Synapsin Determines Memory Strength after Punishment- and Relief-Learning

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Adverse life events can induce two kinds of memory with opposite valence, dependent on timing: “negative” memories for stimuli preceding them and “positive” memories for stimuli experienced at the moment of “relief.” Such punishment memory and relief memory are found in insects, rats, and man. For example, fruit flies (Drosophila melanogaster) avoid an odor after odor-shock training (“forward conditioning” of the odor), whereas after shock-odor training (“backward conditioning” of the odor) they approach it. Do these timing-dependent associative processes share molecular determinants? We focus on the role of Synapsin, a conserved presynaptic phosphoprotein regulating the balance between the reserve pool and the readily releasable pool of synaptic vesicles. We find that a lack of Synapsin leaves task-relevant sensory and motor faculties unaffected. In contrast, both punishment memory and relief memory scores are reduced. These defects reflect a true lessening of associative memory strength, as distortions in nonassociative processing (e.g., susceptibility to handling, adaptation, habituation, sensitization), discrimination ability, and changes in the time course of coincidence detection can be ruled out as alternative explanations. Reductions in punishment- and relief-memory strength are also observed upon an RNAi-mediated knock-down of Synapsin, and are rescued both by acutely restoring Synapsin and by locally restoring it in the mushroom bodies of mutant flies. Thus, both punishment memory and relief memory require the Synapsin protein and in this sense share genetic and molecular determinants. We note that corresponding molecular commonalities between punishment memory and relief memory in humans would constrain pharmacological attempts to selectively interfere with excessive associative punishment memories, e.g., after traumatic experiences.

Key words: Drosophila; memory; pain; punishment; relief; Synapsin

Introduction

Painful, traumatic experiences can have a molding 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 toward “backward conditioning,” that is to memories related to stimuli perceived after a painful event (Moscovitch and LoLordo, 1968; Solomon and Corbit, 1974; Plotkin and Oakley, 1975; Heth, 1976; Wagner, 1981; Wagner and Larew, 1985; Gerber et al., 2014; 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 (Tanigawa 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; Andretta et al., 2010, 2012; Franklin et al., 2013a, b; Felsenberg et al., 2014; 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. 1A).

For punishment learning, reasonably clear working hypotheses exist (see Discussion), 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 memory 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 et al., 2013a). 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 strength. It 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; Südhof, 2004; Greco et al., 2013; Diegelmann et al., 2013a; Sadanandappa et al., 2013; Vasin et al., 2014). Regarding associative processing in Drosophila, Synapsin null mutants show reduced scores in one-trial strong-shock punishment memory, reduced spatial memory in the heat-box paradigm and, as larvae, reduced odor-

Figure 2. Phosphorylation sites of Synapsin and abundance of edited and nonedited Synapsin. LC-MS/MS analysis of experimentally naive, wild-type fly brains to map phosphorylated sites across the Synapsin protein (see also Table 1). Eighty-five LC-MS/MS runs were performed, consisting of a combination of 17 biological and five technical replicates each (coverage of the 4 longest Synapsin protein isoform of 97%). This identified 28 phosphorylated sites of Synapsin: one at tyrosine, 10 at threonine, and 17 at serine. Twenty-three phosphorylated sites were identified for the first time, while five had been reported before (Zhai et al., 2008; Nuwal et al., 2011). Of the seven phosphorylated sites both reported by Nuwal et al. (2011) and covered by the present data, we can confirm three, while we found four of these sites to be nonphosphorylated. Both the edited and the nonedited forms of the Synapsin protein were found. That is, the pre-mRNA of the synapsin transcript is modified from the N-terminal motif RRFS (nonedited) to RGFS (edited) such that the PKA consensus motif RRFS is compromised (Diegelmann et al., 2006). In n/H11005 85 LC-MS/MS runs the nonedited 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 nonedited protein motif (Table 1; 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 before MS 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 aa at the N terminus (Q24546_DROME; www.uniprot.org) was confirmed previously (Nuwal et al., 2011). Blue bars below the sequence indicate peptide-spectra matches (PSMs) 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).
sugar memory (Godenschwege et al., 2004; Michels et al., 2005, 2011; Knapek et al., 2010; Walkinshaw et al., 2015). We reasoned that Synapsin is worthy of study for its role in relief memory 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 memory 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 memory and punishment memory would caution against such approaches.

