microRNAs That Promote or Inhibit Memory Formation in *Drosophila melanogaster*

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ABSTRACT microRNAs (miRNAs) are small noncoding RNAs that regulate gene expression post-transcriptionally. Prior studies have shown that they regulate numerous physiological processes critical for normal development, cellular growth control, and organismal behavior. Here, we systematically surveyed 134 different miRNAs for roles in olfactory learning and memory formation using “sponge” technology to titrate their activity broadly in the *Drosophila melanogaster* central nervous system. We identified at least five different miRNAs involved in memory formation or retention from this large screen, including *miR-9c*, *miR-31a*, *miR-305*, *miR-974*, and *miR-980*. Surprisingly, the titration of some miRNAs increased memory, while the titration of others decreased memory. We performed more detailed experiments on two miRNAs, *miR-974* and *miR-31a*, by mapping their roles to subpopulations of brain neurons and testing the functional involvement in memory of potential mRNA targets through bioinformatics and a RNA interference knockdown approach. This screen offers an important first step toward the comprehensive identification of all miRNAs and their potential targets that serve in gene regulatory networks important for normal learning and memory.

KEYWORDS genetic screen; learning; memory; *Drosophila*; miRNA

INVOLVED in post-transcriptional gene regulation, microRNAs (miRNAs) are a class of small noncoding RNAs (Bushati and Cohen 2007). Prior studies have shown that they serve numerous biological processes, ranging from development to tumorigenesis (Esquela-Kerscher and Slack 2006; Kloosterman and Plasterk 2006; Krützfeldt and Stoffel 2006; Chang and Mendell 2007). miRNAs are transcribed as primary miRNAs (pri-miRNAs) from isolated genes or the introns of protein-coding genes (“mirtrons”) (Filipowicz *et al.* 2008). miRNAs are under regulatory influences similar to protein-coding genes (Krol *et al.* 2010). The pri-miRNAs are then cleaved into precursor miRNAs (pre-miRNAs) by the microprocessor Drosha/Pasha protein complex and transported into the cytoplasm by Exportin5 where they mature through the Dicer/Loquacious protein complex into ~21- to 24-nucleotide miRNA hairpins. These hairpins are

subsequently assembled with Argonaute-containing protein complexes that bind to specific sequences on target messenger RNAs (mRNAs) using primarily a 2-to 8-nucleotide seed region (Bartel 2009). The small size of the target site allows many mRNAs to be recognized and coregulated by each individual miRNA (Bartel 2009). The miRNA complex, once bound, induces post-transcriptional silencing by translational repression and/or mRNA degradation (Filipowicz *et al.* 2008; Bazzini *et al.* 2012; Djuranovic *et al.* 2012).

One important biological process that is understudied relative to miRNA function is learning and memory formation. Among the several known epigenetic processes that allow the nervous system to adapt to environmental signals, the miRNA system is thought to provide relatively rapid and analog control in both time and space over the expression of genomic content (Kosik 2006; Costa-Mattioli *et al.* 2009; McNeill and Van Vactor 2012; Wang *et al.* 2012). To illustrate this point, consider first another fascinating and important epigenetic system: transcriptional control through chromatin changes. Enduring transcriptional regulation through chromatin changes affords a way for neurons to effect long-term, cell-wide, adaptive changes in state (Kramer *et al.* 2011; Gräff and Tsai 2013; Zovkic *et al.* 2013). However, this epigenetic system offers limited ability to control the rapid delivery of genomic material in space, since its speed is limited

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by the requirement for transcription. In addition, newly synthesized mRNAs may be directed to all compartments of the neuron. The miRNA system operates on already synthesized RNAs, potentially altering expression in specific neuronal compartments including axons, dendrites, synapses, and growth cones (McNeill and Van Vactor 2012; Cajigas *et al.* 2012). miRNAs are thus ideally positioned to dynamically regulate gene expression across the spatial dimensions of the neuron. These considerations stress the importance of gaining a broad appreciation of which miRNAs are involved in the regulation of neural circuit function for memory formation.

An important second way by which miRNAs may alter adult learning and memory is through neurodevelopmental roles (Xu *et al.* 2010; Im and Kenny 2012; Saab and Mansuy 2014). Dysregulation of miRNA expression has been described in several human neurodevelopmental diseases (Martino *et al.* 2009; Xu *et al.* 2010; Feng and Feng 2011; Nowak and Michlewski 2013). For example, Xu *et al.* (2008) demonstrated that miR-124a ectopic expression decreases dendritic branching, an effect rescued by the loss of dFMRP expression. In addition, miRNAs in circulating blood are candidate biomarkers for early phases of neurological disease (Wang *et al.* 2008; De Smaele *et al.* 2010; Absalon *et al.* 2013). Moreover, miRNAs may offer promise as therapeutics for disease states using multiple approaches (Sayed and Abdellatif 2011; Im and Kenny 2012). These facts illustrate the importance of first identifying all the miRNAs that are involved in learning or memory formation.

Here, we present the results of a large screen for miRNA functions in olfactory classical conditioning of *Drosophila*. Olfactory classical conditioning is a type of learning that has been well-studied in flies (Davis 2011; Kahsai and Zars 2011). In this paradigm, an odor [the conditioned stimulus (CS)] is paired with a series of electric shocks [the unconditioned stimulus (US)], creating an association between the two stimuli that behaviorally leads to strong aversion of the odor CS by the fly. Multiple brain regions and neuron types have been shown to be involved in olfactory learning, including projection neurons (PNs) of the antennal lobe, mushroom body neurons (MBn), the dorsal paired medial neurons (DPMn), dopaminergic neurons (DAn), and others (Davis 2005, 2011; Busto *et al.* 2010). Thus, the rapid regulation of gene expression by miRNA function is predicted to occur in one or more areas of the fly brain that have been shown to mediate olfactory memory formation.

We tested the potential involvement of 134 miRNAs in intermediate-term memory (ITM) by silencing them individually through development and adulthood using specific complementary oligonucleotides called miRNA sponges (miR-SPs), (Ebert *et al.* 2007; Loya *et al.* 2009; Fulga *et al.* 2015). Our study focused on ITM since changes in performance at this time point capture roles for miRNAs in both short-term memory and ITM as well as acute or developmental roles. Our results identified several different miRNAs important for olfactory memory formation. Surprisingly, competitive inhibition of some miRNAs decreased memory formation, while inhibition of others increased memory formation. Our

results offer a broad initial foundation for further analysis of miRNA function in memory formation.

Materials and Methods

Fly lines

Drosophila melanogaster were raised on the standard medium at room temperature. Crosses were kept at 25° with 70% relative humidity and with a 12 hr dark/light cycle. For the first screen, we crossed *elav¹⁵⁵-gal4* virgin females in a *w^{CS10}* genetic background with either *uas-scramble-miR-SP* or *uas-miR-SP* males in a *w¹¹¹⁸* genetic background (Loya *et al.* 2009; Fulga *et al.* 2015). The sponge constructs and derivative fly lines are described in Fulga *et al.* (2015). The same *uas-scramble-miR-SP* (TTAGAATTTAAACCTCACCATGA) control (either *attP2* or *attP40* insertion) was used with all *miR-SP* lines. For the secondary screen, we crossed *uas-miR-SP* males with virgin females from either the *elav¹⁵⁵-gal4* or *w^{CS10}* lines. The *gal4* drivers that we used in this study were *elav¹⁵⁵-gal4* (Lin and Goodman 1994), *GH146-gal4* (Stocker *et al.* 1997), *MZ604-gal4* (Ito *et al.* 1998; Tanaka *et al.* 2008), *OK107-gal4* (Connolly *et al.* 1996), *c316-gal4* (Waddell *et al.* 2000), *TH-gal4* (Friggi-Grelín *et al.* 2003), *Or83b(orco)-gal4* (Kreher *et al.* 2005), *NP2492-gal4* (Tanaka *et al.* 2008), *Gad-gal4* (Ng *et al.* 2002), *Ddc-gal4* (Li *et al.* 2000), *Cha-gal4* (Kitamoto 2001), *n-syb-gal4* (Pauli *et al.* 2008), and *uas-dicer 2* (Dietzl *et al.* 2007). RNA interference (RNAi) lines were obtained from the KK library of the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl *et al.* 2007).

Behavior

One- to 4-day-old flies were used for the behavioral experiments. Flies were collected ~24 hr prior to conditioning and transferred into fresh food vials ~30 min before training to adapt to the conditions of the behavioral test room (dim red light, 25°, ~75% humidity). Groups of ~65 flies received the standard aversive olfactory conditioning that was previously described (Beck *et al.* 2000). For conditioning, flies were placed into a tube containing a copper grid. There, they received a sequence of two odor stimuli separated by 30 sec of air. The first 1 min of odor (CS+) stimulus was paired with 12 pulses of a 90-V electric shock (US). The second 1 min of odor stimulus was not associated with electric shocks and thus constituted a nonconditioned stimulus (CS-). The odorants used were benzaldehyde (BEN) and 3-octanol (OCT). They were diluted in mineral oil at concentrations of ~0.05 and ~0.2%, respectively. Odorant concentrations were varied slightly to obtain a neutral distribution between the two odors among naive flies. Odors were delivered to the flies in an air stream (rate of ~400 ml/min) produced by bubbling pressurized air through mineral oil laced with odorant. After conditioning, flies were tapped back into their food vials and tested 3 hr later for memory retention. Memory retention was tested by allowing flies to choose for 2 min between an arm in a T-maze containing the

CS+ odor and an arm with the CS− odor. To avoid naive bias for one odor, two groups were trained and tested simultaneously. A first group was trained using BEN as the CS+ paired with the US and then OCT not paired (CS−) with the unconditioned stimulus. The second group was trained with the alternative combination with BEN as CS− and OCT as CS+. Each group (60–70 flies) tested provided a half-performance index (half-PI): $\text{half-PI} = [(\text{number of flies in CS− arm}) - (\text{number of flies in CS+ arm}) / (\text{number of flies in both arms})]$. A final PI was calculated by averaging the two half PIs.

To test odor avoidance, naive flies were allowed to freely distribute for 2 min between the two arms of a T-maze with an odor stream on one side and a non-odorized air stream on the other. To test shock avoidance, naive flies were allowed to distribute for 2 min between the two arms of a T-maze containing copper grids (same as used for training above) with only one side being electrified. An avoidance index was computed with the following formula: $[(\text{number of flies in neutral arm}) - (\text{number of flies in odor/shocked arm}) / (\text{total number of flies in both arms})]$.

