Regulators of Long-Term Memory Revealed by Mushroom Body-Specific Gene Expression Profiling in *Drosophila melanogaster*

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ABSTRACT Memory formation is achieved by genetically tightly controlled molecular pathways that result in a change of synaptic strength and synapse organization. While for short-term memory traces, rapidly acting biochemical pathways are in place, the formation of long-lasting memories requires changes in the transcriptional program of a cell. Although many genes involved in learning and memory formation have been identified, little is known about the genetic mechanisms required for changing the transcriptional program during different phases of long-term memory (LTM) formation. With *Drosophila melanogaster* as a model system, we profiled transcriptomic changes in the mushroom body—a memory center in the fly brain—at distinct time intervals during appetitive olfactory LTM formation using the targeted DamID technique. We describe the gene expression profiles during these phases and tested 33 selected candidate genes for deficits in LTM formation using RNAi knockdown. We identified 10 genes that enhance or decrease memory when knocked-down in the mushroom body. For *vajk-1* and *hacd1*—the two strongest hits—we gained further support for their crucial role in appetitive learning and forgetting. These findings show that profiling gene expression changes in specific cell-types harboring memory traces provides a powerful entry point to identify new genes involved in learning and memory. The presented transcriptomic data may further be used as resource to study genes acting at different memory phases.

KEYWORDS learning; long-term memory; *Drosophila melanogaster*; targeted DamID; transcriptome

ONE of the most intriguing functions of the brain is its ability to form long-term memories (LTMs), which may be stored for days, months, or even a lifetime. The molecular and genetic process underlying LTM formation include *de novo* protein synthesis and corresponding changes in gene regulation, which in turn result in long-lasting changes in synaptic plasticity (Davis and Squire 1984; Tully *et al.* 1994). Initial studies in the Californian sea hare *Aplysia californica* identified the transcription factor cAMP response element binding protein (CREB), which is required for gene regulation of LTM formation (Dash *et al.* 1990; Lee *et al.* 2012). The importance of CREB in LTM formation has been confirmed in vertebrates and invertebrates, indicating its conservation through evolution, supporting the idea that similar pathways control LTM formation (Yin and Tully 1996; Silva *et al.* 1998; Kandel *et al.* 2014). CREB is the most prominent example, but other transcription factors contribute to the regulation of transcription significant for memory and synaptic plasticity (Alberini 2009). New protein synthesis is not only required for LTM formation, but also at later phases after learning. Several studies have shown that reactivated or recalled memories become sensitive to disruption, and that stabilization is again dependent on protein synthesis (Nader *et al.* 2000; Kida *et al.* 2002; Pedreira *et al.* 2002; Lee *et al.* 2012). In addition, a later wave of mRNA and protein synthesis seems to be essential for LTM maintenance (Bekinschtein *et al.* 2007; Katche *et al.* 2010). A recent study in *Drosophila* showed that CREB-dependent transcription is also required for LTM maintenance; however, a different coactivator interacts with CREB in memory formation and maintenance (Hirano *et al.* 2016). Moreover, late memory maintenance becomes independent of CREB, but requires other transcription factors.

Although many genes involved in the acquisition and consolidation of memories have been identified, little is

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known about the genetic bases of LTM formation and maintenance. In *Drosophila*, most studies on LTM focused on the first 24 hr time window after learning. Therefore, our understanding of the genetic and molecular mechanisms of LTM is mostly limited to this early time window.

The mushroom body (MB) represents the main center of olfactory associative memory in the *Drosophila* brain (Heisenberg *et al.* 1985; de Belle and Heisenberg 1994; Dubnau *et al.* 2001). Each MB contains ~2500 neurons, called Kenyon cells (KCs), that receive input from olfactory projection neurons and extend axons to form lobe structures (Aso *et al.* 2009). KCs are classified into three classes, αβ, α′β′ and γ, according to their projection pattern in the lobes (Crittenden *et al.* 1998). Dopaminergic neurons from the protocerebral anterior medial (PAM) cluster convey the sugar reward signal to the MB, where the association of the odor and the reward is taking place (Burke *et al.* 2012; Liu *et al.* 2012). We here use a MB-specific transcriptomic approach to identify genes that are involved in LTM maintenance and forgetting. We made use of the Targeted DamID (TaDa) technique to profile transcription in KCs (Southall *et al.* 2013). TaDa enables cell-type specific gene expression profiling with temporal control. The system employs DNA adenine methyltransferase (Dam) from *Escherichia coli*, which is fused to RNA polymerase II (Pol II). Expression of the fusion protein results in methylation of adenine in the sequence GATC in loci that are bound by Pol II, providing a readout of transcriptional activity. Methylated fragments can specifically be amplified and then sequenced. We prepared and sequenced samples of paired and unpaired trained flies at four time intervals, each with four biological replicates, to analyze gene expression changes within KCs. Differentially expressed genes of these four time intervals after appetitive olfactory conditioning were determined, and 33 candidate genes were selected and tested in a LTM RNAi experiment. Ten RNAi lines that showed a lower or higher 48 hr reward memory performance than the control line were identified. Two genes, *vajk-1* (*CG16886*) and *hacd1* (*CG6746*), were examined in more detail. Knockdown of *hacd1* in the MB resulted in enhanced LTM; however, short-term or middle-term memory was not affected. *vajk-1* knockdown in the MB showed impaired memory at all tested memory phases in knockdown experiments and could be involved in memory formation.

Materials and Methods

Fly strains

Drosophila melanogaster flies were reared on cornmeal medium supplemented with fructose, molasses and yeast. If not mentioned differently, flies were kept at 25° and exposed to a 12 hr light–12 hr dark cycle. For the experiments with *tubGal80^{ts}*, flies were raised at 18° and moved to 29° 5 days before conditioning.

Canton-S was used as wild-type (courtesy of R. Stocker). *UAS-Dam* and *UAS-Dam-Pol II* were obtained from Tony D. Southall (Imperial College London), *c739-Gal4* was obtained from Hiromu Tanimoto (Tohoku University) and *mb247-Gal4* was obtained from Dennis Pauls (University of Würzburg). Used *UAS-RNAi* lines were received from the VDRC stock center (Dietzl *et al.* 2007) or Transgenic RNAi Project (TRiP) collection (Perkins *et al.* 2015) (see Supplemental Material, Table S3 for stock numbers). *c305a-Gal4* (30829), *5-HTR1B-Gal4* (27636), Df(2L)BSC345/CyO (24369), *UAS-Dcr-2* (24644), and *tubGal80^{ts}* (7019) were obtained from the Bloomington stock center. *vajk-1^{LL00558}* contains a PBac insertion in the first coding exon that causes a truncation of the protein. *vajk-1^{LL00558}* was obtained from the Kyoto stock center (140107).

Appetitive olfactory conditioning

The memory apparatus used to conduct the behavior experiments is based on that of Tully and Quinn (1985) and was modified to allow performing four experiments in parallel. Two odors were used; limonene (183164; Sigma-Aldrich) and benzaldehyde (12010; Fluka). Limonene (85 μl) was filled in plastic containers measuring 7 mm in diameter, and 60 μl of benzaldehyde was filled in plastic containers measuring 5 mm in diameter. A vacuum pump adjusted to a flow rate of 7 liter/min was used for odor delivery. Experiments were done at 22–25° and 70–75% relative humidity. Training was performed in dim red light and tests were performed in darkness. Filter paper soaked with a 1.5 M sucrose (84100; Sigma-Aldrich) solution or distilled water was prepared the day before the olfactory conditioning experiments and left to dry at room temperature. At 19–21 hr before conditioning, groups of 60–100 flies (1–4 days old) were put in starvation vials and kept at 18°. Empty fly vials with wet cotton wool on the bottom were used to starve the flies.

For appetitive conditioning, starved flies were loaded in tubes lined with water filter papers. After an initial phase of 90 sec, one of the odors was presented for 120 sec. Then, flies were exposed for 60 sec to nonodorized airflow. During this 60 sec, flies were transferred to tubes lined with sucrose filter papers. Afterward, the second odor was presented for 120 sec. To assess 0 hr memory, flies were tested immediately after conditioning. For 3 and 48 hr memory, flies were put back in starvation vials and were kept at 18° until the test. Flies tested 48 hr after conditioning were put on food for 2 hr at 21–23 hr before the test. One experiment consisted of two reciprocal conditionings, in which the odor paired with sucrose was exchanged.