### Materials and Methods

**Genotypes and rearing of flies.** To compare flies with versus without Synapsin, we compared the deletion mutant syn97CS (Fig. 1) to a Canton-S (CS) wild-type strain. The syn97CS strain had undergone 13 outcrossing steps to this very CS wild-type strain to yield effectively identical genetic backgrounds as described previously (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/H11002 for the deletion mutant and synapsin/H11001 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 w1118):

- elav-Gal4 [X] (strain c155 from Lin and Goodman, 1994)
- mb247-Gal4 [III], synapsin/H11002, which was generated by using mb247-Gal4 (Schulz et al., 1996), recomb...
bined into the synapsin mutant background; UAS-syn[III], synapsin[H11002], which was generated on the basis of Löhr et al. (2002); tub-Gal80ts[II]; UAS-syn[III], synapsin[H11002] (Michels et al., 2011); and UAS-RNAi-syn[III].

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 elav-Gal4/+; UAS-RNAi-syn/+ flies. As a driver control, elav-Gal4 females were crossed to males without any transgene, yielding single-heterozygous elav-Gal4/+; Rescue: To rescue Synapsin, female mb247-Gal4[III], synapsin/H11001 flies were crossed to male UAS-syn[III], synapsin/H11002, yielding double-heterozygous mb247-Gal4/UAS-syn flies in the homozygous synapsin mutant background. As a driver control, female mb247-Gal4, synapsin/H11001 were crossed to male synapsin/H11002, yielding single-heterozygous mb247-Gal4/+ in the homozygous synapsin mutant background. As an effector control, synapsin/H11001 females were crossed to male UAS-syn, synapsin/H11002 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-Gal80ts. We crossed female mb247-Gal4[III], synapsin/H11001 to male tub-Gal80ts[II]; UAS-syn[III], synapsin/H11002, yielding triple-heterozygous tub-Gal80ts/+; mb247-Gal4/UAS-syn in the homozygous synapsin mutant background. As an effector control we used the offspring of female synapsin/H11002 and male tub-Gal80ts; UAS-syn, synapsin/H11002 to yield double-heterozygous tub-Gal80ts/+; UAS-syn/H11001, in the homozygous synapsin mutant background. As a driver

Figure 3. Memory strength is lessened, rather than features of coincidence detection modulated, in mutant flies lacking Synapsin. A, When using only one ISI for relief learning and one ISI for punishment learning, less strong scores in both relief memory and punishment memory could be a result of a lessening in strength of the associative memory (A1), a narrowed dynamic range of associative coincidence detection (A2), a narrowed 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− and synapsin+ mutant flies. C, The median 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.

Other details as in Figure 1. C, The median 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.
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 (data 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 on a 16/8 h light/dark cycle. Unless mentioned otherwise, 1- to 5-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 d to allow inactivation of Gal80ts and thus expression of Synapsin (Induced), or retained at 18°C for 4 d (Control).

Throughout, we used flies in groups of 100, handled in a tube system based on Tully and Quinn (1985) and 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) or 110 μl limonene (LM; CAS number 5989-27-5; Sigma-Aldrich) were applied in Teflon containers of 5 mm or 7 mm diameter, respectively.

Learning experiments and behavioral controls. For punishment learning and for relief learning, flies received six training trials. In the following example, BA is the to-be-learned odor and LM is the control odor. At time 0:00, flies were loaded into the experimental setup, which took ~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 six 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 a 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 et al., 2009b).

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 ISI 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 setup 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 as # in the following equation) within each arm was counted. The relative preference between BA and LM (PREF) was then calculated as follows:

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

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 (Tully and Quinn (1985); Gerber and Stocker (2007) feature discussions of why these PIs yield measures of associative memory, cleared of nonassociative effects):

$$\text{PI} = \frac{(\text{PREF}_{\text{BA}} - \text{BA to-be-learned}) - \text{PREF}_{\text{LM}} - \text{LM to-be-learned})}{2}$$

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 setup. 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; 10 s 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 (PREFSHOCK) was calculated to provide negative values for avoidance of the electrified arm:

$$\text{PREFSHOCK} = \frac{(\text{#Electrified arm} - \text{#Non-electrified arm}) \times 100/#_{\text{Total}}}{2}$$

To assess olfactory behavior, the flies were loaded into the experimental setup. 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 (PREFODOR) was calculated as follows:

$$\text{PREFBA} = \frac{(\text{#BA} - \text{#LM}) \times 100/#_{\text{Total}}}{2}$$

$$\text{PREFLM} = \frac{(\text{#LM} - \text{#BA}) \times 100/#_{\text{Total}}}{2}$$

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; 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 toward BA and LM was measured as described in the preceding paragraph (Eqs. 4a,b).