Data analysis

For the primary screen, 3-hr memory performance of each *elav^{c155}-gal4/+>uas-miR-SP/+* genotype was compared to a control *elav^{c155}-gal4/+>uas-scramble-miR-SP/+* genotype. Two successive and independent statistical approaches were utilized to select putative hits. First, the *scramble-miR-SP* genotype score was compared to the *miR-SP* genotype using a two-tailed, two-sample Student *t*-test. We chose to use the Student *t*-test because there is no minimum for sample size and performance indices within a line are known to follow a normal distribution (Tully *et al.* 1994). We also found that the performance indices of the control *elav^{c155}-gal4/+>uas-scramble-miR-SP/+* flies in this study followed a normal distribution ($n = 280$, $\mu = 0.48$, $\sigma = 0.13$, Kolmogorov–Smirnov test, $P > 0.1$). The structure of the screen using successive steps would also eliminate false positives selected initially. Second, the average of all the *scramble-miR-SP* scores across the primary screen was used as a population control value to account for day-to-day variability of *scramble-miR-SP* scores inherent in any behavioral study. The performance of the *miR-SP* flies was compared to the population control value using a two-tailed, one-sample Student *t*-test. Significance was set for P -values < 0.05 , and, when reached, we assigned a score of 1 to the *miR-SP*. The P -values < 0.1 were treated as a trend to minimize false negatives from using a fewer number of biological replicates ($n = 4$) compared to what is often needed ($n = 6–8$) in more focused studies. For trends, we assigned a score of 0.5. Lines exhibiting no effect were deemed “neutral” with a score of 0. The scores obtained using the two statistical approaches were then summed and the lines ranked according to their scores. When two inserts (*attP40* and *attP2*) were available for a given *miR-SP*, both lines were tested by different experimenters. We later combined the scores obtained for the two *miR-SP* inserts

and ranked the lines according to those scores. The maximum score obtainable was 4. Lines scoring > 0.5 were retained for the secondary screen.

For the secondary screen, the same two statistical approaches were used with six biological replicates. Each *elav^{c155}-gal4/+>uas-miR-SP/+* genotype performance score was compared to the corresponding *+/+>uas-miR-SP/+* genotype score. When available, the *attP40* and *attP2* inserts were tested by different experimenters. We subsequently combined the scores from the primary and secondary screens. The maximal score obtainable was 8, and we retained lines with a score of ≥ 4 for the third and last screen.

Statistics

Excel Stat and Prism were used for data analysis. Performance indices (PIs) followed a normal distribution (Figure 1, B and C), so two-tailed, Student *t*-tests were used to compare the two groups. To compare one group to the population mean value, a one-sample, two-tailed Student’s *t*-test was used. For multiple group comparisons, ANOVA followed by Bonferroni post-hoc tests was used. Proportions were compared using a χ^2 test. Distributions were compared using the Kolmogorov–Smirnov or the D’Agostino and Pearson omnibus normality test when possible. Correlation was assessed with Pearson or Spearman correlation coefficients depending on normality of the samples. Significance was set at $\alpha = 0.05$.

Results

A genetic screen targeting 134 miRNAs identified at least five that are involved in the biology of memory formation

We tested the involvement of 134 different miRNAs in the biology of olfactory classical conditioning by attenuating their function with a library of transgenic miRNA sponges (miR-SPs, Supporting Information Figure S1, A–C). We anticipated that this behavioral screen would produce substantial variability in memory scores leading to significant numbers of false positives and negatives in the initial screen for several reasons. First, behavioral assays are inherently variable and require extensive replication and statistical analyses to identify *bona fide* differences. Second, because of the significant labor involved in a behavioral screen, we limited the testing in the initial screen to four biological replicates for each line. Third, the miR-SP transgenes produce a partial loss of function rather than a complete loss of function, since the mechanism of competitive inhibition requires hybridization of the miR-SPs to their target miRNAs (Figure S1C). The extent of hybridization and inhibition depends on numerous factors, including the relative expression level of the miRNA targets and the miR-SPs, the efficiency of stable hybridization of the miR-SPs to endogenous miRNAs in the cellular context, and the possible existence of compensatory mechanisms. Fourth, miRNAs provide analog modulation of the expression of their target mRNAs (McNeill

Figure 1 Initial screen of 134 miR-SPs. (A) Selected data diagramming the logic for selecting putative hits in the primary screen. PIs were calculated for the progeny (*elav^{c155}-gal4>uas-miR-SP*) of each miR-SP tested in either the *attP40* or *attP2* sites. A control genotype with a scrambled sequence (*elav^{c155}-gal4>uas-scr-miR-SP*) was tested with each experimental group. In addition, the average 3-hr memory score for the *scr-miR-SP* control was calculated across the primary screen. This produced two probability values for each miR-SP, one obtained by comparing each miR-SP to the performance index of the *scr-miR-SP* control tested in parallel, and the other to the performance average of the *scr-miR-SP* control across the screen (average *scr-miR-SP*). A score of 1 was assigned for P -values <0.05 . A score of 0.5 was assigned to P -values <0.1 but >0.05 to include trends. A score of 0 was assigned to P -values >0.1 (neutral) (see Table S1). The data shown are the mean $\pm$ SEM with $n = 4$. Probabilities are calculated from two-tailed, one- or two-sample Student t -tests. (B) Three-hour memory PIs of flies carrying miR-SP *attP40* inserts driven by *elav^{c155}-gal4* follow a normal distribution. The observed distribution of PIs was compared to a theoretical one with similar parameters ($\mu = 0.49$ and $\sigma = 0.13$) using a Kolmogorov–Smirnov test. A random normal distribution with parameters similar to the observed distribution was generated and superimposed on the observed distribution for better visualization. (C) Three-hour memory PIs of flies carrying miR-SPs *attP2* inserts driven by *elav^{c155}-gal4* follow a normal distribution. The observed distribution of the values was compared to a theoretical distribution with the same parameters ($\mu = 0.45$ and $\sigma = 0.11$) using Kolmogorov–Smirnov test. (D) The numbers of the miR-SP lines classed initially as increasing, decreasing, showing a trend, or with no effect (neutral) as compared to each line’s paired control (vs. *scr-miR-SP*) or to the *scr-miR-SP* average score across the screen (vs. average *scr-miR-SP*). The *attP40* charts for the two comparisons (top row) and the *attP2* charts for these comparisons (bottom row) are combined in the third column of charts. An asterisk (*) indicates lines with significant effect on 3-hr memory. The proportions in various charts were compared using χ^2 tests. There was no significant difference observed between the proportions found in various phenotypic classes obtained for the *attP40* or *attP2* sponges using the two statistical approaches, yet there was a significant difference observed between the proportions found in various phenotypic classes obtained for the *attP40* and *attP2* sponges within each statistical approach, indicating a differential efficacy of the *attP40* vs. *attP2* insertions. (E) Comparison of 3-hr memory PI distribution between miR-SPs inserted into either the *attP40* or the *attP2* genomic sites. Distributions were compared using Kolmogorov–Smirnov test. (F) Average 3-hr memory PIs for the *scr-miR-SP* controls and miR-SPs when inserted in the *attP40* or *attP2* genomic sites. Results are mean $\pm$ SEM with $n = 28$ –95, two-tailed, two-sample Student t -test; * $P < 0.05$.

and Van Vactor 2012) so that even a strong knockdown of an miRNA might have only modest effects on the expression level of its mRNA targets. Thus, the screen was extraordinarily demanding in requiring the identification of authentic hits using a partial loss-of-function approach of a regulatory

mechanism that might influence the inherently variable readout of conditioned behavior.

Because of these constraints, we adopted a stepwise and liberal approach for selecting putative hits, initially accepting lines with significant differences from the control but also

those that exhibited a trend ($0.05 < P\text{-value} < 0.1$). The miR-SP library contains lines with *uas-miR-SP* transgenes inserted at the *attP40* site on the second chromosome and lines with the same *uas-miR-SP* transgenes inserted at the *attP2* site on the third chromosome (Figure S1A). We tested both the *attP40* and the *attP2* insertions for each miR-SP. Individual miR-SPs were expressed in the developing and adult nervous system using the neuron-specific *gal4* driver, *elav^{C155}-gal4*. Homozygous *elav^{C155}-gal4* flies were crossed with each homozygous *uas-miR-SP* fly line, and F_1 progeny containing both transgenes (*elav^{C155}-gal4/+>uas-miR-SP/+*) were tested for 3-hr memory. We evaluated the effects of each miR-SP line in two different ways (Figure 1A). First, the average memory performance of each *elav^{C155}-gal4/uas-miR-SP* group was compared to a control group using a scramble miRNA sponge (*uas-scr-miR-SP*) tested in parallel using four biological replicates for both the experimental and the control groups. However, given the substantial behavioral variability of both experimental and control groups across days of experimentation using $n = 4$, we also compared the average performance of each miR-SP group to the scramble control group scores averaged across the screen (Figure 1A). The behavioral performance of each miR-SP in both the *attP40* and the *attP2* sites, when both were available, was included in our initial selection of putative hits (Figure 1 and Table S1).

Table S1 lists the 134 miRNAs of the 144 high-confidence miRNA sequences described in miRBase (<http://www.mirbase.org/>) that were tested (Griffiths-Jones 2004; Ruby *et al.* 2007). One hundred and fifteen *attP40* insertions of the 134 were tested for memory because 17 miR-SPs were not available in the collection and 2 crosses failed to produce progeny (Table S1). One hundred and fourteen *attP2* insertions were tested; 17 *attP2* miR-SPs were not available and 3 failed to produce progeny. Thus, 39 miRNAs were tested using only the *attP40* or *attP2* insertion that was available.

The 3-hr PIs for the *attP40* miR-SP inserts exhibited a normal distribution with an average PI of 0.49 (Figure 1B). Twenty-one of the miR-SPs significantly modulated memory scores compared to the *scr-miR-SP* controls, with 8 lines decreasing memory and, surprisingly, 13 lines increasing it (Figure 1D, top row, left). Twelve lines exhibited a trend (Figure 1D). Similar numerical results were obtained by comparison to the averaged *scr-miR-SP* control, with 24 lines significantly modulating memory and 7 lines exhibiting a trend (Figure 1D, top row, center). Among the significantly different group, 19 lines increased memory and 5 decreased it (Figure 1D). For 13 of the lines, significant differences were obtained using both statistical approaches with 8 lines increasing memory and 5 decreasing it (Figure 1D, top row, right).

The PIs for the *attP2* miR-SP inserts also followed a normal distribution with an averaged value of 0.45 (Figure 1C). Compared to the *scr-miR-SP* control, 11 miR-SPs significantly modulated memory and 10 exhibited a trend (Figure 1D, bottom row, left). Two of the significantly different miR-SPs increased memory and 9 decreased it (Figure 1D). When the averaged *scr-miR-SP* PI score was used in the

analysis, 19 lines were determined to significantly modulate memory and 8 lines exhibited a trend (Figure 1D, bottom row, center). Among the significantly different group, 5 miR-SPs increased memory and 14 decreased it (Figure 1D). Four miR-SPs were significant using both statistical approaches, and, among them, 3 miR-SP lines decreased memory and 1 increased it (Figure 1D, bottom row, right).

