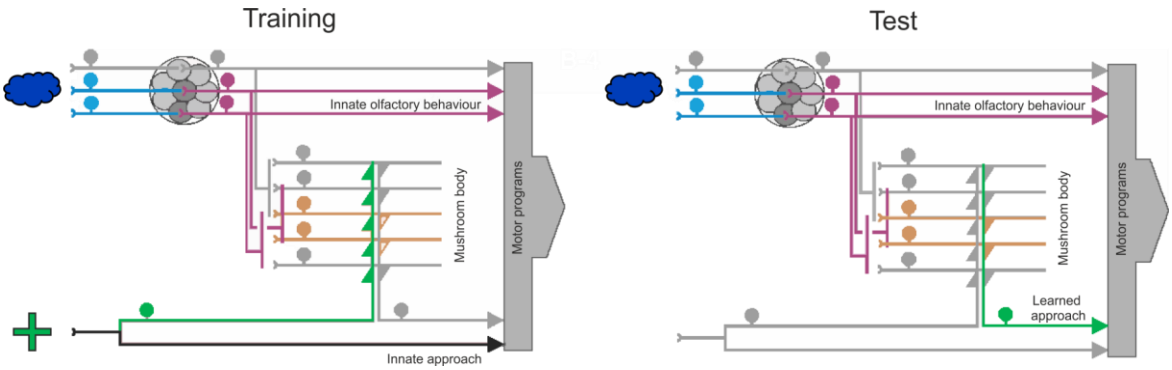

Figure S10. Simplified circuit-level working hypothesis of the events during odor-reward training and test. Odors (blue cloud) are coded combinatorially along ascending olfactory pathways up to the mushroom body Kenyon cells (orange). Intersecting modulatory neurons convey a reward signal (green). Coincidence of odor-evoked activity in the mushroom body Kenyon cells with activity from these modulatory neurons leads to plasticity in the output synapses of the mushroom body Kenyon cells. Processing along these modified synapses then is the basis for learned avoidance upon testing (modified from Gerber et al. 2014). No learned behavior is observed if the testing odor too strongly deviates from the training odor in intensity and/or quality (Chen et al. 2011; Mishra et al. 2013), arguably because of insufficient overlap in the set of Kenyon cells relative to the trained odor.