Statistical analyses of behavioral data. Nonparametric statistics was used throughout, Kruskal–Wallis or Mann–Whitney U tests were used to compare multiple or two groups of flies, respectively. To test for differences from zero, we used the one-sample sign tests. 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 of <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 performed according to Gloor et al. (1993), using material from individual flies. The primer binding sites were upstream (primer 1: 5’-AGAAATTGTGCTGAGTT-3’), within (primer 2: 5’-CGGTTGTCGTTTTGTT-3’), or downstream (primer 3: 5’-CCTCTAATTGCGCTGTG-3’) of the deletion (Fig. 1).

**Immunohistochemistry and Western blotting.** For whole-mount immunohistochemistry, brains were dissected in Ringer’s solution and fixed for 2 h in 4% formaldehyde with PBS containing 0.3% Triton X-100 (PBST) as the solvent. Samples were blocked in 3% normal goat serum (Jackson ImmunoResearch) to detect the primary antibody. All incubations steps were followed by multiple PBST washes. Incubations were incubated overnight with an Alexa 488-coupled goat anti-mouse Ig (diluted 1:200 in PBST; Invitrogen) to detect the primary antibody. All incubation steps were followed by multiple washing procedures (1 × TBST). Visualization was achieved with the ECL Western blot detection reagents (GE Healthcare Life Sciences).

**Analysis of Synapsin phosphorylation by LC-MS/MS.** Brains of experimentally naive adult *Drosophila* were dissected in Ringer’s solution containing halotere and phosphatase inhibitor cocktail, EDTA-free (1:100; Thermo Fisher Scientific) 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 of water containing 8 M freshly denized urea. Tissue and cell destruction was achieved by means of a microglass 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$_4$HCO$_3$ buffer, pH 8.0, containing 2 mM dithiothreitol and 1% (w/v) RapiGest SF surfactant (Waters) to obtain a final concentration of 2 μM urea and 0.75% RapiGest. After gently shaking at room temperature for 60 min, 10 μM 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) followed by incubation at room temperature for 12 h. After complete digestion, RapiGest was hydrolyzed by adding trifluoroacetic 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), 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). Proteome analysis was performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro; Thermo Scientific) equipped with an EASY-nLC ultra HPLC (Thermo Scientific). Samples were resolubilized in 50 μl of 0.1% TFA and 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 (Di- onex). 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 (FTMS) 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: FTMS (resolution 60,000; m/z range 400–2000) and 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). FDR was set to <1%. Phospho-sites were accepted as confident for motif-specific phosphorylations was performed using the NetPhosK 1.0 Server (Blom et al., 2004).

Results

Synapsin is a phosphoprotein

Given that Drosophila Synapsin has been described as a phospho-protein (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 naive, synapsin− wild-type flies the Synapsin protein features 28 phosphorylated sites and that these sites encompass consensus motifs for 11 kinases (Fig. 2, Table 1).

We further note that proteins corresponding to both the edited and the nonedited version of Synapsin (Diegelmann et al., 2006) were found to be expressed (Fig. 2, Table 1).

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

Mutant flies lacking Synapsin (synapsin−; Fig. 1B, C) are deficient in both relief memory and punishment memory scores (Fig. 1D). 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-memory and punishment-memory strength indeed lessened?

Given the known dependence of conditioned valence on the timing between odor and shock (the ISI; Gerber et al., 2014), there are actually five scenarios as to how a lack of Synapsin could lead to defects in both relief memory and punishment memory. That is, suppose only one ISI each was chosen for relief training and punishment training (Fig. 3, vertical arrows). Under such conditions all scenarios, but the one in Figure 3A1, predict that the respective maximum of the relief-memory 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. 3A1). Therefore, we decided to compare the full ISI function between synapsin− wild-type and synapsin− mutant flies.

As shown in Figure 3, B 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 = 62, 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-memory and relief-learning memory strength is lessened, corresponding to the scenario in Figure 3A. This argues that a lack of Synapsin entails a lessening in strength of associative memory rather than an alteration in the temporal properties of coincidence detection (Fig. 3A2–A5).

Task-relevant sensory and motor faculties are intact in synapsin mutant flies

To ascertain whether the lessening in strength of both relief memory and punishment memory in the synapsin mutant is actually secondary to some sort of sensory or motor impairment, we test whether behavior toward 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. 4A; $U = 104, p > 0.05$; $N = 16, 16$). Likewise, synapsin wild-type and synapsin mutant flies do not differ in their behavior toward BA or LM (Fig. 4B; $U = 116, 158, p > 0.05$ in both cases; $N = 19, 16, 20, 16$). We conclude that avoidance of the shock as well as both the odors in question is indistinguishable between genotypes, in experimentally naive animals.