The proportion of miR-SP lines that significantly modulated memory appeared to be somewhat different for the *attP40* vs. *attP2* insertion sites (Figure 1D). To test for an authentic difference, we compared the fraction of lines that increased, decreased, exhibited a trend, or were neutral for *attP40* and *attP2* inserts using the two statistical approaches (Figure 1D). We found that the two insertion sites were indeed different in their effects. This held true when either the *scr-miR-SP* control (χ^2 test, $P = 0.03$) or the averaged *scr-miR-SP* control value (χ^2 test, $P = 0.006$) was considered. This may be due to a different expression level from inserts at the *attP40* vs. *attP2* sites. To show this in a second way, we compared the PI distribution of the 95 lines for which two inserts were available (Figure 1E). The average of the 95 PI values for all the miR-SP was significantly different between the two insertion sites (Figure 1F).

For seven miR-SPs, a significant effect or trend was observed with both *attP40* and *attP2* inserts (Table S2 and Figure S2A). To integrate the data collected into a single variable for comparing the miR-SPs, we assigned a score of 1.0 for $P\text{-values} < 0.05$ and a score of 0.5 for $0.05 < P\text{-values} < 0.1$ for the four comparisons made for each miR-SP, yielding lines with scores from 0 to 4 (Figure 1A and Table S1). Seventy-one of the miR-SPs modulated memory (score ≥ 0.5) for either the *attP40* or the *attP2* insert when compared to the paired *scr-miR-SP* control or the *scr-miR-SP* PI averaged across the screen. We passed 53 of the lines with a score of ≥ 1.0 (Table S1) into a secondary screen.

In the secondary screen, we employed an alternative control to test the phenotypic dependency on the presence of both a *uas-miR-SP* and the *elav^{C155}-gal4* driver (Figure 2, A and B and Table S3). Leakiness of any *uas-miR-SP* insertion would render it difficult for further study. Each *uas-miR-SP* line was crossed to either *elav^{C155}-gal4* or *w^{CS10}* flies (control). The results of this secondary screen were analyzed using the same two statistical approaches described above (Figure 2, A and B).

The 3-hr PIs for the miR-SPs inserted in the *attP40* locus followed a normal distribution with a mean of 0.5 (Figure 2C). For the 53 lines of interest, 49 *attP40* inserts were tested; 4 *attP40* inserts were not available (Table S3 and Figure 2A). Ten of the *elav^{C155}-gal4*-driven miR-SPs significantly modulated 3-hr memory compared to the corresponding $+/+>uas-miR-SP/+$ control genotype, with 8 increasing and 2 decreasing memory (Figure 2E, left). Six lines exhibited a trend, and 33 lines were neutral. An equivalent proportion (χ^2 test, $P = 0.09$) was obtained when the PI scores were compared with the average PI of all tests of the $+/+>uas-miR-SP/+$ control, with 11 miR-SPs increasing and

Figure 2 Secondary screen. (A) Selected data for the rescreen of miR-SP *attP40* inserts. Three-hour memory performance was tested for each miR-SP expressed in the central nervous system (*elav^{c155}-gal4*). Each miR-SP was crossed to *w^{CS10}* flies, and the progeny were used as a control for the corresponding miR-SP line. An average control score was computed as the average PI for all control flies across the secondary screen (average +>miR-SP). Two probability values were computed for each miR-SP, one comparing each miR-SP to its paired control and the second comparing the miR-SP PI to the average control. *P*-values <0.05 were considered significant. *P*-values <0.1 but >0.05 were considered a trend. Neutral lines failed to reach any of our criteria. The data shown are the mean $\pm$ SEM with *n* = 6. Probabilities are calculated from two-tailed, one- or two-sample Student *t*-tests. (B) Selected data for the rescreen of *attP2* miR-SP inserts. (C) Distribution of 3-hr memory performance for the miR-SPs *attP40* inserts. (D) Distribution of 3-hr memory performance for the *attP2* miR-SPs inserts. (E) Proportion of *attP40* miR-SP lines increasing, decreasing, showing a trend, or neutral for 3-hr memory compared to each line's paired control (left: vs. +>miR-SP). (Right) Proportions compared to the control scores averaged across the secondary screen. Proportions were compared using χ^2 tests. (F) Proportion of *attP2* miR-SP lines increasing, decreasing, showing a trend, or neutral relative to 3-hr memory.

9 decreasing memory (Figure 2E, right). Five miR-SPs gave $0.5 < P$ -values < 0.1 and 24 were neutral. Finally, 8 of the miR-SPs exhibited significantly increased or decreased memory performance using both statistical approaches (Table S4 and Figure S2B).

The average 3-hr memory performance was 0.47 for the *attP2* inserts, and the PIs followed a normal distribution (Figure 2D). Forty-four miR-SPs were tested with nine *attP2* inserts being unavailable (Table S3 and Figure 2B). Twelve *elav^{c155}-gal4*-driven miR-SPs significantly increased memory and one decreased it compared to the corresponding +/+>uas-miR-SP/+ control genotype (Figure 2F, left). These proportions were again similar (χ^2 test, *P* = 0.63) when compared with the average PI score of the control across the secondary screen: 16 miR-SPs significantly increased and none decreased memory (Figure 2F, right). Nine miR-SPs significantly modulated memory using both statistical approaches (Table S4 and Figure S2B).

We combined the results of the two successive screens to rank the miR-SPs according to their performance relative to the *scr-miR-SP* and the +/+>uas-miR-SP controls, including

both the *attP40* and *attP2* inserts (Table S1 and Table S3). From this we chose 16 lines (Table S5) that were retested in parallel with both *gal4* and *uas* controls. This step was added to validate the most reproducible and strongest lines for further analyses. We compared the 3-hr performance of each miR-SP driven by *elav^{c155}-gal4* (*elav^{c155}-gal4/+>uas-miR-SP/+*) with the *scr-miR-SP* crossed with *elav^{c155}-gal4* (*elav^{c155}-gal4/+>uas-scr-miR-SP/+*) and *uas-miR-SP* crossed with *w^{CS10}* flies (+/+>uas-miR-SP/+). From the 16 miR-SP retested, 5 miR-SPs significantly modulated 3-hr memory compared to both controls (Figure 3 and Figure S3). The five miRNAs identified were *miR-9c*, *miR-31a*, *miR-305*, *miR-974*, and *miR-980*. Four of these miR-SPs reduced memory (*miR-9c*, *miR-31a*, *miR-305*, *miR-974*) and one (*miR-980*) increased memory. We present additional characterization below on *miR-31a* and *miR-974*.

microRNA-31a is required in cholinergic neurons for optimal 3-hr memory

The behavioral genetic screen described above utilized the pan-neuronal *gal4* driver, *elav^{c155}-gal4*. A critical issue

Figure 3 Final screen. Each *miR-SP* candidate was crossed with the *elav^{c155}-gal4* driver to reduce the corresponding miRNA expression in the CNS. The *miR-SP* was crossed to *w^{CS10}* flies as a first control. A *scr-miR-SP* was also crossed to *elav^{c155}-gal4* as a second control. Three-hour memory was evaluated after training. The five lines presented here showed 3-hr memory performance significantly modulated compared to both controls. Results are presented as the mean $\pm$ SEM with $n = 6$ –12. Analysis of variance followed by Bonferroni post-hoc tests. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$.

stemming from these results concerns the brain regions and cell types that require the normal expression of *miR-31a* for normal 3-hr memory. To address this, we employed 10 different *gal4* lines that drive robust expression in limited regions of the adult brain, including portions of the olfactory nervous system that are involved in olfactory memory along with drivers for cell-type-specific expression (Figure 4A; Davis 2005, 2011). We also retested the *gal4* driver, *elav^{c155}-gal4*, in this spatial and cell-type mapping study. The *gal4*-driven expression of *miR-31a-SP* (in the *attP40* site) had no effect on 3-hr memory compared to the associated scrambled control in any specific neuronal population tested except for those associated with *Cha-gal4*, a *gal4* line driven by the promoter of choline acetyltransferase (Figure 4A; Kitamoto 2001). We reproduced this result using a *miR-31a-SP* line containing both *attP40* and *attP2* inserts (Figure 4B). The odor and shock avoidance of cholinergic neuron- or CNS-expressed *miR-31a-SP* were not significantly different from the control (Figure 4, C and D). We conclude from these data that normal expression of *miR-31a* is required in cholinergic neurons outside of those represented by the spatially restricted *gal4* drivers (Figure 4A) for normal 3-hr memory and not for normal odor or shock perception. Further studies are required to identify the subpopulation of cholinergic neurons involved.

For each miRNA, dozens to hundreds of potential RNA targets are predicted using *in silico* approaches (Bartel 2009). Predicted targets are often validated by quantitative RT-PCR, sensor assays, direct pull-down, or target protector experiments (Hsu *et al.* 2009; Staton and Giraldez 2011; Connelly and Deiters 2014). Such experiments may reveal interactions between a given miRNA and its predicted target, but they fail to reveal whether the interaction is functional in the biological process of interest. Moreover, target

mRNAs that are inhibited from translation by their miRNA but not degraded would fail to exhibit changes in expression (Griggs *et al.* 2013). Consequently, we chose to test the involvement of *miR-31a* potential targets using an RNAi knockdown approach to obtain insights into the network of genes functionally required for normal 3-hr memory that may be potentially regulated by the microRNA. The *miR-SPs* are predicted to buffer their corresponding miRNAs, which in principle should lead to overexpression of their direct mRNA targets (Figure S1C). Thus, the phenotypes observed with any *miR-SP* in theory can be induced by the overexpression of the proteins post-transcriptionally regulated by targeted miRNAs. The knockdown of authentic targets of *miR-31a* might produce the opposite phenotype as the *miR-31a-SP* if the behavioral phenotype is linearly related to mRNA and protein expression levels. Alternatively, the inhibition of authentic targets of *miR-31a* might produce the same phenotype as the *miR-31a-SP* if the behavioral phenotype emerges due to either reduced or increased expression from optimum level. The RNAi knockdown approach was facilitated by the existence of large libraries of conditional *uas-RNAi* lines that exist for nearly every *Drosophila* gene (<http://stockcenter.vdrc.at/control/main>).

We used TargetScan for *in silico* prediction of potential mRNA targets of *miR-31a* (Lewis *et al.* 2003). The algorithm predicts miRNA targets by searching for sequences complementary between the seed region of the miRNA and the 3' UTR of selected mRNAs, taking into account the local sequence environment (Bartel 2009). Using this approach, 57 putative *Drosophila* mRNA targets were predicted for *miR-31a* (Table S6). We expressed the *uas-RNAi*'s using *n-syb-gal4*, a second pan-neuronal and strong *gal4* driver, for 38 of the putative targets. Sixteen targets had no *uas-RNAi* line available in the VDRC KK library, and three *uas-RNAi*'s failed to produce progeny when expressed in the CNS. Among the 38 lines screened, 15 were identified as potential hits based on the distribution of PI values, selecting those lines deviating from the mean by >1 SD (Figure 5 and Table S6). Of the 15 lines that were retested with another $n = 4$, four were identified as potential mRNA targets that decrease 3-hr memory by >1 SD from the mean (Table S7). All four potential targets decrease 3-hr memory, functioning as downstream targets of *miR-31a*, whose increased or decreased expression perturbs memory, or in the biology of memory formation independently of *miR-31a*. However, some of these lines exhibit a collapsed-wing phenotype in conjunction with *n-syb-gal4* (Table S7), leaving open the possibility that the behavioral deficit is due to poor fitness rather than a specific effect on memory processes.