For the unpaired training protocol, flies were loaded in tubes lined with sucrose filter papers, and, after 120 sec, transferred to tubes lined with water filter papers; 2 min after the sucrose, flies were exposed for 120 sec to the first odor, 60 sec to nonodorized airflow and 120 sec to the second odor.

Memory tests

For the memory test, flies were loaded into a sliding compartment and moved to a two-arm choice point where they could

choose between benzaldehyde and limonene. After 120 sec, gates were closed and the number of flies within each arm was counted. A preference index was calculated as follows:

$$\mathrm{PREF} = \Big(\big(\mathrm{N}_{\text{paired odor}} - \mathrm{N}_{\text{control odor}} \big) 100 \Big) \Big/ \mathrm{N}_{\text{total}}$$

The preference indices from the two reciprocal groups were averaged to calculate a memory performance index (PI).

Sensory tests

Sensory tests were performed with the same apparatus as the memory tests. To measure sucrose response, flies could choose between a tube lined with a sucrose filter paper and a tube lined with a water filter paper for 120 sec. A sucrose preference index (PrefI) was calculated with this formula:

$$\mathrm{PrefI} = \Big(\big(\mathrm{N}_{\text{sucrose}} - \mathrm{N}_{\text{water}} \big) 100 \Big) \Big/ \mathrm{N}_{\text{total}}$$

For the odor avoidance tests, flies could choose between a tube with an odor container attached (filled with benzaldehyde or limonene) and a tube with an empty plastic container attached. Flies in each tube were counted after 120 sec and an odor preference index was calculated:

$$\mathrm{PrefI} = \Big(\big(\mathrm{N}_{\text{air}} - \mathrm{N}_{\text{odor}} \big) 100 \Big) \Big/ \mathrm{N}_{\text{total}}$$

Targeted DamID

UAS-Dam and *UAS-Dam-Pol II* flies were crossed to *tubGal80^{ts}*; *mb247-Gal4*. Offspring of those crosses were reared at 18° and trained with the standard (paired) or unpaired olfactory appetitive conditioning paradigm. Expression of Dam and Dam-Pol II was induced by shifting the flies to 29°. Four time intervals were used: T1–T4. For T1, flies were moved to 29° 3 hr before conditioning, were trained at 25°, and moved back to 29° for 12 hr. For T2–T4, animals were trained at 18° and shifted to 29° 12–24 hr (T2), 24–48 hr (T3), or 48–72 hr (T4) after conditioning. At the end of the 29°-time interval, flies were frozen in liquid nitrogen and heads were collected. Extraction of genomic DNA from the fly heads (50–100 per sample), amplification of methylated fragments, DNA purification, and sonication was performed according to Marshall *et al.* (2016). After sonication, DamID adaptors were removed by digesting overnight at 37° with 5 units of Sau3AI (R0169S; NEB). The sequencing libraries were prepared according to the Illumina TruSeq nano DNA library protocol. The samples were sequenced using NGS (Illumina HiSeq3000) at an average of ~30 million paired-end reads per sample.

Targeted DamID analysis

Low-quality bases of the sequencing reads were trimmed using Trimmomatic (Bolger *et al.* 2014) and the remaining good quality reads were mapped to the *D. melanogaster* genome (Release 6.05; Hoskins *et al.* 2015) using Bowtie2 (Langmead and Salzberg 2012). Next, the damidseq_pipeline software was used to process the data (Marshall and Brand 2015) and to generate log2 ratio files for each pairwise comparison between Dam-Pol II and Dam-only for each time point separately. For each gene, the log2 ratio was calculated and a false discovery rate (FDR) assigned. The FDR value was generated via 50,000 simulations and represents the probability of having a given expression for a given gene based on the length of the gene and the total number of GATC sites present in this gene. The expression level of each gene was defined based on the weighted log2 ratio comparing the number of reads mapping on the Dam-Pol2 experiment to the number of reads mapping on the background (Dam-only experiment) and taking into account the number of GATC sites per gene as well as the length of the annotated gene. Each gene with a log2 ratio > 0 (*i.e.*, significantly higher mapping on Dam-Pol2 sample compared to the background) and a FDR value < 0.05 were considered as expressed for a given time point and a given pairwise comparison. For each time point, we generated 16 expression values resulting from comparing the four Dam-Pol2 replicates to the four Dam-only replicates. This range of 16 log2 expression values was then compared between paired and unpaired trained flies across the four time points using a Student's *t*-test and a linear model distribution.

Statistics

To compare the PrefIs or PIs of two groups, the Welch two sample *t*-test was used. To test if PI mean values are different from zero, the one sample Student's *t*-test was used. Statistical analyses and graphical representation of the data were performed using R version 3.4.1 (R Foundation for Statistical Computing).

Data availability

Generated fly strains are available upon request. Sequencing data can be accessed on BioProject (PRJNA419677). Supplemental material is available at Figshare: https://doi.org/10.25386/genetics.6580280.

Results

Assessing gene expression profiles during LTM formation

To study temporal gene expression changes in the mushroom body during LTM formation we used the Targeted DamID technique, an adaptation of the DNA adenine methyltransferase identification (DamID) technique (van Steensel and Henikoff 2000; Southall *et al.* 2013). This technique employs an *E. coli* DNA adenine methyltransferase (Dam), which is fused to a DNA-associated protein of interest. The bacterial Dam methylates adenine in the GATC sequence, tagging the regions of the genome where the Dam-fusion protein has interacted with DNA. TaDa allows temporally controlled expression of Dam in a cell- or tissue-specific fashion. We used TaDa to profile RNA pol II binding, which allows the identification of actively transcribed loci and therefore provides an indirect readout of gene expression. The Dam-Pol II fusion

protein (*UAS-Dam-Pol II*) was expressed in KCs of the mushroom body—a brain center for olfactory associative memory—using the *mb247-Gal4* (*MB-Gal4*) driver (Heisenberg *et al.* 1985; de Belle and Heisenberg 1994; Dubnau *et al.* 2001). *mb247-Gal4* drives expression of UAS-target transgenes in the α, β, and γ lobes of the mushroom body (Zars *et al.* 2000; Aso *et al.* 2009). In order to control for unspecific methylation we compared the expression of Dam-Pol II with UAS-Dam. Temporally restricted expression was achieved using the temperature sensitive *tubulin-Gal80^{ts}* (McGuire *et al.* 2003).

To study LTM, flies were trained in a classical olfactory conditioning paradigm, using sucrose as a positive reinforcer. Animals were sequentially exposed to two odorants, one of which was paired with sucrose. Following the learning procedure, flies preferentially moved toward the paired odor. A single trial of appetitive olfactory conditioning is capable of inducing LTM that lasts for days (Krashes and Waddell 2008; Colomb *et al.* 2009). In an unpaired training protocol, in which odors and sucrose were presented temporally separated, flies did not form odor memories (Figure 1, A and B). To gain insight into the transcriptional changes underlying the formation, consolidation, and maintenance of LTM, we performed TaDa sequencing analysis during different time intervals after training. Gene expression was compared between flies that were trained to associate sucrose with an odor and control flies that received sucrose and odors unpaired in time. The experiment was designed as a time course with four time intervals after the conditioning protocol (Figure 1D). Time interval 1 (T1) included the first 12 hr after training. Flies were moved to 29° 3 hr before olfactory conditioning to induce expression of Dam-Pol II or Dam-only in the MB. The second time interval (T2) was 12–24 hr, T3 was 24–48 hr, and T4 was 48–72 hr after training. For each time point we used four biological replicates for experiment and control (Dam-Pol II and Dam-only) as well as for paired and unpaired training. At the end of the induction time interval, the heads of the flies were collected. Genomic DNA was extracted from heads and digested with the restriction enzyme *Dpn*I, which cuts at adenine-methylated GATC sites. Methylated fragments were PCR amplified and DNA was prepared and sequenced using Illumina HiSeq3000 with ∼30 million paired-end reads per sample on average (Figure 1C). Next, we calculated the log2 ratio between Dam-Pol II and Dam-only samples. A positive log2 ratio of Dam-Pol II / Dam-only implies that GATC sites were preferentially methylated in Dam-Pol II samples compared to Dam-only background methylation, indicating active transcription at given gene locus (Figure 1C). We further calculated for each gene from a given condition at a given time interval expression values and FDRs. The FDR value represents the probability of having a given expression for a given gene based on the length of the gene and the total number of GATC sites present in this gene. Based on the expression level and the FDR value, we identified genes as expressed in case of a positive log2 ratio and a significant FDR value (FDR < 0.05). To find differentially expressed genes between paired and unpaired trained groups, calculated expression values were compared and *P*-values determined with a Student's *t*-test. A total of 506 differentially expressed genes were discovered. At T1 we identified 86 differentially expressed genes. Of those, 46 were upregulated and 40 were downregulated in the paired group compared to the unpaired group (Figure 1E). For T2, 115 genes were significantly higher expressed in the paired trained group, but we did not detect significant downregulation at this time point. In total, 56 differentially upregulated genes and 45 downregulated genes were found at T3. At the last time interval (T4), we identified 75 genes with higher and 202 genes with lower median expression in the paired conditioned group (Table S1). Most of the genes were differentially upregulated or downregulated at a single time interval. However, 69 genes were found to be differentially expressed at multiple time intervals, and 18 different patterns of differentially regulated genes over the four time intervals were observed, of which 11 contain differentially regulated genes at more than one time interval. A prominent pattern with 33 genes include genes that are higher expressed at T2 and lower expressed at T4 in the paired trained group compared to the unpaired trained group (Figure S1 and Table S2).