These kinds of control procedures 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 naive animals only shows that at the beginning of the learning experiment the mutants are normal in sensorimotor ability; whether these faculties are still intact at the moment of the test remains unclear (Prétat, 1998; Michels et al., 2005; see discussion in Gerber and Stocker, 2007; Knapek et al., 2010). For example, the handling during the experiment, exposure to odors, and/or exposure to the shocks during training can nonassociatively alter olfactory behavior (Prétat, 1998; Boyle and Cobb, 2005; Colomb et al., 2007; Knapek et al., 2010; Sadananadappa 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 isoamyl acetate and 1-butanol, synapsin wild-type and synapsin mutant flies differ in olfactory behavior after such exposure (H. Tanimoto, 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. 5A) or the odors are omitted (shock exposure, Fig. 5D). After this kind of treatment, we test whether synapsin wild-type and synapsin mutant flies differ in their behavior toward either odor. We do not find any between-genotype differences in these tests (Fig. 5B, 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 naive animals (Fig. 4B, C), and the testing of animals after sham training (Fig. 5) feature a test situation where the flies chose between an odor arm versus a blank arm of the T-maze. However, both relief memory and punishment memory are assessed in a choice situation between the trained odor and the control odor (Fig. 1A). The rationale, throughout the literature, for not running the control procedures with two odors as well is that if both odors were (approximately) equally salient, one might obtain choice scores of approximately zero in both wild-type and mutant flies, but possibly 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 the argument 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 nondiscriminatory tasks, i.e., if one-odor versions of the task are used (Fig. 6A). However, also in these nondiscriminatory tasks, deficits in relief memory and punishment memory are found (Fig. 6B; $U$ 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 learning and punishment learning reflect defects in
associative memory and are unrelated to odor discrimination ability.

RNAi-mediated knock-down phenocopies the relief memory and punishment memory defect

We next test whether the defect of the synapsin mutant flies in relief memory 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 (knockdown).

A reduction in Synapsin levels (Fig. 7A) leads to reduced relief memory in the knock-down flies compared with both genetic controls (Fig. 7B; U = 19, 38, p < 0.05/3 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 knockdown flies.

Knock-down flies also show reduced punishment memory compared with both genetic control flies (Fig. 7C; U = 47, 48, p < 0.05/3 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 knockdown) 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 memory and punishment memory.

Locally restoring Synapsin restores relief memory 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 memory and/or punishment memory.

The expression of Synapsin in the rescue flies is indeed restricted to the mushroom bodies (Fig. 8A). Upon such local expression, we find that both relief memory and punishment memory are fully rescued: after relief learning, rescue flies perform better than both control genotypes (Fig. 8B; U = 26, 48, p < 0.05/3 in both cases; N = 17, 15, 15, 15), and actually do as well as...
### A

|   | Control        | Synapsin | Cell bodies | Merge |
|---|----------------|----------|-------------|-------|
| 1 | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) | ![Image](image4.png) |
| 2 | ![Image](image5.png) | ![Image](image6.png) | ![Image](image7.png) | ![Image](image8.png) |
| 3 | ![Image](image9.png) | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| 4 | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) | ![Image](image16.png) |
| 5 | ![Image](image17.png) | ![Image](image18.png) | ![Image](image19.png) | ![Image](image20.png) |
| 6 | ![Image](image21.png) | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| 7 | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) | ![Image](image28.png) |
| 8 | ![Image](image29.png) | ![Image](image30.png) | ![Image](image31.png) | ![Image](image32.png) |

|   | UAS-syn | tub-Gal80<sup>ns</sup> | mb247-Gal4 | synapsin |
|---|---------|-----------------------|------------|----------|
| 1 | -       | +                     | -          | -        |
| 2 | -       | +                     | -          | +        |
| 3 | -       | +                     | -          | +        |
| 4 | -       | +                     | -          | -        |

### B

![Graph](image5.png)

### C

![Graph](image6.png)
synapsin\textsuperscript{+} 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\textsuperscript{−} mutant defect in relief memory.

Also after punishment learning, rescue flies perform better than either kind of control flies (Fig. 8C; \(U = 150, 185, p < 0.05/3\) in both cases; \(N = 26, 26, 26, 26\)). Indeed, their punishment-memory scores are as high as those of synapsin\textsuperscript{+} 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\textsuperscript{−} mutant defect in punishment memory is also fully restored upon restoring Synapsin locally in the mushroom body.