Competitive inhibition of *miR-974* in olfactory receptor neurons and MB-V2 neurons increases 3-hr memory performance

We also investigated the spatial disruption of *miR-974* that modulates 3-hr memory (Figure 6). We tested seven different *gal4* transgenes that drive expression in different subsets

Figure 4 *MiR-31a-SP* impairs 3-hr memory acting in cholinergic neurons. (A) *miR-31a-SP attP40* expression reduced 3-hr memory when expressed in cholinergic neurons. *miR-31a-SP* was expressed in specific cell types and in different brain regions including parts of the olfactory system using different *gal4* lines. Expression domain of the *gal4* drivers: CNS, central nervous system; PN, projection neurons; CC, central complex; MBn, mushroom body neurons; DPMn, dorsal paired medial neurons; DAn, dopaminergic neurons; ORN, olfactory receptor neurons; MB-V2, mushroom body extrinsic neurons V2; GABAn, GABAergic neurons; 5-HT, serotonergic neurons; Ach, cholinergic neurons. Three-hour memory performance for progeny of each *gal4* line crossed with the *uas-miR-31a-SP* was compared to progeny from a cross with the *scr-miR-SP* control. Results are presented as the mean $\pm$ SEM with $n = 6-23$. Two-tailed, two-sample Student *t*-tests, * $P < 0.05$. (B) *miR-31a-SP attP40* single and double inserts reduced 3-hr memory. For the double insert, a double *scr-miR-SP* insert (*attP40* and *attP2*) was used as a control. Results are presented as the mean $\pm$ SEM with $n = 6$. Two-tailed, two-sample Student *t*-test, ** $P < 0.01$, * $P < 0.05$. (C) *miR-31a-SP* had no significant effect on odor and shock avoidance when expressed with *elav^{c155}-gal4*. Results are presented as the mean $\pm$ SEM with $n = 6$. (D) *MiR-31a-SP* had no significant effect on odor and shock avoidance when expressed in cholinergic neurons. Results are the mean $\pm$ SEM with $n = 6$.

of brain neurons and found that expression of *miR-974-SP* had no significant effect. These *gal4* lines drive expression in some projection, central complex, mushroom body, and dopaminergic neurons and in both of the dorsal paired medial neurons (Figure 6A). Surprisingly, *miR-974-SP* expression significantly increased memory when expressed in olfactory receptor neurons and in mushroom body-V2 neurons, a phenotype opposite of that observed when expressed throughout the CNS. Although surprising, such a result is plausible if memory increase, generated by local expression of *miR-974-SP*, is masked by global expression in other brain areas that impair memory performance (see Discussion). We subsequently verified the CNS-wide effect of impairing 3-hr memory using *elav^{c155}-gal4* (Figure 6B) and tested odor and shock avoidance to ensure that flies expressing *uas-miR-974-SP* exhibited no significant difference from control in sensory parameters (Figure 6C). The simplest conclusion from these analyses is that a reduction in *miR-974* abundance in olfactory receptor neurons and mushroom body-V2 neurons might increase 3-hr memory, but a global reduction of this miRNA impairs memory.

We screened 16 predicted mRNAs of *miR-974* downstream targets (Table S8) for their involvement in 3-hr memory. Five of these predicted targets passed the first screen and one was confirmed in a rescreen (Table S7 and Table S8). The confirmed RNAi line is against *Fas1*, a cell

adhesion molecule (Table S7). This line also exhibited the collapsed-wing phenotype.

Discussion

We report here the first systematic screen for individual miRNA involvement in memory formation using miRNA sponges, a loss-of-function approach. We tested 134 miRNAs using miRNA-SP in two genomic locations in successive screens. At present, 256 putative miRNA sequences are available in the miRBase for *D. melanogaster* (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=dme) of which 144 are listed as being of high confidence. Therefore, our screen encompassed >90% of the high-confidence miRNAs described so far. The screen identified five miRNAs—*miR-9c*, *miR-31a*, *miR-974*, *miR-305*, and *miR-980*—that reproducibly altered memory by either disrupting the neuronal physiology underlying memory formation or altering the development of the nervous system. Most interestingly, some miRNA-SPs impaired memory while others enhanced it. We partially mapped the effects for two miRNA-SPs to cell types or brain regions using a panel of *gal4* drivers, used an *in silico* approach to predict miRNA gene targets, and tested some of the predicted mRNA targets using an RNAi approach that offered a direct way to interrogate the functional involvement of putative miRNA targets in ITM. All

Figure 5 Distribution of 3-hr memory performance for the RNAi candidates of *miR-31a* and *miR-974* when expressed in the CNS. Distribution of 3-hr memory performance of the *uas-RNAi* candidates when crossed with *uas-dcr2;n-syb-gal4* along the theoretical normal distribution with the same parameters and using a Kolmogorov-Smirnov test. Crosses for which the PI was below or above 1 SD from the mean were retested.

together, our results offer important and broad insights toward understanding the roles of miRNAs in memory storage and offer a valuable resource on which to base further studies.

We believe that our ranking method offers value for genetic screens because it is progressive, additive, simple, and straightforward. This method provided a rank according to several parameters: (1) the genomic localization of the sponge insert to compensate for possible genomic position effects, (2) the use of two different control crosses to ensure specificity of the sponge and eliminate false positives due to leaky expression, and (3) the successive and multiple ($\times 5$) tests of the same line; all of which provided confidence for our final selections. Our experimental approach also offers advantages: (1) two different researchers tested the lines, countering possible individual bias; (2) the parameters employed were given equal weights; and (3) sponge lines were ranked rather than eliminated.

Although five miRNAs were identified as important for memory formation measured at 3 hr after conditioning, it is likely that false negatives exist among the 129 that failed at one or more points in our extensive and stringent screening pipeline. Learned behavior is an extraordinarily complex phenotype for a genetic screen, the assay for memory formation is labor-intensive and time-consuming, and we utilized a partial loss-of-function approach such that positives with more subtle effects may have been missed. The participation of some miRNAs was missed because memory formation is not a unitary process. For example, *miR-276a* has been shown to be necessary for long-term memory formation but dispensable for short-term memory (Li *et al.* 2013). We focused our study on aversive classical conditioning, being thereby blind to miRNAs that might have a specific involvement in appetitive conditioning. Moreover, the miRNA-SPs function by competitive inhibition, so the effectiveness of any given miRNA-SP will vary according to the expression level of the miRNA-SP and its corresponding miRNA. However, our approach offers significant advantages over a genomic knockout methodology by facilitating the subsequent cell type and spatial mapping of the effects, an important consid-

Figure 6 *miR-974-SP* increases 3 h memory when expressed in olfactory receptor or mushroom body-V2 neurons. (A) *miR-974-SP* expression improved 3-hr memory when expressed in OR and MB-V2 neurons. *miR-974-SP* was expressed in several brain regions including parts of the olfactory system using several different *gal4* drivers. Detail of the brain regions encompassed by the *gal4* drivers: CNS, central nervous system; PN, projection neurons; CC, central complex; MBn, mushroom body neurons; DPMn, dorsal paired medial neurons; DAN, dopaminergic neurons; ORN, olfactory receptor neurons; MB-V2, mushroom body extrinsic neurons V2. Three-hour memory performance of each *gal4* driver crossed with the *uas-miR-974-SP* was compared to crosses with the *scr-miR-SP* control. Results are the mean $\pm$ SEM with $n = 16$ –18. Two-tailed, two-sample Student *t*-tests, $**P < 0.01$. (B) The *miR-974-SP attP40* insert reduced 3-hr memory in an independent experiment when driven by *elav^{C155}-gal4*. Results are the mean $\pm$ SEM with $n = 6$. Two-sample, two-tailed Student *t*-tests, $*P < 0.05$. (C) *miR-974-SP* had no effect on odor and shock avoidances when expressed in the CNS with the *elav^{C155}-gal4* driver. Results are the mean $\pm$ SEM with $n = 6$ –12.

eration given that the process of memory formation is distributed across multiple cells types within the brain (Davis 2011). Furthermore, conditional inhibition offers protection against the accumulation of genetic modifiers or physiological compensation that can occur across generations with chronic knockouts.

An unexpected observation made from our screen that may be important for all genetic experiments that employ site-specific transgene integration was that sponges inserted into the *attP40* site appeared more potent than those inserted at the *attP2* site. This is despite prior studies showing that the expression level of a luciferase reporter inserted in either the *attP40* or *attP2* locus was similar in the nervous system of larvae (Markstein *et al.* 2008). The most likely explanation for this is that the local environment of one of

the insertion sites influences expression, at least in the adult brain, despite the presence of insulator sequences in the construct. Our ranking approach considered both insertions without bias.

Little is known about the five miRNAs identified to be involved in memory formation, and additional studies will be necessary to obtain deeper insights. Nevertheless, several of those identified have been studied in other systems and found to be involved in neuroplasticity and brain disorders. *miR-9c* is part of a conserved family found from flies to humans (Yuva-Aydemir *et al.* 2011). It is highly expressed in the brain (Delaloy *et al.* 2010), and an unpublished report quoted by Bredy *et al.* seems to indicate that increased *miR-9* regulates dendritic arborization and cognitive abilities in mice (Bredy *et al.* 2011). Other reports indicate that *miR-9* is elevated after alcohol exposure (Pietrzykowski *et al.* 2008) and is involved in dementia (Hebert *et al.* 2008) and polyglutamine disease (Packer *et al.* 2008). In addition, *miR-9* expression is downregulated in patients with Alzheimer's and Huntington's disease (Cogswell *et al.* 2008; Packer *et al.* 2008; Maciotta *et al.* 2013).

miR-31a is part of a family with one ortholog in humans (Gerlach *et al.* 2009). It was first described as a tumor suppressor with expression levels varying according to metastatic state (Valastyan and Weinberg 2010). Our data show that *miR-31a* is necessary for optimal 3-hr memory performance in the CNS and more specifically in cholinergic neurons. The *Cha-gal4* driver covers a large number of neurons in the adult brain. We hypothesize that the population responsible for the phenotype is included within the *Cha-gal4* expression domain, but is outside of the neuronal populations included in the battery of the more specific *gal4* lines employed. Further studies will be necessary to identify the specific subpopulation of cholinergic neurons in which *miR-31a* is necessary. We tested 3-hr memory in a large group of potential *miR-31a* target mRNAs and showed that four modulated memory. This, of course, is indirect evidence for potential mRNA targets but offers a list for further study.

miR-974 has been described in five species of *Drosophila* (Gerlach *et al.* 2009). Our data show that reducing its expression in the whole nervous system reduces memory. Intriguingly, the effect was opposite when *mir-974* was silenced in ORN and MB-V2 neurons. This unusual observation prompts three possible explanations. First, *miR-974-SP* may have a negative effect on memory when expressed in a neural compartment that we did not test, nullifying the positive effects of expression in ORN or MB-V2 neurons. Second, the increasing effects on memory when expressed locally may be nullified by expression throughout the CNS through systems interactions. Third, the *miR-SP* expression level induced by the different *gal4* drivers might induce different phenotypes. Further studies will be necessary to choose between those explanations. This miRNA has 16 targets predicted by TargetScan with one (*Fas-I*) participating in 3-hr memory. *Fas1* is a cell-adhesion molecule involved in axonal guidance (Zinn *et al.* 1988). The *fas1* mutant exhibits

an increased number of terminal branches and varicosities at the neuromuscular junction (Zhong and Shanley 1995). Structural or functional changes in the adult brain due to abnormal *Fas1* expression may underlie the observed changes in memory.