Candidate RNAi screen for LTM defects

To identify genes that regulate LTM formation, maintenance, and forgetting, we screened candidate genes for LTM defects using MB-specific *UAS-RNAi*. We selected a total of 33 candidate genes based on their expression profile in the MB during LTM formation. Selected candidate genes could be positive regulators that form or stabilize memories, as well as negative regulators that hinder formation and maintenance or actively remove memories. We therefore generated a ranked list of differentially upregulated and downregulated genes for different time intervals (Table S1). The ranking was based on the median gene expression differences between the paired and unpaired group. For T3 and T4, the six highest ranked genes as upregulated and downregulated were selected. Since no significant downregulated genes were identified for T2, we selected the top 10 ranked upregulated genes at T2.

Since we were particularly interested in the late phase of memory formation and maintenance, we did not select the top ranked genes from T1, assuming that differentially expressed genes from this time interval are preferentially implicated in the early memory formation process. We further selected eight genes according to gene ontology (GO) terms (Ashburner *et al.* 2000; The Gene Ontology Consortium 2017): transcription factor activity (*E(spl)mβ-HLH*, *Cdk7* and *Hsf*), actin cytoskeleton organization (*Vps4* and *capt*), cellular component of dendrites (*Mmp1*); synaptic vesicle docking (*Syx8*), and a gene implicated in LTM (*Hn*).

In 2015, Walkinshaw and colleagues performed a large genetic screen analysis, in which 42 genes that enhance memory were identified amongst 3200 RNAi lines tested for 3 hr memory. Three of these genes (*amon*, *prt*, and *hacd1*) were differentially expressed at T2, T3, or T4 in our

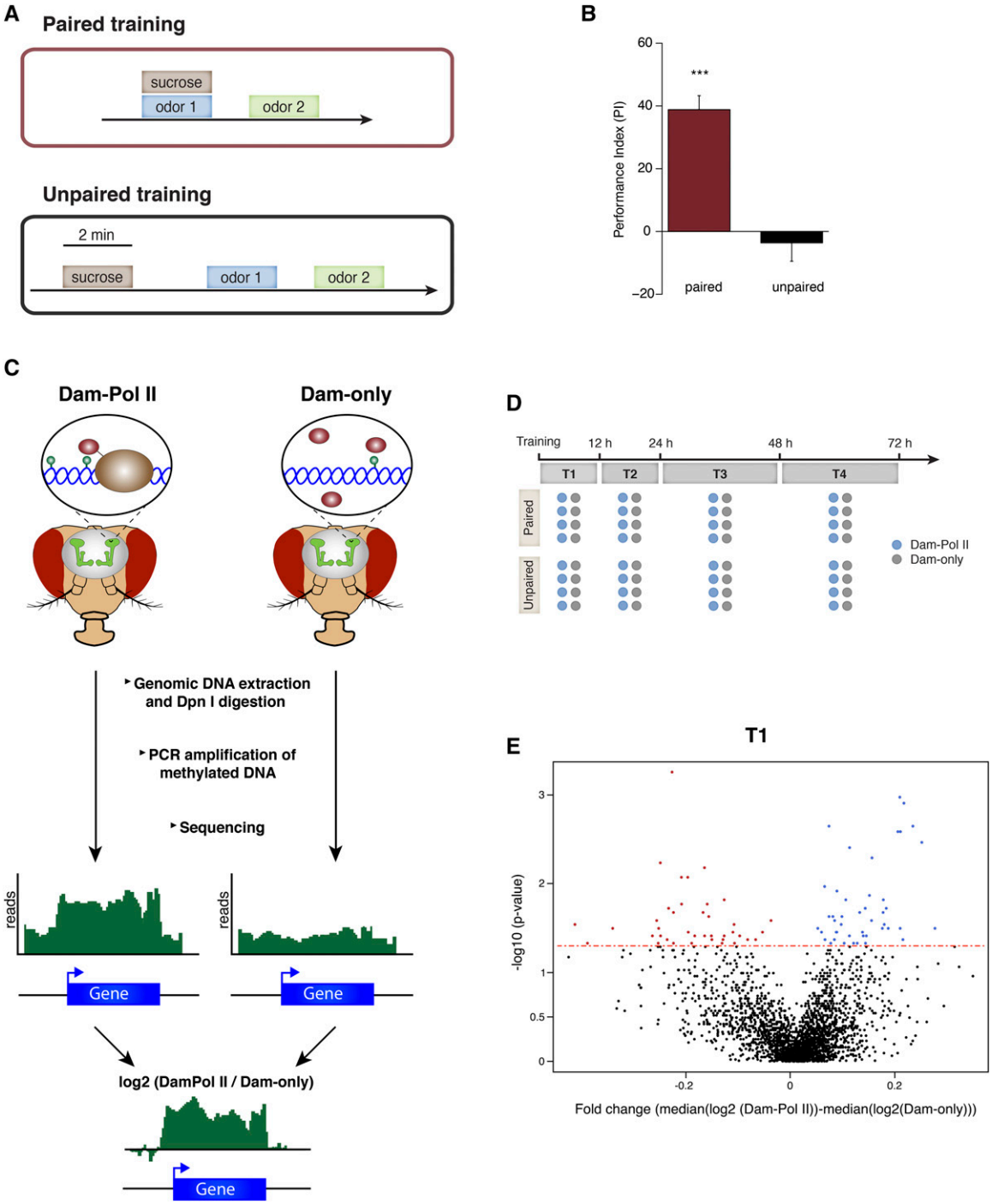

Figure 1 Monitoring gene expression changes after memory formation. (A) Illustration showing the two learning paradigms used. For the paired training, flies were allowed to feed on sucrose during odor 1 presentation. For the unpaired training, the sucrose feeding was separated from the odor presentations. (B) Paired trained wild-type flies displayed LTM measured after 24 hr. Flies exposed to the unpaired training did not show a changed odor preference. Bar graphs represent the mean, and error bars represent the SEM. $n = 8$. *** $P < 0.001$ (one-sample *t*-test). (C) Schematic representation of the targeted DamID (TaDa) experimental pipeline. A MB-specific Gal4 driver line was used to drive expression of *Dam-Pol II* and *Dam-only*. Genomic DNA from heads was extracted and digested with the methylation-sensitive restriction enzyme *Dpn*I. Methylated sequences were then amplified by PCR and sequenced. The resulting reads were mapped to a reference genome and the log2 ratio of Dam-Pol II/Dam-only was calculated. A positive ratio indicates transcription by the RNA Pol II. (D) Schematic illustration of the experimental design to profile gene expression after memory formation. Transcription was monitored at four time intervals (T1–T4) after training. Four replicates for *Dam-Pol II* and *Dam-only* expressing flies were conducted at each time interval, with flies trained in a paired and flies trained in an unpaired conditioning paradigm. (E) Representative volcano plot showing differentially regulated genes between paired and unpaired trained flies at the first time interval (T1). Median log2 fold changes are plotted against −log10 (*P*-value). Genes with a significant fold change between paired and unpaired are colored. Red indicates significantly higher expression in the unpaired group and blue indicates significantly higher expression in the paired group. The dashed red line indicates a *P*-value of 0.05.

experiment, which we added to our selection of candidate genes. Those three genes could possibly also be negative regulators of LTM (Walkinshaw *et al.* 2015).