We conclude that restoring Synapsin in the set of cells covered by mb247-Gal4, covering the mushroom body \(\alpha, \beta, \gamma\) lobes, and with faint if any background expression (Aso et al., 2009; Fig. 8A), is sufficient to restore the defects in both relief memory and punishment memory, which ensue upon a lack of Synapsin.

Acutely and locally restoring Synapsin restores relief memory 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\textsuperscript{14} we combined the expression of Gal80\textsuperscript{15}, a temperature-dependent inhibitor of Gal4, together with mb247-Gal4 as a driver and UAS-syn as an effector. Thus, the induction of Synapsin expression in the mushroom bodies can be achieved by raising the temperature acutely only during adulthood, which inactivates Gal80\textsuperscript{15}, releases the inhibition of Gal4, and allows Synapsin expression in the mb247-Gal4 pattern (compare Figs. 9A10, 10A10). As compared with constitutive expression of Synapsin, such induced expression is notably faint (compare Figs. 8A22, 10A22).

In control conditions without induction, relief-memory scores in the experimental genotype are less than in the synapsin\textsuperscript{+} wild-type flies (Fig. 9B; \(U = 46, p < 0.05/3; N = 21, 21\)), and not different from effector control 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\textsuperscript{+} wild-type flies (Fig. 10B; \(U = 151, p > 0.05/3; N = 16, 16\)). 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 compared with synapsin\textsuperscript{−} wild-type flies (Fig. 10C; \(U = 231, p > 0.05/3; N = 25, 22\)); these flies indeed show stronger punishment-memory scores than both the effector control and driver control (\(U = 135, 117, p < 0.05/3\) in both cases, \(N = 22, 22\)). Without induction, punishment memory remains at levels less than in synapsin\textsuperscript{+} wild-type flies (Fig. 9C; \(U = 38; p < 0.05/3, N = 15, 12\)) 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\textsuperscript{−} mutant defects in both relief memory and punishment memory.

Discussion

We report Synapsin as a determinant for memory strength in a behavioral assay of timing-dependent associative plasticity (Figs. 1, 3, 6–9). The defects upon a lack of Synapsin do not reflect any task-relevant sensorimotor impairment, as manifest either in naïve responsiveness to odors or shock (Fig. 4) or in responsiveness to the odors after sham-training procedures (Fig. 5). Also, the outcrossing regimen for the mutant, the RNAi-phenocopy of the mutant phenotype, and the spatially and 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 memory and punishment memory toward generally more negative valence (Yarali et al., 2009b). 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 of Drosophila, 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 knockdown of Synapsin. The observation that this defect is partial is in agreement with what Godenschwege et al. (2004) and Knapke 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 (Knapke 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 et al. (2013b) have found. The emerging scenario is 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 memory and punishment memory (for the stimuli experienced, respectively, after and before the trauma), an amnesic cold-shock treatment would leave only a punishment memory.

Our observation that restoring Synapsin in the Synapsin null mutant fully restores both relief- and punishment-memory scores (Fig. 8) 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. 10), and in a spatially specific way, in the mushroom body (Figs. 8, 10). Notably, this was the case for both relief memory and punishment memory. This prompts the question of how the fly brain is organized on the cellular and/or molecular level to establish memories that support opposite behaviors (i.e., conditioned avoidance vs approach). For such a discussion, a brief sketch of the current working hypothesis on punishment learning is warranted (for review, see 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 et al., 2014a, b).

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; Schweizer 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 signaling 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). Nontrained odors do not support conditioned avoidance, unless sufficiently similar in quality and/or intensity to the trained one (Yarali et al., 2009a; 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 learning and reward learning may engage different sets of mushroom body neurons as well as different input and output neurons, as if the mushroom body was 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 et al., 2014a, b). With respect to the mb-input neurons, those dopaminergic neurons that are required for punishment learning, defined, for example, by the TH-Ga4 driver, are dispensable for relief-memory (Yarali and Gerber, 2010). It is not known which TH-Ga4-negative mb-input neurons and which mb-output neurons are participating in relief learning. Whether different subsets of the mushroom body neurons harbor the memory trace after relief learning and punishment learning likewise remains to be determined.

Alternatively, relief memory 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. 2, Table 1; Michels et al., 2011; Niwalk et al., 2011; Sadanandappa et al., 2013; Diegelmann et al., 2013a), it is conceivable that different kinases and/or phosphorylation sites of the Synapsin protein could be used during relief learning 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 learning and punishment learning, in particular as the proteins corresponding to both the edited and the nonedited version are indeed expressed (Fig. 2, Table 1).

To summarize, the current results regarding Synapsin point to shared genetic and molecular determinants for relief memory 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 memory 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.

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