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microRNAs That Promote or Inhibit Memory Formation in *Drosophila melanogaster*

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Figure S1 MicroRNA 'sponges' are conditional genetic constructs inserted in *Drosophila melanogaster* genome coding for complementary oligonucleotides targeting specific miRNAs.

(A) The 'sponge' conditional construct was inserted into the *attP40* locus (left arm, 25C6) on the 2nd chromosome in a site-specific manner using phage phiC31 integrase. The same construct was inserted into the *attP2* locus (left arm, 68A4) on the 3rd chromosome (Loya *et al.* 2009 ; Fulga *et al.* 2014).

(B) The 'sponge' insert is comprised of 20 repeats of a complementary sequence to the target miRNA. Repeats are separated from each other by a random sequence of 4 nucleotides. The 'sponge' sequences are located in a RNA transcript that also codes for the fluorescent protein mCherry. Ten repeats of the Gal4 binding sequence, *uas*, are upstream of the mCherry open reading frame. An *attB* sequence is present to allow site-specific integration via recombination induced by phage phiC31 integrase with an *attP* site. Gypsy insulators insure minimal interactions of the whole construct with the surrounding sequence environment.

(C) Under physiological conditions, a miRNA recognizes a specific population of mRNAs based, in part, on the recognition of a specific sequence in the 3'UTR region of the mRNA. The seed sequence, between 6 and 8 nucleotides, is bound by the miRNAs to reduce gene expression. The miRNA-SP was built with sequence complementarity with the miRNA to interfere with its normal interactions with mRNA targets.

Figure S2 Performance index correlations between the initial screen (vs *scr-miR-SP*) and the secondary screen (vs *+/miR-SP*) and between the *attP40* and *attP2* inserts.

(A) Correlation of Performances Indices (PIs) was assessed between the *miR-SP attP40* and *attP2* inserts for the *miR-SP* lines significantly or showing a trend to affect 3 h memory with either or both statistical approaches for the initial screen vs the *scr-miR-SP*. Pearson's correlation coefficient (R) was calculated between samples since both were following a Normal distribution. PIs are significantly correlated between the *attP40* and *attP2* inserts. Lower left quadrant includes decreasing lines while upper right increasing ones. *MiR-SP* lines showing discrepant effects between inserts were discarded for the analysis (black lined dots, upper left and lower right quadrants).

(B) Correlation of PIs was assessed between the initial screen (vs *scr-miR-SP*) and the secondary screen (vs *+/miR-SP*) for the *miR-SP* lines significantly or showing a trend to affect 3 h memory with either or both statistical approaches for the *attP40* insertion site. PIs are significantly correlated between the initial and the secondary screen.

Figure S3 Performance index correlations between the three successive screens for the five *miR-SP* lines significantly decreasing or increasing memory.

(A) Correlation of performance indices (PIs) was assessed between the initial (vs *scr-miR-SP*) and the secondary (vs *+/miR-SP*) screen for the five *miR-SP* lines significantly modulating memory when expressed in the CNS. Pearson's correlation coefficient (R) was calculated between samples since both were following a Normal distribution. PIs are significantly correlated between the initial and the secondary screen.

(B) Correlation of PIs was assessed between the initial and the final screen. Spearman's rank correlation coefficient (r) was calculated between samples since the sample from the final screen was not following a Normal distribution. PIs are significantly correlated between the first and the secondary screen. R^2 is also presented.

(C) Correlation of PIs was assessed between the secondary and the final screen. Spearman's rank correlation coefficient (r) was calculated between samples since the sample from the final screen was not following a Normal distribution. PIs are highly correlated but not significantly between the secondary and the final screen. R^2 is also presented.

Table S1 Three-hour memory performances after miRNAs ‘sponging’ in the central nervous system

a target miRNA	b PI	attP40 insert			attP2 insert				f score
		d ratio	p-value		PI	ratio	p-value		
			d vs scr- miR-SP	e vs avg scr-miR-SP			vs scr- miR-SP	vs avg scr- miR-SP	
bantam	0.59	1.44	0.1115	0.3591	0.49	1.05	0.568	0.379	-
let-7	0.56	1.21	0.2718	0.3903	NA	NA	NA	NA	-
mir-1	0.35	1.05	0.807	0.170	0.63	1.31	0.1318	0.064,#	0.5
mir-10	0.59	1.19	0.212	0.133	0.44	1.38	0.1728	0.2559	-
mir-100	0.69	1.30	0.0247,*	0.0108,*	0.51	1.06	0.608	0.437	2
mir-1000	0.49	0.92	0.32	0.482	NA	NA	NA	NA	-
mir-1001	0.47	1.29	0.31	0.944	NA	NA	NA	NA	-
mir-1002	0.66	1.26	0.029,*	0.023,*	0.27	0.54	0.0069,*	0.0052,*	4
mir-1003	NA	NA	NA	NA	0.32	0.88	0.633	0.186	-
mir-1004	NA	NA	NA	NA	0.48	0.93	0.631	0.700	-
mir-1005	0.44	1.27	0.499	0.836	0.44	0.88	0.6037	0.6079	-
mir-1006	0.5	1.35	0.348	0.9996	0.28	0.81	0.426	0.043,*	1
mir-1007	0.49	1.42	0.167	0.695	0.33	0.89	0.6942	0.1385	-
mir-1009	0.58	1.57	0.0175,*	0.2186	0.68	1.03	0.65	0.010,*	2
mir-1010	0.49	1.42	0.068,#	0.520	0.42	1.14	0.5031	0.3722	0.5
mir-1011	0.6	1.37	0.174	0.005,*	NA	NA	NA	NA	1
mir-1012	0.69	1.05	0.514	0.010,*	0.6	1.13	0.4627	0.0952,#	1.5
mir-1013	0.63	1.45	0.055,#	0.035,*	0.5	0.96	0.9001	0.7822	1.5
mir-1014	0.5	1.09	0.1705	0.9992	NA	NA	NA	NA	-
mir-1015	0.33	0.62	0.1462	0.0958,#	0.47	0.71	0.022,*	0.907	1.5
mir-1016	0.65	1.58	0.009,*	0.027,*	0.43	0.81	0.4158	0.3742	2
mir-11	0.44	0.92	0.7026	0.4754	0.39	0.79	0.065,#	0.169	0.5
mir-12	0.51	1.03	-	-	0.31	0.98	0.9705	0.2063	-
mir-124	0.52	1.24	0.267	0.146	0.6	1.39	0.2039	0.2425	-
mir-125	0.56	1.04	0.78	0.114	0.5	0.94	0.8084	0.9681	-
mir-133	0.57	1.38	0.092,#	0.005,*	NA	NA	NA	NA	1.5
mir-137	0.57	0.82	0.2939	0.4947	0.41	0.77	0.34	0.706	-
mir-13a	0.52	1.56	0.034,*	0.421	0.62	0.89	0.3506	0.0439,*	2
mir-13b	NA	NA	NA	NA	0.69	1.25	0.1604	0.0044,*	1
mir-14	0.4	0.87	0.4354	0.1638	NA	NA	NA	NA	-
mir-184	0.66	1.21	0.089,#	0.004,*	0.529	0.76	0.0717,#	0.2785	2
mir-190	0.43	0.79	0.2471	0.3429	0.59	1.32	0.081,#	0.112	0.5
mir-193	0.53	0.97	0.8335	0.5665	0.43	0.97	0.818	0.506	-

<i>mir-219</i>	NA	NA	NA	NA	0.46	1.05	0.639	0.506	-
<i>mir-252</i>	0.575	1.05	0.796	0.2815	0.52	1.17	0.329	0.402	-
<i>mir-263a</i>	0.44	1.33	0.3367	0.9365	0.5	1.14	0.533	0.742	-
<i>mir-263b</i>	0.46	0.87	0.5784	0.7745	0.59	1.01	0.957	0.372	-
<i>mir-274</i>	NA	NA	NA	NA	0.49	1.29	0.3372	0.9283	-
<i>mir-275</i>	0.709	1.53	0.0473,*	0.0351,*	NA	NA	NA	NA	2
<i>mir-276</i>	0.54	1.41	0.075,#	0.145	0.36	0.78	0.4113	0.1862	0.5
<i>mir-276a</i>	0.455	0.98	0.9472	0.5621	0.49	1.27	0.173	0.419	-
<i>mir-276b</i>	0.53	1.39	0.168	0.393	0.27	0.57	0.1575	0.0822,#	0.5
<i>mir-277</i>	0.775	1.23	0.1708	0.0071,*	0.44	0.78	0.18	0.825	1
<i>mir-278</i>	0.287	0.50	0.004,*	0.052,#	0.28	0.46	0.0697,#	0.2204	2
<i>mir-279</i>	0.493	0.78	0.2509	0.9371	0.62	1.10	0.285	0.022,*	1
<i>mir-281</i>	0.41	1.17	0.148	0.184	0.48	1.20	0.5716	0.8808	-
<i>mir-2811</i>	0.48	1.20	0.5123	0.7336	0.52	1.45	0.01,*	0.229	1
<i>mir-2812</i>	0.62	1.75	0.013,*	0.113	NA	NA	NA	NA	1
<i>mir-282</i>	0.44	1.24	0.062,#	0.525	0.477	1.19	0.5075	0.6224	0.5
<i>mir-283</i>	0.33	0.83	0.6174	0.1458	0.36	0.84	0.279	0.072,#	0.5
<i>mir-284</i>	0.49	1.13	0.526	0.746	0.38	0.77	0.3521	0.2363	-
<i>mir-285</i>	NP	NP	NP	NP	0.46	1.07	0.649	0.978	-
<i>mir-286</i>	0.57	1.08	0.4071	0.141	0.47	1.07	0.814	0.8882	-
<i>mir-287</i>	NA	NA	NA	NA	0.45	0.91	0.6787	0.5444	-
<i>mir-288</i>	NA	NA	NA	NA	0.44	1.03	0.868	0.818	-
<i>mir-289</i>	0.72	1.23	0.0909,#	0.011,*	NA	NA	NA	NA	1.5
<i>mir-2a</i>	0.49	0.87	0.48	0.8337	NA	NA	NA	NA	-
<i>mir-2b</i>	0.55	1.35	0.1879	0.5812	0.39	0.82	0.035,*	0.067,#	1.5
<i>mir-2c</i>	NA	NA	NA	NA	0.49	1.19	0.3082	0.7875	-
<i>mir-3</i>	0.5	1.30	0.183	0.607	0.44	0.94	0.7172	0.355	-
<i>mir-303</i>	NA	NA	NA	NA	0.37	0.87	0.618	0.444	-
<i>mir-304</i>	0.51	1.21	0.148	0.348	0.63	1.09	0.4171	0.0201,*	1
<i>mir-305</i>	0.17	0.29	0.0012,*	0.006,*	0.38	0.88	0.275	0.100	2
<i>mir-306</i>	0.48	1.12	0.5946	0.5432	0.4	0.76	0.031,*	0.277	1
<i>mir-307</i>	0.39	0.90	0.679	0.453	NP	NP	NP	NP	-
<i>mir-309</i>	0.558	0.96	0.7136	0.2233	0.5	1.15	0.558	0.697	-
<i>mir-310</i>	0.43	0.99	0.975	0.753	0.562	1.21	0.3887	0.3014	-
<i>mir-311</i>	0.577	1.24	0.2932	0.2516	0.36	0.83	0.267	0.013,*	1
<i>mir-312</i>	0.5	1.12	0.324	0.404	0.56	1.20	0.3944	0.4211	-
<i>mir-313</i>	0.48	0.89	0.4309	0.8042	0.47	1.04	0.76	0.926	-
<i>mir-314</i>	0.36	0.81	0.117	0.074,#	0.43	0.80	0.1087	0.2319	0.5
<i>mir-315</i>	0.216	0.40	0.044,*	0.1042	0.54	1.42	0.073,#	0.280	1.5