Flies expressing *UAS-RNAi* under the control of *mb247-Gal4* were trained using the same appetitive olfactory learning paradigm as above for TaDa experiment, and LTM performance was assessed 48 hr later. We chose to test for 48 hr memory to identify genes that are involved in LTM. To increase efficiency of RNAi we coexpressed a *UAS-Dcr2* transgene (Dietzl *et al.* 2007). The microRNA (miRNA) mir-282 was inhibited with a miRNA sponge construct (Fulga *et al.* 2015).

As a positive control, we included a *UAS-RNAi* knockdown against the adenylyl cyclase *rutabaga* (*rut*), which is required for memory formation (Blum *et al.* 2009). We indeed observed that a MB-specific knock-down of *rut* resulted in impaired memory performance (Figure 2A). Three *mb247-Gal4/UAS-RNAi* crosses did not produce a sufficient number of adult offspring, and could therefore not be tested for memory performance. We assume that this was not due to the effect of RNAi in the MB, but rather resulted from a high mortality rate based on the genetic background of the stock. The offspring of the remaining lines did not display visible developmental defects. Tested *UAS-RNAi* lines with ± 1 SD from the PI of the driver line *MB-Gal4* were selected as positive hits. Of the 30 RNAi lines tested, 20 showed no significant changes in memory performance, while 1 showed a decreased memory performance, and 9 an increased 48 hr memory performance (Figure 2A and Table S3). None of these genes were previously reported to be involved in LTM, and they therefore represented new genes potentially regulating LTM. To further support the function of these 10 genes in LTM, we used a second different *UAS-RNAi* line and tested the *UAS-RNAi* parental lines, as well as the Gal4 driver line. The experimental flies (*MB-Gal4/UAS-RNAi*) were compared to the parental control lines. For three genes (*vajk-1*, *CG12338*, and *hacd1*), experimental lines performed significantly differently from Gal4 and UAS control lines using two independent RNAi lines. Four genes could be confirmed with one RNAi construct, but the experimental group of the second construct was not significantly different from both parental controls. Knockdown of *Cpr64Aa* and *mir-282* resulted in a higher 48 h memory than in control lines. However, no second construct against those genes was available. The experimental groups of the gene *obst-A* did not show a significantly different memory score from the parental control groups for both RNAi constructs, although there was a clear trend visible and *P*-values were only marginally higher than 0.05. (Figure 2B and Table S3).

We examined how the 10 genes that showed an altered LTM performance when inhibited in the MB performed in the TaDa experiment. Our list of 10 hits contains significantly differentially expressed genes from all the different time intervals. Seven genes were significantly higher or lower expressed at two time intervals after paired training, while three genes were significantly different only at one time interval. The biggest median gene expression differences between the paired and the unpaired trained groups were observed 12–24 hr after training. For T2, eight genes showed a higher expression value in paired trained flies, which formed a LTM. Smaller expression changes were detected at T1 and T4, in which most of the genes were lower expressed in the paired group (Figure S2).

Knockdown of Hacd1 in the MB increases LTM

We next further assessed the top hit with increased or decreased 48 hr memory in more detail. We first analyzed *hacd1*, which encodes 3-hydroxyacyl-CoA dehydratase—an enzyme in lipid metabolism required for catalyzing very long-chain fatty acid (VLCFA) (Denic and Weissman 2007; Ikeda *et al.* 2008). A previous study had provided genetic evidence for *hacd1* in VLCFA biosynthesis, since RNAi knockdown of *hacd1* in oenocytes led to a strong decrease in cuticular hydrocarbon levels (Wicker-Thomas *et al.* 2015). In our gene expression profiling experiment, *hacd1* was expressed lower at T1 and higher at T2 in KCs of paired trained flies. At T4, *hacd1* was expressed significantly lower in animals that received paired training (Figure S2). In the initial MB-specific RNAi screen, *hacd1* showed the highest LTM score, which was confirmed by a second *UAS-RNAi* line (Figure 2). We wondered if this enhanced memory is specific to LTM or also manifests itself in earlier memory phases. Thus, in addition to LTM (48 hr), *mb247-Gal4/UAS-hacd1-RNAi* was tested for middle-term memory (3 hr) and short-term memory, measured directly after training (0 hr). No significant differences between the experimental and the control groups were detected for 0 and 3 hr memory, indicating that short-term and middle-term memory are not affected by *hacd1-RNAi* knockdown (Figure 3A). However, we observed again a significantly higher PI in *hacd1-RNAi* expressing flies compared to control parental lines, supporting a role for *hacd1* in LTM.

Expression of *hacd1-RNAi* was temporally restricted using *tubGal80^{ts}* (McGuire *et al.* 2003). Flies were raised at 18° and moved after hatching to 29° for 5 days to induce expression of the RNAi construct. Late knockdown of *hacd1* in the MB also resulted in a LTM enhancement phenotype measured 48 hr after appetitive conditioning (Figure 3B).

KCs can be classified into three morphologically and functionally distinct cell types. The axons of those three main classes extend into different lobes named αβ, α′β′, and γ (Crittenden *et al.* 1998). We specifically expressed *hacd1-RNAi* in one of the three major MB subdivisions and tested LTM performance of the flies. No significant differences were observed between experimental and control flies (P-value $>$ 0.05). However, a clear trend for memory enhancement was visible in flies expressing *hacd1-RNAi* in MB αβ or MB α′β′ neurons (Figure 3C).

Knockdown of vajk-1 in the MB results in learning defects

The *vajk-1* gene is located within the Nimrod gene cluster on chromosome 2, the largest syntenic unit in the genome (Somogyi *et al.* 2010; Cinege *et al.* 2017). Genes of the Nimrod cluster have been suggested to contribute to innate