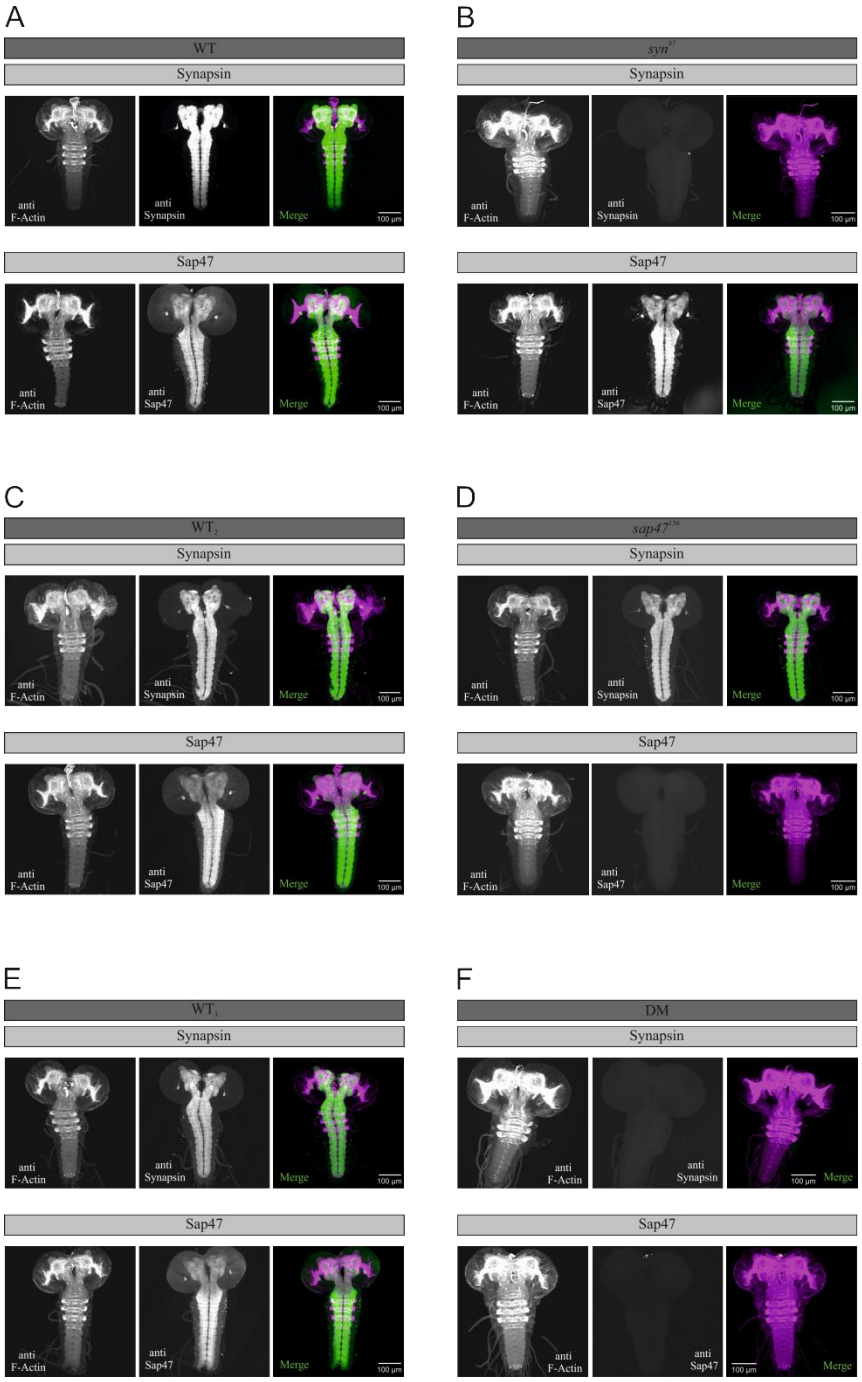

Figure S11. Whole mounts of larval brains. **(A)** For the wild-type WT strain, the top row of tiles shows whole mount preparations of the larval brain hemispheres and ventral nerve cord. These are stained with anti F-actin for orientation (left tile) and with anti-Synapsin (middle tile). The rightmost tile shows the merge (magenta: anti F-actin, green: anti Synapsin). The bottom row of tiles shows the same, but for the Sap47 protein. Note that both the Synapsin and the Sap47 protein, if expressed, are expressed throughout the larval nervous system. **(B)** Same as in (A), but for the syn^{97} mutant, which lacks the Synapsin protein but expresses Sap47. **(C, D)** Same as in (A, B), showing that the wild-type WT_2 strain expresses both Synapsin and Sap47, while the $sap47^{156}$ mutant expresses Synapsin but lacks the Sap47 protein. **(E, F)** Same as in (A, B), showing that the wild-type WT_3 strain expresses both Synapsin and Sap47, while the double mutant (DM) lacks both these proteins. All antibodies used are the same as in Figure 5F-K. Scale bar: 100 µm.

Danksagung

Ein besonderer Dank gilt meinem Doktorvater Prof. Dr. Bertram Gerber, der meinen beruflichen Lebensweg über einige Jahre begleitet hat. Vielen Dank für die gemeinsamen Jahre, die angenehme Zusammenarbeit, sowie die vielzähligen, wertvollen Erfahrungen und die tolle Unterstützung. Ich habe viel gelernt.

Während meines Promotionsstudiums durfte ich Teil der Arbeitsgruppe Gerber sein. Dies erfüllt mich mit großer Freude. Ich danke allen Kollegen aus dem Team für die schöne Zeit, das tolle Miteinander und die Unterstützung, wobei ein besonderer Dank Birgit, Timo und Michael gilt.

Meine liebe Familie bestreitet meinen Lebensweg nun schon seit über 30 Jahren mit mir. Für den enormen Rückhalt und die verlässliche Hilfe während meines Studiums und meiner Promotion danke ich euch von Herzen. Besonders möchte ich meinen lieben Eltern danken: es ist schön zu wissen, dass es jemanden gibt, der immer hinter einem steht.

Selbstständigkeitserklärung

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Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation mit dem Titel:

„Associative memory in *Drosophila melanogaster*: Synapsin as a study case"

selbstständig verfasst, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 04.03.2016

Lebenslauf

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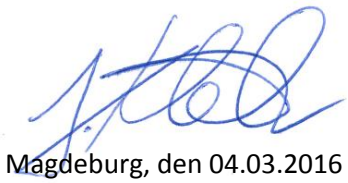

Magdeburg, den 04.03.2016