<i>mir-316</i>	0.5	1.18	0.04,*	0.169	0.363	1.13	0.6688	0.2212	1
<i>mir-317</i>	0.554	1.72	0.0552,#	0.6091	NA	NA	NA	NA	0.5
<i>mir-31a</i>	0.35	0.63	0.049,*	0.017,*	0.36	0.71	0.174	0.329	2
<i>mir-31b</i>	0.48	0.87	0.4475	0.7689	0.45	0.89	0.434	0.900	-
<i>mir-33</i>	0.68	1.33	0.041,*	0.017,*	0.39	0.77	0.3518	0.2893	2
<i>mir-34</i>	0.46	0.96	0.667	0.978	0.4	0.79	0.235	0.0023,*	1
<i>mir-375</i>	0.41	1.07	0.731	0.459	0.2	0.62	0.1192	0.0193,*	1
<i>mir-4</i>	0.63	1.42	0.223	0.1642	0.488	0.18	0.8936	0.9341	-
<i>mir-5</i>	0.49	1.29	0.235	0.681	0.4	0.87	0.4425	0.2058	-
<i>mir-6</i>	0.39	0.82	0.331	0.416	0.48	0.99	0.9828	0.8906	-
<i>mir-7</i>	NA	NA	NA	NA	0.4	0.72	0.144	0.0865,#	0.5
<i>mir-79</i>	0.65	1.19	0.2049	0.0051,*	0.45	0.94	0.435	0.768	1
<i>mir-8</i>	0.34	0.90	0.571	0.122	0.55	0.99	0.9673	0.6606	-
<i>mir-87</i>	0.29	0.69	0.297	0.132	0.24	0.47	0.0232,*	0.0072,*	2
<i>mir-927</i>	0.248	0.77	0.5855	0.1448	NA	NA	NA	NA	-
<i>mir-929</i>	0.47	1.25	0.161	0.766	NP	NP	NP	NP	-
<i>mir-92a</i>	0.67	1.60	0.039,*	0.036,*	0.67	1.76	0.0188,*	0.0331,*	4
<i>mir-92b</i>	0.34	0.71	0.22	0.318	0.5	0.94	0.5431	0.901	-
<i>mir-932</i>	0.56	1.01	0.961	0.4079	NA	NA	NA	NA	-
<i>mir-956</i>	0.71	1.27	0.1714	0.0475,*	0.37	0.97	0.832	0.047,*	2
<i>mir-957</i>	0.42	0.99	0.97	0.664	0.47	0.84	0.4141	0.7352	
<i>mir-958</i>	0.61	1.18	0.3142	0.1068	0.3	0.71	0.056,#	0.040,*	1.5
<i>mir-959</i>	0.48	1.01	0.947	0.778	NA	NA	NA	NA	-
<i>mir-960</i>	0.26	0.50	0.0319,*	0.0301,*	0.44	0.93	0.527	0.647	2
<i>mir-961</i>	0.47	1.00	0.988	0.823	NP	NP	NP	NP	-
<i>mir-962</i>	NA	NA	NA	NA	0.4	0.85	0.145	0.159	-
<i>mir-964</i>	0.45	0.87	0.464	0.817	0.42	0.81	0.3085	0.233	-
<i>mir-965</i>	0.455	0.88	0.4877	0.4379	0.38	0.74	0.147	0.134	-
<i>mir-966</i>	0.32	0.63	0.082,#	0.078,#	0.395	0.99	0.8772	0.2723	1
<i>mir-968</i>	0.53	1.32	0.0149,*	0.3211	0.49	0.95	0.78	0.534	1
<i>mir-969</i>	0.53	1.07	0.746	0.387	0.44	1.10	0.716	0.4788	-
<i>mir-970</i>	0.38	0.96	0.5422	0.061,#	0.5	1.14	0.643	0.747	0.5
<i>mir-971</i>	NA	NA	NA	NA	0.475	1.22	0.4332	0.747	-
<i>mir-972</i>	0.57	1.46	0.0925,#	0.2084	0.47	0.96	0.826	0.829	0.5
<i>mir-973</i>	0.48	0.97	0.837	0.667	0.32	0.82	0.4785	0.0108,*	1
<i>mir-974</i>	0.24	0.48	0.056,#	0.071,#	NA	NA	NA	NA	1
<i>mir-975</i>	0.58	1.49	0.1179	0.3317	0.4	0.79	0.37	0.543	-
<i>mir-976</i>	0.44	1.06	0.697	0.794	0.59	0.94	0.4943	0.0249,*	1
<i>mir-977</i>	0.2	0.31	0.0026,*	0.0242,*	0.35	0.69	0.109	0.126	2

<i>mir-978</i>	NP	NP	NP	NP	0.37	0.58	0.0134,*	0.1026	1
<i>mir-979</i>	0.64	1.14	-	-	0.65	1.28	0.184	0.073,#	0.5
<i>mir-980</i>	0.63	1.31	0.147	0.012,*	0.53	0.95	0.6272	0.4198	1
<i>mir-981</i>	0.22	0.39	0.0279,*	0.0863,#	0.3	0.62	0.087,#	0.017,*	3
<i>mir-982</i>	0.54	1.12	0.558	0.176	0.36	0.64	0.0774,#	0.1994	0.5
<i>mir-983</i>	0.53	0.92	0.697	0.395	0.38	0.59	0.0409,*	0.2836	1
<i>mir-984</i>	0.5	1.03	0.866	0.459	0.62	0.97	0.8062	0.1755	-
<i>mir-985</i>	NA	NA	NA	NA	0.67	1.05	0.8088	0.1056	-
<i>mir-987</i>	NA	NA	NA	NA	0.29	0.69	0.04,*	0.026,*	2
<i>mir-988</i>	0.69	1.64	0.001,*	0.005,*	0.55	0.93	0.4359	0.2986	2
<i>mir-989</i>	0.43	0.73	0.0884,#	0.4264	0.49	1.18	0.232	0.557	0.5
<i>mir-990</i>	0.39	1.01	0.928	0.160	0.39	0.75	0.2965	0.1738	-
<i>mir-991</i>	NA	NA	NA	NA	0.52	1.34	0.147	0.501	-
<i>mir-992</i>	NA	NA	NA	NA	0.32	0.62	0.2369	0.2151	-
<i>mir-994</i>	0.53	0.90	0.622	0.8272	0.49	1.27	0.079,#	0.511	0.5
<i>mir-995</i>	0.52	1.06	0.731	0.374	0.39	0.75	0.2539	0.0469,*	1
<i>mir-996</i>	NA	NA	NA	NA	0.33	0.67	0.18	0.288	
<i>mir-998</i>	0.57	1.15	0.22	0.045,*	0.44	0.85	0.5482	0.4858	1
<i>mir-999</i>	0.58	1.16	0.459	0.4027	0.32	0.64	0.081,#	0.146	0.5
<i>mir-9b</i>	0.28	0.83	0.655	0.204	0.45	0.86	0.4355	0.6464	-
<i>mir-9c</i>	0.26	0.48	0.0388,*	0.0484,*	0.19	0.41	0.018,*	0.051,#	3.5
<i>mir-iab-4as-3p</i>	0.47	1.02	0.5181	0.5382	NA	NA	NA	NA	-
<i>mir-iab-4as-5p</i>	0.76	1.15	0.039,*	0.002,*	0.46	1.00	0.2491	0.636	2

The *attP40* insertion site is located on the second chromosome and *attP2* on the third (see Figure S1A). ^aTarget miRNA: miRNA targeted by the miR-SP. ^bPI: 3 h memory performance index for each miR-SP when expressed in the central nervous system (*elav^{C155}-gal4/+>uas-miR-SP/+* cross). NA: the *uas-miR-SP* construct was not available for this *locus*. NP: no progeny was obtained when the miR-SP was crossed with *elav^{C155}-gal4*. ^cRatio: ratio computed between the 3 h memory performance of each miR-SP cross and the corresponding *scr-miR-SP* control cross. ^d*Scr-miR-SP*: computed p-value when comparing the performance of each *elav^{C155}-gal4/+>uas-miR-SP/+* cross to an *elav^{C155}-gal4/+>uas-scr-miR-SP/+* control cross (two-tailed, two-sample Student t-tests, n=4, *: p<0.05, #: p<0.1). ^eAvg *scr-miR-SP*: computed p-value comparing the performance of each *elav^{C155}-gal4/+>uas-miR-SP/+* assay to the averaged performance of all *elav^{C155}-gal4/+>uas-scr-miR-SP/+* control assays (two-tailed, one-sample Student t-tests, n=4, *: p<0.05, #: p<0.1). ^fScore: simplified presentation of the statistic p-values for the 2 controls and 2 inserts, 1: p-value < 0.05, 0.5: 0.05 < p-value < 0.1 and 0: p-values > 0.1.