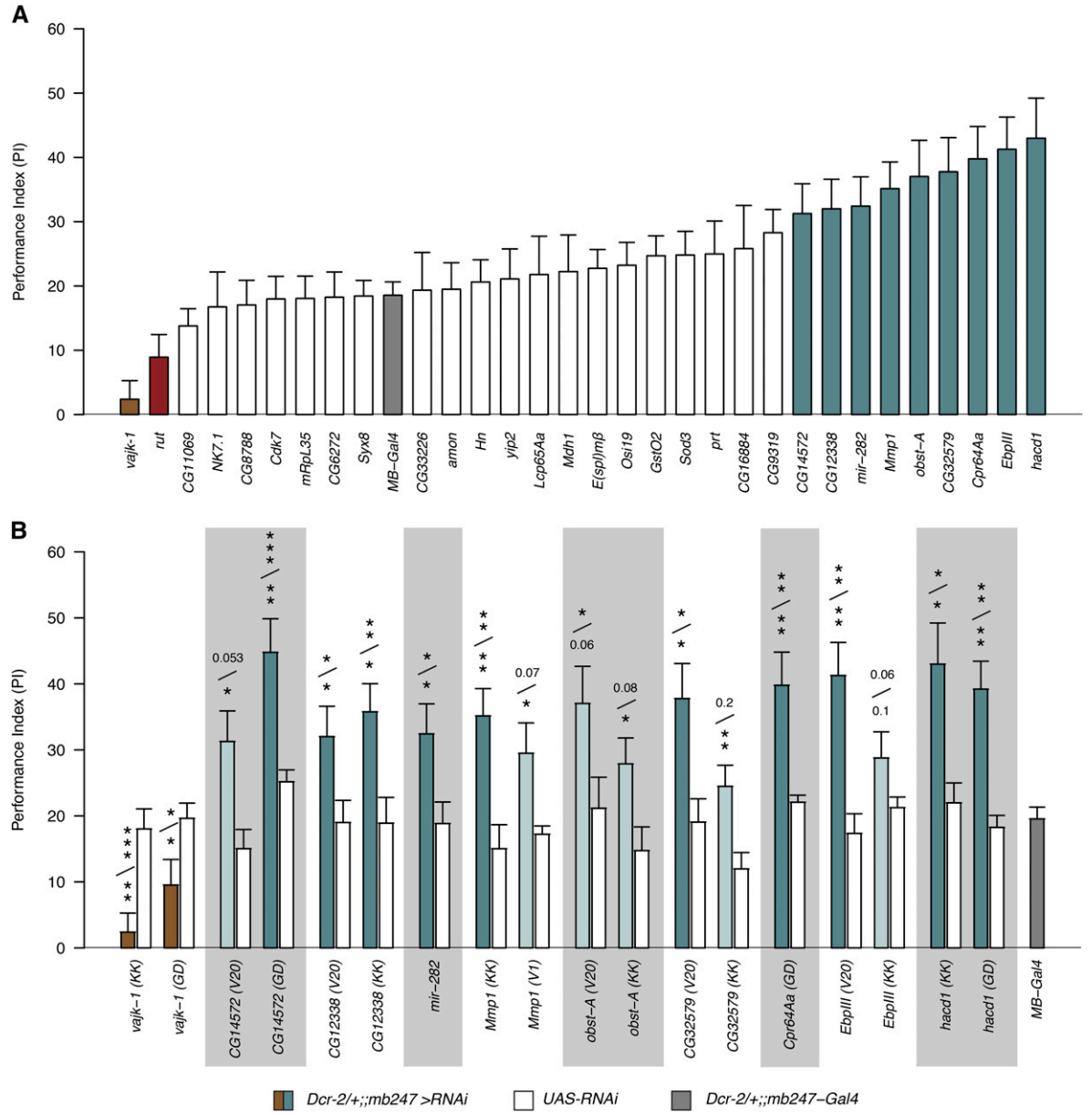

Figure 2 The 48 hr memory RNAi screen. Flies were trained in an appetitive olfactory conditioning paradigm and memory was assessed after 48 hr. RNAi constructs were used to inhibit gene products of the candidate genes. (A) RNAi lines with at least ±1 SD from the PI of the driver line *MB-Gal4* (highlighted in gray) were defined as hits. Nine RNAi lines with a higher memory performance (highlighted in teal blue), and one RNAi line with a lower memory performance (highlighted in brown) were identified. *rutabaga* (*rut*) was added as a positive control (highlighted in red); n = 6–8 for experimental lines, n = 20 for *MB-Gal4*. (B) Additional RNAi lines targeting the hits were used to verify the memory phenotypes. Parental *UAS-RNAi* lines and the *MB-Gal4* line were tested along and used as controls. Dark colors represent a significant difference from the parental Gal4 and UAS lines. Upper asterisks and numbers indicate the *P*-value of the comparison to *MB-Gal4* and lower asterisks and numbers the *P*-value of the comparison to the *UAS-RNAi* line; n = 6–9 for *UAS-RNAi* and *MB >RNAi*, n = 24 for *MB-Gal4*. Bar graphs represent the mean and error bars represent the SEM. Asterisks denote significant difference between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Numbers signify *P*-values (Welch two sample *t*-test).

immune response (Kurucz *et al.* 2007). In our transcriptomics experiment, *vajk-1* was significantly lower expressed at T1 and significantly higher expressed at T2 in the MB of paired trained flies compared to unpaired trained flies (Figure S2). MB-specific expression of *UAS-vajk-1-RNAi* resulted in a low 48 hr memory performance, which was confirmed by a second *UAS-RNAi* line (Figure 2). We next assessed if *vajk-1* knockdown affects only LTM by testing short-term and middle-term memory. We found that *mb247-Gal4/UAS-vajk-1-RNAi* flies showed impaired short-, middle- and LTM by displaying significantly lower memory scores at all three time points, when compared to parental control strains (Figure 4A). The finding that *UAS-vajk-1-RNAi* expressing flies showed reduced memory immediately after learning, suggests that *vajk-1* may be involved in memory formation. Reduced learning capability may also result from developmental defects

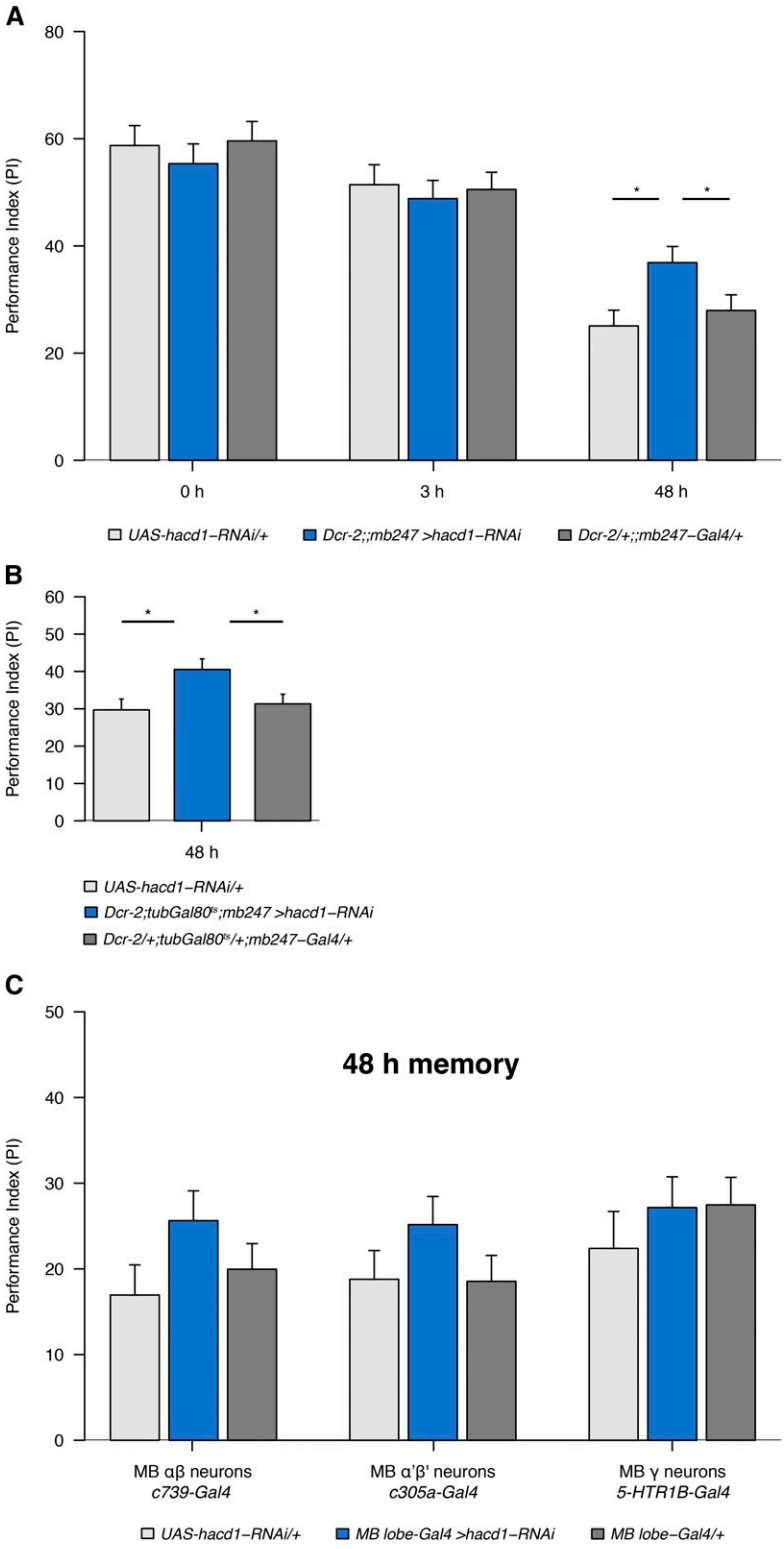

Figure 3 Knockdown of *hacd1* in the MB enhances 48 hr memory. (A) Flies expressing *hacd1-RNAi* in KCs were tested along with controls for their memory performance immediately after conditioning (0 hr) or after a period of 3 and 48 hr. Memory measured 2 days after training was significantly enhanced in *hacd1-RNAi* expressing flies compared to parental lines. PIs did not differ between groups after 0 and 3 hr; n = 9–12. (B) RNAi expression was temporally restricted with *tubGal80^{ts}*. Flies were moved for 5 days to 29° to active expression of *hacd1-RNAi* before conditioning. A higher 48 hr memory performance was measured in flies expressing *hacd1-RNAi* in the MB, compared to parental control lines; n = 10. (C) *hacd1-RNAi* was expressed in three different MB subsets (αβ, α′β′, and γ) and memory was assessed 48 hr after training. Memory performance did not significantly differ between groups; n = 7–8. Bar graphs represent the mean and error bars represent the SEM. Asterisks denote significant difference between groups (* $P < 0.05$).

in MB formation or defects in sensory input. We therefore performed sensory tests with *mb247-Gal4/UAS-vajk-1-RNAi* flies and the parental lines. All the tested lines moved away from the presented odors (benzaldehyde or limonene), and no significant difference between the groups was observed (Figure 4, B and C). *UAS-vajk-1-RNAi* expression also had no influence on the sugar attraction behavior. Sucrose response was not different from the control lines (Figure 4D). To examine if developmental defects cause the observed memory impairment, we temporally restricted RNAi expression with *tubGal80^{ts}* (McGuire *et al.* 2003). Memory was assessed 0 and 48 hr after memory formation. Knockdown of *vajk-1* in the MB

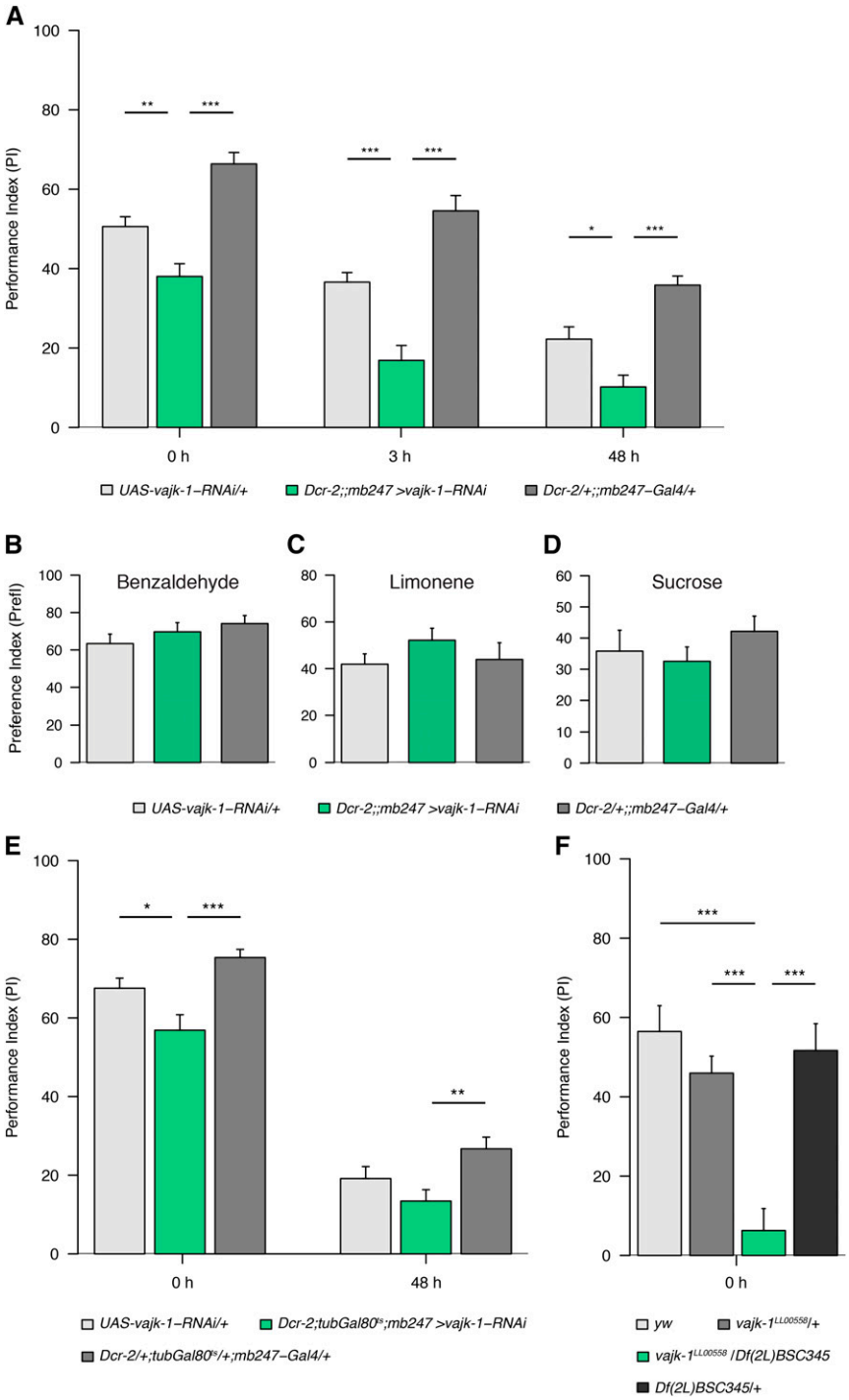

Figure 4 Expression of *vajk-1-RNAi* in KCs causes memory impairment. (A) Flies were trained in an appetitive olfactory learning paradigm and tested 0, 3, or 48 hr later. Inhibiting *vajk-1* in the MB resulted in reduced memory performance at all measured time points compared to parental lines; n = 9–13. (B and C) Benzaldehyde and limonene odor avoidance of the parental lines and the *vajk-1-RNAi* line crossed to *mb247-Gal4* were tested. No significant differences between the groups were observed; n = 8–9. (D) Flies with RNAi inhibited *vajk-1* performed not significantly different from the control lines in a sucrose response test; n = 12–13. (E) A late knockdown of *vajk-1* was achieved by shifting the flies after hatching to 29° to activate expression of *vajk-1-RNAi*. Knockdown of *vajk-1* resulted in reduced learning performance directly after training compared to parental lines. At 48 hr after training, *vajk-1-RNAi* expressing flies displayed a lower memory performance index than the parental Gal4 control line, but did not significantly differ from the parental UAS control line (P-value = 0.18); n = 11–16. (F) *vajk-1*^{LL00558} over a deficiency showed impaired short-term memory (0 hr). The heterozygous mutant and deficiency line displayed unaffected 0 hr memory; n = 6–8. Bar graphs represent the mean and error bars represent the SEM. Asterisks denote significant difference between groups (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

induced reduced learning, measured directly after conditioning. For 48 hr memory, flies expressing *UAS-vajk-1-RNAi* showed a lower performance than the parental Gal4 control, but the memory score was not significantly different from the parental UAS control (P-value = 0.18, Welch two sample t-test) (Figure 4E). Next, we used *vajk-1*^{LL00558}—a mutant line for vajk-1. This line contains a PBac insertion in the first coding exon, which most likely results in a truncated protein or nonsense mediated RNA decay. We tested *vajk-1*^{LL00558} over a deficiency for short-term memory. Mutants displayed a drastically reduced memory, while controls showed ordinary memory scores (Figure 4F).

To test the requirement of *vajk-1* in the three major KC classes, characterized Gal4 lines were used to target RNAi to distinct MB lobe neurons. First, flies were tested for short-term memory. We observed a significant reduction in 0 hr memory of flies with inhibited *vajk-1* in MB γ neurons compared to control groups. Furthermore, *vajk-1-RNAi* expressing flies in MB α′β′ neurons displayed a lower memory than the parental line *c305a-Gal4*, while the difference to *UAS-vajk-1-*

RNAi was not significant (*P*-value = 0.06). vajk-1 knockdown in the αβ KCs did not affect formation of short-term memory. Performance indices did not differ between the tested groups (Figure 5A). Subsequently, we tested *vajk-1* necessity in MB lobes for LTM, measured 48 hr after appetitive olfactory conditioning. Animals expressing *vajk-1-RNAi* in αβ KCs showed a decreased 48 hr memory performance, which was significantly different from the Gal4 control, but not from the UAS control (*P*-value = 0.07). Considerably impaired LTM was observed when *vajk-1* was inhibited in α′β′ KCs, while flies expressing *vajk-1-RNAi* in γ KCs were able to form LTM similar to the concurrent tested control groups (Figure 5B).