Table S2 List of the miRNA-SPs modulating 3 h memory for either and both insertion sites using both statistical approaches for the initial screen

miRNA target	3 h PI
^a <i>attP40</i> (p-value < 0.05)	
<i>miR-100</i>	increased
<i>miR-1002*</i>	increased
<i>miR-1016</i>	increased
<i>miR-275[#]</i>	increased
<i>miR-305</i>	increased
<i>miR-31a</i>	increased
<i>miR-33</i>	increased
<i>miR-92a</i>	increased
<i>miR-960</i>	decreased
<i>miR-977</i>	decreased
<i>miR-988</i>	increased
<i>miR-9c</i>	decreased
<i>miR-iab-4as-3p</i>	increased
^b <i>attP2</i> (p-value < 0.05)	
<i>miR-1002*</i>	decreased
<i>miR-87</i>	decreased
<i>miR-92a</i>	increased
<i>miR-987[#]</i>	decreased
^c <i>attP40</i> and <i>attP2</i> (p-value < 0.1)	
<i>miR-1009</i>	increased
<i>miR-1012</i>	increased
<i>miR-1015</i>	decreased
<i>miR-278</i>	decreased
<i>miR-92a</i>	increased
<i>miR-981</i>	decreased
<i>miR-9c</i>	decreased

^aList of the 13 miR-SP lines inserted into the *attP40* site that significantly modulated 3 h memory using both statistical approaches. ^bThe four miR-SPs lines inserted at the *attP2* site that significantly modulated 3 h memory performance using both statistical approaches.

^cThe seven miR-SP lines showing at least a trend (p-value < 0.1) in the same direction (increasing or decreasing) with both inserts. *Lines showing opposite effects on memory between *attP40* and *attP2* inserts for this screen. [#]Lines for which only one insert was available.

Table S3 Secondary screen for 3 h memory performance by ‘sponging’ 53 miRNAs

a <i>miRNA</i> target	<i>attP40</i> insert				<i>attP2</i> insert				
	bPI	c <i>ratio</i>	p-value		PI	<i>ratio</i>	p-value		f <i>score</i>
			d <i>vs +>miR-SP</i>	e <i>vs avg +>miR-SP</i>			vs <i>+>miR-SP</i>	vs <i>avg +>miR-SP</i>	
<i>mir-100</i>	0.50	1.16	0.5265	0.4043	0.62	1.59	0.001,*	0.0003,*	2
<i>mir-1002</i>	0.51	1.21	0.089,#	0.653	0.43	1.43	0.1800	0.0301,*	1.5
<i>mir-1006</i>	0.53	1.43	0.0965,#	0.2399	0.45	1.96	0.009,*	0.219	1.5
<i>mir-1009</i>	0.64	1.12	0.2980	0.0593,#	0.49	1.36	0.8565	0.0845,#	1
<i>mir-1011</i>	0.65	1.02	0.8930	0.002,*	NA	NA	NA	NA	1
<i>mir-1012</i>	0.40	1.10	0.4344	0.3225	0.65	1.41	0.053,#	0.0063,*	1.5
<i>mir-1013</i>	0.76	1.23	0.008,*	< 0.0001,*	0.46	1.05	0.8526	0.3554	2
<i>mir-1015</i>	0.35	1.10	0.7619	0.2328	0.39	0.76	0.3520	0.990	-
<i>mir-1016</i>	0.76	1.01	0.9640	0.0001,*	0.43	1.53	0.0423,*	0.2688	2
<i>mir-133</i>	0.42	1.05	0.7375	0.8577	NA	NA	NA	NA	-
<i>mir-13a</i>	0.52	1.41	0.2271	0.3156	0.31	0.94	0.7520	0.237	-
<i>mir-13b</i>	NA	NA	NA	NA	0.58	1.29	0.054,#	0.0065,*	1.5
<i>mir-184</i>	0.69	1.35	0.027,*	0.0036,*	0.46	0.96	0.8882	0.1450	2
<i>mir-275</i>	0.59	0.87	0.075,#	0.0228,*	NA	NA	NA	NA	1.5
<i>mir-277</i>	0.62	1.34	0.1468	0.0028,*	0.53	1.15	0.4500	0.0527,#	1.5
<i>mir-278</i>	0.46	0.97	0.9000	0.6831	0.48	1.41	0.088,#	0.134	0.5
<i>mir-279</i>	0.44	0.85	0.2370	0.174	0.55	1.53	0.1649	0.0057,*	1
<i>mir-2811</i>	0.61	1.45	0.045,*	0.156	NA	NA	NA	NA	1
<i>mir-2812</i>	0.48	1.50	0.1840	0.503	NA	NA	NA	NA	-
<i>mir-289</i>	0.65	1.10	0.2420	0.0056,*	NA	NA	NA	NA	1
<i>mir-2b</i>	0.43	0.93	0.6380	0.103	0.44	1.29	0.1228	0.0851,#	0.5
<i>mir-304</i>	0.53	1.23	0.1170	0.533	0.59	1.37	0.0589,#	0.0072,*	1.5
<i>mir-305</i>	0.28	0.52	0.0056,*	0.0099,*	0.31	0.97	0.5400	0.436	2
<i>mir-306</i>	0.53	1.38	0.04,*	0.0639,#	0.52	1.37	0.044,*	0.017,*	3.5
<i>mir-311</i>	0.63	1.54	0.037,*	0.0494,*	0.4	1.60	0.2488	0.6883	2
<i>mir-315</i>	0.43	0.74	0.052,#	0.9665	0.61	1.53	0.005,*	0.0061,*	2.5
<i>mir-316</i>	0.35	0.68	0.1003	0.0152,*	0.69	1.30	0.032,*	0.001,*	3
<i>mir-31a</i>	0.28	0.97	0.9530	0.02156,*	0.26	0.65	0.0494,*	0.1474	2
<i>mir-33</i>	0.46	1.04	0.7900	0.5954	0.41	2.05	0.046,*	0.013,*	2
<i>mir-34</i>	0.51	0.96	0.8450	0.873	NA	NA	NA	NA	-
<i>mir-375</i>	0.53	1.43	0.1396	0.0807,#	0.46	1.12	0.6510	0.323	0.5
<i>mir-79</i>	0.47	0.78	0.3344	0.7082	NA	NA	NA	NA	-
<i>mir-87</i>	0.32	0.73	0.2200	0.0418,*	0.28	0.77	0.3875	0.2055	1

<i>mir-92a</i>	0.52	0.90	0.1350	0.0146,*	0.63	1.75	0.011,*	0.0121,*	3
<i>mir-956</i>	NA	NA	NA	NA	0.43	1.05	0.8941	0.2423	-
<i>mir-958</i>	0.70	1.27	0.016,*	0.0001,*	0.36	0.95	0.6397	0.9632	2
<i>mir-960</i>	0.44	1.19	0.1810	0.217	0.43	0.89	0.6403	0.2905	-
<i>mir-966</i>	0.42	0.93	0.5220	0.0612,#	0.47	1.24	0.4080	0.1763	0.5
<i>mir-968</i>	0.46	1.18	0.1000	0.3568	0.67	1.60	0.015,*	0.0045,*	2
<i>mir-973</i>	0.42	1.10	0.6299	0.8718	0.67	1.52	0.002,*	0.0006,*	2
<i>mir-974</i>	0.27	0.47	0.006,*	0.0116,*	NA	NA	NA	NA	2
<i>mir-976</i>	0.35	1.17	0.2398	0.0714,#	0.72	1.24	0.087,#	0.0016,*	2
<i>mir-977</i>	0.50	1.35	0.3129	0.1088	0.41	0.89	0.5980	0.706	-
<i>mir-978</i>	NA	NA	NA	NA	0.36	1.13	0.7830	0.678	-
<i>mir-980</i>	0.59	1.37	0.057,#	0.0006,*	0.64	1.23	0.06,#	0.0005,*	3
<i>mir-981</i>	0.33	0.83	0.4200	0.0126,*	0.27	1.50	0.3275	0.2551	1
<i>mir-983</i>	0.48	0.96	0.9110	0.302	0.34	1.00	0.9907	0.8315	-
<i>mir-987</i>	NA	NA	NA	NA	0.4	0.90	0.6370	0.5411	-
<i>mir-988</i>	0.59	1.39	0.0378,*	0.037,*	0.28	1.56	0.6110	0.0599,#	2.5
<i>mir-995</i>	0.47	1.28	0.052,#	0.1987	0.44	1.16	0.5190	0.459	0.5
<i>mir-998</i>	0.57	0.84	0.3130	0.0152,*	0.49	1.20	0.3290	0.0724,#	1.5
<i>mir-9c</i>	0.37	0.98	0.9370	0.2351	0.44	2.00	0.015,*	0.401	1
<i>mir-iab-4as-5p</i>	0.58	1.48	0.0098,*	0.0031,*	0.56	1.47	0.014,*	0.0061,*	4

^aTarget miRNA: miRNA targeted by the miR-SP. ^bPI: 3 h memory performance index for each miR-SP when expressed in the central nervous system (*elav^{c155}-gal4/+>uas-miR-SP/+* cross). NA: indicates that the *uas-miR-SP* construct was not available for this locus. ^cRatio: ratio computed between the 3 h memory performances of each *elav^{c155}-gal4/+>uas-miR-SP/+* cross and the corresponding *+/+>uas-miR-SP/+* control cross. ^d*+>miR-SP*: computed p-value when comparing the performance of each *elav^{c155}-gal4/+>uas-miR-SP/+* cross to an *+/+>uas-miR-SP/+* control cross (two-tailed, two-sample Student t-tests, n=6, *: p<0.05, #: p<0.1). ^eAvg *+>miR-SP*: computed p-value comparing the performances of each *elav^{c155}-gal4/+>uas-miR-SP/+* cross to the averaged performance of all *+/+>uas-miR-SP/+* control crosses (two-tailed, one-sample Student t-tests, n=6, *: p<0.05, #: p<0.1). ^fScore: simplified presentation of the statistic p-values for the 2 controls and 2 inserts, 1: p-value < 0.05, 0.5: 0.05 < p-value < 0.1 and 0: p-values > 0.1.

Table S4 List of the miRNA-SPs modulating 3 h memory for each insert with both statistical approaches for the secondary screen

miRNA target	3 h PI
^a <i>attP40</i> (p-value < 0.05)	
<i>miR-1013</i>	increased
<i>miR-184</i>	increased
<i>miR-305</i>	decreased
<i>miR-311</i>	increased
<i>miR-958</i>	increased
<i>miR-974[#]</i>	decreased
<i>miR-988</i>	increased
<i>miR-iab-4as-3p</i>	increased
^b <i>attP2</i> (p-value < 0.05)	
<i>miR-100</i>	increased
<i>miR-306</i>	increased
<i>miR-315</i>	increased
<i>miR-316[*]</i>	increased
<i>miR-33</i>	increased
<i>miR-92a[*]</i>	increased
<i>miR-968</i>	increased
<i>miR-973</i>	increased
<i>miR-iab-4as-5p</i>	increased

^aList of the eight miR-SP lines inserted into the *attP40* site that significantly modulated 3 h memory using both statistical approaches. ^bThe nine miR-SP lines inserted at the *attP2* site that significantly modulated 3 h memory performance using both statistical approaches.

^{*}Lines showing opposite effects on memory between *attP40* and *attP2* inserts. [#]Lines for which only one insert was available.

Table S5 List of the 16 miR-SPs kept for the final screen

target miRNA	attP40 insert					attP2 insert					sum	f _{global score}
	screen 1		screen 2			screen 1		screen 2				
	^a vs scr- <i>miR-SP</i>	^b vs avg <i>scr-miR-SP</i>	^c vs +> <i>miR-SP</i>	^d vs avg <i>+>miR-SP</i>	^e sum	<i>vs scr- miR-SP</i>	<i>vs avg scr- miR-SP</i>	<i>vs +>miR- SP</i>	<i>vs avg +>miR-SP</i>			
<i>mir-100</i>	1	1	0	0	2	0	0	1	1	2	4	
<i>mir-1002</i>	1	1	0.5	0	2.5	1	1	0	1	3	5.5	
<i>mir-13b</i>	NA	NA	NA	NA	-	0	1	0.5	1	2.5	2.5/4	
<i>mir-275</i>	1	1	0.5	1	3.5	NA	NA	NA	NA	-	3.5/4	
<i>mir-289</i>	0.5	1	0	1	2.5	NA	NA	NA	NA	-	2.5/4	
<i>mir-305</i>	1	1	1	1	4	0	0	0	0	0	4	
<i>mir-315</i>	1	0	0.5	0	1.5	0.5	0	1	1	2.5	4	
<i>mir-316</i>	1	0	0	1	2	0	0	1	1	2	4	
<i>mir-31a</i>	1	1	0	1	3	0	0	1	0	1	4	
<i>mir-92a</i>	1	1	0	1	3	1	1	1	1	4	7	
<i>mir-974</i>	0.5	0.5	1	1	3	NA	NA	NA	NA	-	3/4	
<i>mir-980</i>	0	1	0.5	1	2.5	0	0	1	1	1.5	4	
<i>mir-981</i>	1	0.5	0	1	2.5	0.5	1	0	0	2	4.5	
<i>mir-988</i>	1	1	1	1	4	0	0	0	1	1	5	
<i>mir-9c</i>	1	1	0	0	2	1	0.5	1	0	2.5	4.5	
<i>mir-iab-4as-5p</i>	1	1	1	1	4	0	0	1	1	2	6	

For the primary screen, each miR-SP was crossed with *elav^{C155}-gal4* and compared with the control cross using *scr-miR-SP*. For the secondary screen, the cross between *w^{CS10}* flies and each miR-SP was used as a control.

Simplified score for the p-values with 1 = p-value < 0.05; 0.5 = 0.05 < p-value < 0.1 and 0 = p-values > 0.1 when comparing the performances of each *elav^{C155}-gal4/+>uas-miR-SP/+* cross to: ^a*+>uas-scr-miR-SP/+* control, ^b*avg scr-miR-SP*, ^c*+>miR-SP* paired control, and ^dthe avg scores for *+>miR-SP* across the secondary screen. ^eThe sum of the scores for the 2 screens and ^fthe global score which is the sum of all scores for both screen and both inserts. Only lines with scores ≥ 4 were kept (or ≥ 2 when one insert was available) for testing with both controls at the same time.

Table S6 Three-hour memory performance of *miR-31a* predicted gene targets when silenced in the central nervous system using RNAi

Gene Name	^a ID	^b RNAi No. (KK)	3 h PI
<i>CG11247</i>	CG11247	no KK	-
<i>wls</i>	CG6210	103812	0.35±0.09
<i>Egfr</i>	CG10079	107130	no progeny
<i>mid</i>	CG6634	105411	0.44±0.14
<i>repo</i>	CG31240	no KK	-
<i>sha</i>	CG13209	104474	0.47±0.11
<i>CG1136</i>	CG1136	107455	0.49±0.04
<i>CG16947</i>	CG16947	108103	0.35±0.14
<i>CG17390</i>	CG17390	no iRNA	-
<i>mask</i>	CG33106	103411	0.35±0.03
<i>CG7852</i>	CG7852	103582	no progeny
<i>CG13287</i>	CG13287	109806	0.2±0.07*
<i>CG30429</i>	CG30429	103389	0.23±0.17
<i>cpo</i>	CG31243	no KK	-
<i>E(bx)</i>	CG32346	109831	0.27±0.07
<i>Hr38</i>	CG1864	104178	0.54±0.11*
<i>lin</i>	CG11770	100039	0±0.05*
<i>qkr54B</i>	CG4816	100702	0.53±0.03*
<i>Sb</i>	CG4316	108455	0.24±0.01
<i>CG14223</i>	CG14223	no KK	-
<i>CG14767</i>	CG14767	105373	0.55±0.04*
<i>CG14837</i>	CG14837	107466	0.19±0.06*
<i>CG18641</i>	CG18641	102771	0.17±0.11*
<i>CG32446</i>	CG32446	104437	0.35±0.07
<i>CG3280</i>	CG3280	no KK	-
<i>CG8757</i>	CG8757	102128	0.61±0.03*
<i>CG9698</i>	CG9698	100678	0.29±0.05
<i>ago</i>	CG15010	100356	0.56±0.04
<i>Awh</i>	CG1072	108409	0.22±0.09*
<i>Best2</i>	CG10173	no KK	-
<i>chn</i>	CG11798	108695	0.28±0.05
<i>ewg</i>	CG3114	no KK	-
<i>MTA1-like</i>	CG2244	110632	0.26±0.002
<i>Osi7</i>	CG1153	100174	0.27±0.05
<i>pgant6</i>	CG2103	106203	0.46±0.08

<i>Pp1-87B</i>	CG5650	no KK	-
<i>puc</i>	CG7850	no KK	-
<i>Tsp42El</i>	CG12840	107538	0.19±0.04*
<i>CG10326</i>	CG10326	104790	0.57±0.1*
<i>CG1103</i>	CG1103	no iRNA	-
<i>CG13046</i>	CG13046	no KK	-
<i>CG14082</i>	CG14082	no KK	-
<i>CG14304</i>	CG14304	106221	0.54±0.06*
<i>CG14961</i>	CG14961	106865	no progeny
<i>CG14995</i>	CG14995	109283	0.58±0.14*
<i>CG15544</i>	CG15544	no KK	-
<i>CG15822</i>	CG15822	100293	0.38±0.1
<i>CG17104</i>	CG17104	107804	0.45±0.03
<i>CG2121</i>	CG2121	109845	0.39±0.08
<i>CG32044</i>	CG32044	no iRNA	-
<i>CG34441</i>	CG34441	103649	0.44±0.09
<i>CG4615</i>	CG4615	106673	0.62±0.05*
<i>CG5021</i>	CG5021	105709	0.46±0.05
<i>CG5869</i>	CG5869	101994	0.16±0.06*
<i>CG6118</i>	CG6118	102587	0.34±0.09
<i>CG7369</i>	CG7369	100824	0.33±0.11
<i>CG9636</i>	CG9636	no KK	-

^aID: The CG ID for each of the 57 *mRNAs* predicted by TargetScan to be *miR-31a* targets. ^bThe RNAi transformant ID in the KK library from the Vienna Drosophila RNAi Center targeting the gene listed. No RNAi line from the KK library was available for 16 of the predicted targets. The 3 h performance score (PI) of each individual *uas-RNAi* when crossed with the *uas-dcr2;n-syb-gal4* driver. *Indicate the lines for which the PI deviated from the mean by more than one standard deviation. These lines were selected for a second, independent test. Results are the mean ± standard error of the mean with n=4.

Table S7 *MiR-31a* and *miR-974* potential gene targets repeatedly altering 3 h memory when silenced in the CNS

Gene Name	^a ID	^b RNAi No (KK)	3 h PI	Molecular Function	Biological Process	^c Collapsed wing phenotype
<i>miR-31a</i>						
<i>CG13287</i>	CG13287	109806	-0.03±0.18	sequence-specific DNA binding transcription factor activity	transcription	no
<i>lin</i>	CG11770	100039	0.07±0.03	unknown	system development	yes
<i>CG18641</i>	CG18641	102771	0.09±0.07	lipase activity	lipid metabolic process	no
<i>CG5869</i>	CG5869	101994	0.21±0.032	actin binding	gliogenesis	yes
<i>mir-974</i>						
<i>Fas1</i>	CG6588	101779	0.17±0.05	molecule binding	cell adhesion	yes

Four of the 38 potential gene targets for *mir-31a* repeatedly altered 3 h when silenced in the CNS using RNAi. One of the 16 potential targets of *miR-974* altered 3 h memory. Three hours memory scores failed under our criterion (mean $\pm$ one standard deviation) after two independent memory tests. ^aID: The CG ID for each of the mRNAs predicted by TargetScan to be *miRNA* targets. ^bThe RNAi transformant ID in the KK library from the Vienna Drosophila RNAi Center targeting the gene listed. The 3 h performance score (PI) of each individual *uas-RNAi* when crossed with the *uas-dcr2;n-syb-gal4* driver. Results are the mean $\pm$ standard error of the mean with n=4. ^cRNAi lines exhibiting a collapsed wing phenotype when crossed with the *uas-dcr2;n-syb-gal4* driver.

Table S8 Three-hour memory performance of *miR-974* potential gene targets when silenced in the central nervous system using RNAi

Gene Name	^a ID	^b RNAi No. (KK)	3 hr PI
<i>cpo</i>	CG31243	no KK	-
<i>Fas1</i>	CG6588	101779	0.11±0.069*
<i>Ptp61F</i>	CG9181	108888	0.57±0.068*
<i>CG14306</i>	CG14306	106368	0.33±0.141
<i>CG6739</i>	CG6739	108197	0.19±0.034*
<i>Dnr1</i>	CG12489	106453	0.44±0.137
<i>Elongin-B</i>	CG4204	101542	0.39±0.05
<i>Fmr1</i>	CG6203	110800	0.19±0.031*
<i>Sec61alpha</i>	CG9539	109660	-
<i>CG13958</i>	CG13958	no KK	-
<i>CG40577</i>	CG40577	no RNAi	-
<i>grh</i>	CG5058	109135	0.4±0.167
<i>kek3</i>	CG4192	no KK	-
<i>nAcRalpha-30D</i>	CG4128	101571	0.51±0.07*
<i>Takl2</i>	CG4803	104701	0.4±0.06
<i>CG32970</i>	CG32970	101561	0.27±0.15

^aID: The CG ID for each of the 16 *mRNAs* predicted by TargetScan to be *miR-974* targets.

^bThe RNAi transformant ID in the KK library from the Vienna Drosophila RNAi Center targeting the gene listed. No RNAi line from the KK library was available for five of the predicted targets. The 3 h performance score (PI) of each individual *uas-RNAi* when crossed with the *uas-dcr2;n-syb-gal4*; driver. *Indicates the lines for which the PI deviated from the mean by more than one standard deviation. These lines were selected for a second, independent test. Results are the mean ± standard error of the mean with n=4.