Discussion

Appetitive olfactory learning using sugar as reward forms memories that last for several days after training. To monitor the transcriptional program that occurs in the MB, we used a transcriptomics approach identifying actively transcribed genetic loci during four time intervals after training. Based on the analysis of MB gene expression profiles, we performed a MB-specific candidate RNAi screen, which identified 10 genes that exhibited altered 48 hr memory performance in an RNAi knockdown experiment. The hits were confirmed by testing independent *UAS-RNAi* lines and parental control lines. The two genes, *vajk-1* and *hacd1*—top candidates of increased and decreased 48 hr memory—were further tested in more detail.

Transcriptomics characterization of gene expression profiles during LTM in the MB

Genetic tools available in *Drosophila* provide a fruitful basis to study genetic mechanisms required for different phases of learning and memory formation (Keene and Waddell 2007). Here, we used the TaDa technique, which enabled us to specifically profile gene expression in the MB, without the requirement of isolating cells from this brain structure. Our findings show that TaDa with Dam-Pol II is a valuable technique to measure gene expression in a specific cell population in the *Drosophila* brain. TaDa is a quite recent technique and has not been used before to study memory-related gene expression changes. The bioinformatics analysis of sequencing data allowed us to identify changes in the transcriptome at the whole-genome level during different phases of memory formation between paired and unpaired trained flies. A functional RNAi screen supports the validity of this technical approach (see below). While we specifically focused here on the later stages during LTM formation and forgetting, the transcriptome data further allows studies on genetic aspects of memory initiation, thus providing a valuable resource for future functional studies.

We used appetitive olfactory conditioning to induce LTM and to study its genetic basis. However, many different forms of LTMs exist, and induced changes in the transcriptional program may not be identical. Aversive olfactory conditioning with electric shock as negative reinforcer is the most commonly used learning form in *Drosophila*. The aversive memory is similar to the appetitive memory, but also major differences exist. A single appetitive conditioning session is sufficient to form LTM, while formation of aversive LTM requires spaced training that consists of 5–10 conditioning sessions with a 15-min rest interval in between each. Moreover, flies have to be starved prior to reward learning, since the motivational drive is essential for memory formation and retrieval (Tully and Quinn 1985; Krashes and Waddell 2008; Colomb *et al.* 2009). Thus, some of our identified genes might be specific to olfactory reward memory, while others are commonly used during the formation and maintenance of long-lasting memories. We compared our list of differentially regulated genes during memory formation with genes identified in a similar experiment for aversive LTM. In this study, differentially expressed transcripts of flies subjected to spaced or massed training were analyzed. A total of 42 confirmed differentially expressed genes 0, 6 or 24 hr after conditioning were discovered (Dubnau *et al.* 2003); only 2 of those genes were found in our experiment. However, major technical and experimental differences exist, which makes a comparison difficult. Interestingly, in the study of Dubnau *et al.* (2003), most of the identified genes were unique to a single time point, which is similar to our results. We found that 86% of the genes were upregulated or downregulated at one time interval. Whether transcriptional changes are restricted to a specific time phase for most of the genes, or whether this is due to measurement noise, is unclear. We found that 7 of our 10 identified hits from the behavior RNAi screen were differentially transcribed at multiple time intervals. Thus, genes found at several time points seem to be good candidates for LTM regulation.

RNAi-based identification of novel genes in regulating LTM forgetting

From the selected 33 candidate genes for the *UAS-RNAi* screen for LTM, we identified one hit with lower and nine hits with higher 48 hr memory performance; thus, roughly 30% of the selected candidates were identified as hits. Since RNAi lines may identify false positive genes by off-target effects, we further used a second *UAS-RNAi* line for eight of the hits. Moreover, the parental Gal4 and UAS lines were tested. We validated nine genes, which showed a changed LTM performance compared to parental controls, at least with one construct used to inhibit gene products.

It is important to note that *UAS-RNAi* induced knockdown may reduce protein levels only partly, and therefore cause a hypomorphic phenotype resulting in a false negative call. It seems likely that more candidate genes may actually cause a behavioral phenotype when stronger gene inactivation is used. In the future, other techniques may be used to study these genes, including Minos transposon insertions to tag and knockdown proteins (Nagarkar-Jaiswal *et al.* 2015), the generation of transgenic CRISPR/Cas9 to generate mutations in the locus (Xu *et al.* 2015), or *UAS-ORF* lines for overexpression experiments (Bischof *et al.* 2013).

Interestingly, we found nine genes with enhanced LTM performance. Reducing the effect of the microRNA mir-282,

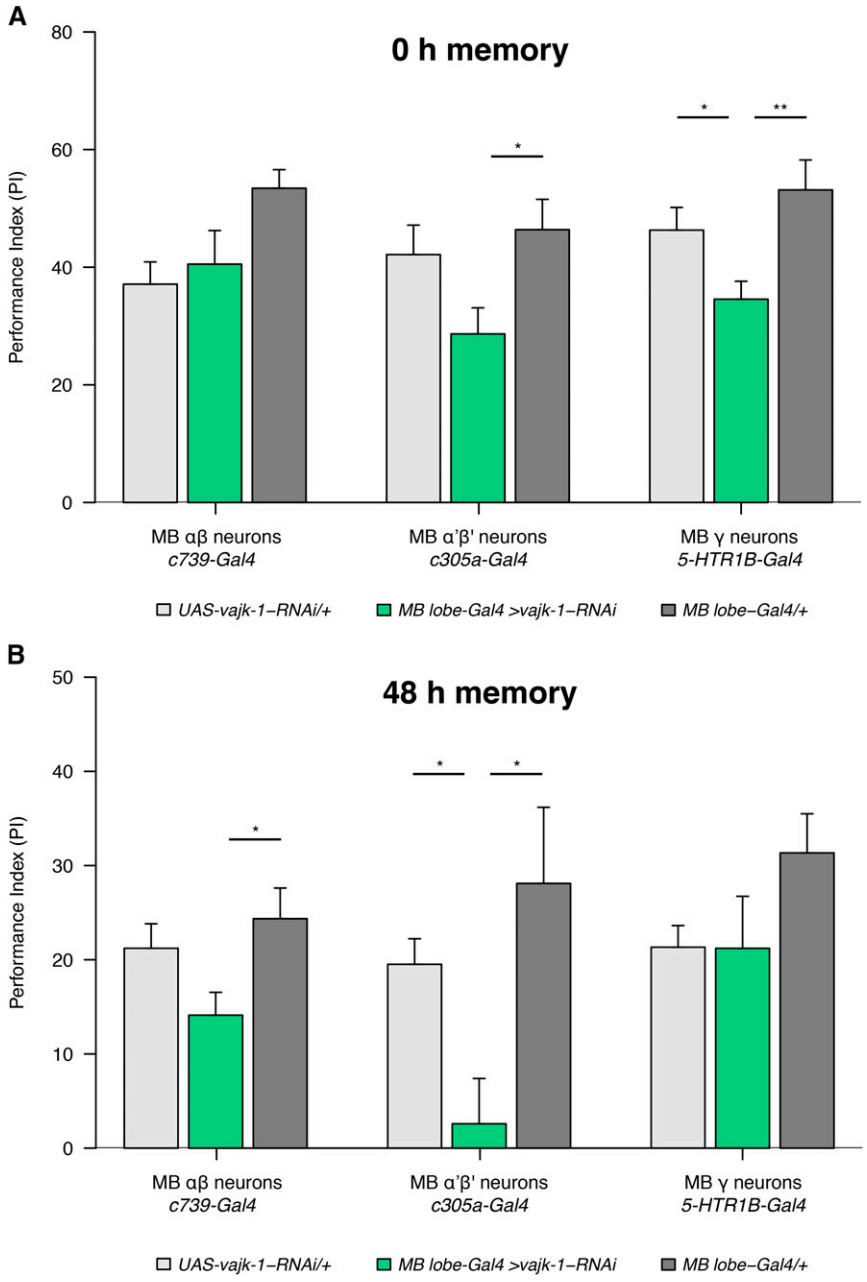

Figure 5 *vajk-1* is required in different MB lobe neurons for different memory phases. Gal4 lines driving expression in distinct MB subtypes (αβ, α′β′, and γ) were used to express *vajk-1-RNAi* and to test for short-term memory (0 hr, A) and LTM (48 hr, B). (A) vajk-1 knockdown in MB γ neurons resulted in lower 0 hr memory than in parental controls. Flies with inhibited *vajk-1* in MB α′β′ neurons showed a significantly reduced memory compared to *c305a-Gal4*, but not compared to *UAS-vajk-1-RNAi*; n = 7–11. (B) Inhibiting vajk-1 in MB γ neurons had no effect on LTM, while *vajk-1-RNAi* expression in MB α′β′ neurons resulted in impaired 48 hr memory. LTM performance of *vajk-1-RNAi* expressing flies in MB αβ neurons was significantly different from *c739-Gal4* flies, but not from *UAS-vajk-1-RNAi*. n = 6–9. Bar graphs represent the mean and error bars represent the SEM. Asterisks denote significant difference between groups (* $P < 0.05$, ** $P < 0.01$).

by using a miRNA sponge construct, showed increased 48 hr memory when expressed in KCs (Fulga *et al.* 2015). Other miRNAs have previously been identified in learning and memory formation in *Drosophila*. While inhibiting mir-980 showed enhanced short-term and middle-term memory, mir-276a has been described to be necessary for LTM formation (Li *et al.* 2013; Guven-Ozkan *et al.* 2016). Interestingly while both genes regulated neuronal excitability, the molecular mechanisms that regulate learning and memory appear distinct. The autism susceptibility gene, *A2bp1*, was identified as target of mir-980 causing memory enhancement, while mir-276a interferes with memory formation by regulating dopamine receptor expression (Li *et al.* 2013; Guven-Ozkan *et al.* 2016).

While the role for mir-282 in learning and memory formation was unknown, a recent study identified the adenylyl cyclase *rutabaga* as target gene of mir-282 (Vilmos *et al.* 2013). Moreover, using the microRNA.org resource for miRNA target prediction, we found that 5 of the top 25 target genes are reported to be involved in learning and memory (Betel *et al.* 2008, 2010) (Table S4).

CG12338 encodes a protein that was suggested to be involved in the D-amino acid metabolic process (Gaudet *et al.* 2011). The mouse homolog *D-amino acid oxidase* (*Dao*) has a critical role in spatial memory. Mutant mice lacking DAO performed significantly better than wild-type mice in the Morris water maze test (Maekawa *et al.* 2005). *Dao* mutant mice have a higher D-amino-acid concentration in the brain,

which possibly enhances *N*-methyl-D-aspartate (NMDA) receptor response and thereby facilitates spatial learning (Hashimoto *et al.* 1993; Morikawa *et al.* 2001). Our observation that knock-down of *CG12338* caused enhanced memory suggests that a similar mechanism may be involved in regulating memory in *Drosophila*.

Two of our memory-enhancing hits regulate extracellular matrix organization: *obstructor-A* (*obst-A*) and *Matrix metalloproteinase 1* (*Mmp1*). Obst-A was shown to be required for ECM dynamics and coordination of ECM protection (Petkau *et al.* 2012). Mmp1 belongs to a conserved family of extracellular proteases that cleave protein components of the ECM. Mmp1 can mediate matrix remodeling and is required for degrading severed dendrites during metamorphosis (Kuo *et al.* 2005; Glasheen *et al.* 2010). In rats, it was observed that MMP-3 and -9 increased learning-dependent and inhibition altered long-term potentiation and learning capacity (Meighan *et al.* 2006). *Cuticular protein 64Aa* (*Cpr64Aa*), another gene that showed a higher 48 hr memory performance in the RNAi screen, is also reported to be a cellular component of the ECM. The ECM is a dynamic structure that can alter the synaptic efficiency, thus contributing to synaptic plasticity (Wlodarczyk *et al.* 2011; Frischknecht and Gundelfinger 2012). Specialized structures of stable and accumulated ECM molecules called perineuronal nets (PNNs) were found around certain neurons in the mammalian brain, where they play a critical role in control of plasticity (Härtig *et al.* 1992; Pizzorusso *et al.* 2002). PNNs were shown to participate in memory mechanisms, and modifications of PNNs can enhance LTM (Gogolla *et al.* 2009; Hylin *et al.* 2013; Romberg *et al.* 2013). Digestion of PNNs mediated prolonged long-term object recognition memory, and the same prolongation was observed in mice lacking an essential PNNs component (Romberg *et al.* 2013). It has been suggested that LTMs could be stored and maintained in neurons surrounding ECM structures (Tsien 2013). Our results suggest that, in *Drosophila* ECM, proteins could also contribute to memory maintenance. The discovered genes will serve as a valuable starting point for future studies of the molecular mechanisms underlying LTM.

Identification of vajk-1 and hacd1 as learning and memory genes

Expression of *UAS-vajk-1-RNAi* in the MB caused memory impairment. The *vajk-1* gene is located together with two homologous genes (*vajk-2* and *vajk-3*) in a large intron of *Ance-3*, which is part of the Nimrod cluster. However, *vajk* genes are not related to the Nimrod genes, which are involved in the innate immune defense. The *vajk* gene members are conserved in insects, but their function is unknown (Somogyi *et al.* 2010; Cinege *et al.* 2017). Our results suggest that *vajk-1* could be involved in the memory formation process.

MB αβ, α′β′, and γ neurons have distinct roles in different memory phases. For MB α′β′ neurons, an essential role in early memory consolidation has been suggested. γ KCs are particularly important for early memory phases, while αβ KCs have a main role in LTM (Perisse *et al.* 2013; Guven-Ozkan and Davis 2014). It has been proposed that appetitive short-term and LTMs are formed independently and in parallel in the MB (Trannoy *et al.* 2011). Those findings are in agreement with the results of our *vajk-1* knockdown experiment in specific MB lobe neurons. No effect on 0 hr memory was observed when *vajk-1-RNAi* was expressed in MB αβ neurons, and 48 hr memory was intact after knockdown in γ neurons.

We found that RNAi knockdown of *hacd1* in KCs resulted in enhanced 48 hr memory, but 0 and 3 hr memory were unaffected. One of 42 identified genes that showed increased 3 hr memory after RNAi knockdown was *hacd1* (Walkinshaw *et al.* 2015). Our results did not show memory enhancement at 3 hr, but did so at 48 hr after memory formation. This discrepancy is probably due to the use of a different driver line or learning paradigm. We used a MB driver line and conditioned the flies in an appetitive paradigm. In contrast, Walkinshaw *et al.* (2015) used the panneuronal driver *Nsyb-Gal4* and aversive olfactory conditioning.

hacd1 is involved in the synthesis of VLCFA and catalyzes the dehydration of the 3-hydroxyacyl-CoA (Wicker-Thomas *et al.* 2015). *hacd1* is evolutionarily conserved among eukaryotes, but little is known about its function. Mammals have two homologs: *HACD1* and *HACD2*. Expression of *HACD2* was shown to be ubiquitous, whereas *HACD1* was found in heart and muscle cells and linked to certain muscle diseases and arrhythmogenic right ventricular dysplasia (Li *et al.* 2000; Wang *et al.* 2004; Pelé *et al.* 2005). The yeast homolog *PHS1* is also involved in the fatty acid elongation process, being responsible for the third step in the VLFA synthesis cycle (Denic and Weissman 2007). Moreover, it was proposed that *PHS1* is part of the endoplasmic reticulum membrane and possesses six transmembrane domains (Kihara *et al.* 2008), and that it could be implicated in protein trafficking (Yu *et al.* 2006).

How *hacd1* could be implicated in LTM is currently unknown. However, various reports show that fatty acids and their mediators have numerous functions in the brain, including roles in learning and memory. It has been shown that overexpression of the *fatty-acid binding protein* (*Fabp*) in fruit flies increased LTM consolidation (Gerstner *et al.* 2011). Also in mammals, proteins involved in fatty acid metabolism can act on memory. It has been demonstrated that deletion of monoacylglycerol lipase caused memory enhancement in mice (Pan *et al.* 2011), and that inhibition of fatty acid amide hydrolase enhanced learning in rats (Mazzola *et al.* 2009).

Aside from a role in lipid metabolism, the *Drosophila* Hacd1 protein could also be part of a signaling cascade, since it contains a protein-tyrosine phosphatase-like (PTPLA) domain that catalyzes the removal of a phosphate group attached to tyrosine. However, it has not been tested if this domain is functional. Future studies will be required to reveal the molecular mechanism of Hacd1 in LTM regulation.

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