SUPPLEMENTAL RESULTS

Figure S1. Odor and Shock Avoidance for *miR-980SP* and *miR-980* Overexpression Flies, Related to Figure 1 and Figure 2

Odor and shock avoidances represent the behavioral output of computations that occur in multiple areas of the brain. The use of drivers with restricted expression in the brain may produce either a gain or loss in the behavior being measured. Broadly expressing drivers may also produce a gain or loss in the behavior being measured or alternatively, no change, if per chance a gain produced by one restricted area is counterbalanced by a loss in another. Very specific mushroom body drivers like *R13F02-GAL4* likely have no effect since ablation of these cells does not alter odor or shock avoidance behavior (de Belle and Heisenberg, 1994).

(A) The *c155-GAL4>UAS-miR-980SP* flies avoided ben, oct and electric shock pulses at similar avoidance indices as the *c155>UAS-scrambled* flies *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6.

(B) *NP3544* flies avoided ben, oct and electric shock pulses at similar levels to *wCS10* control. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6.

(C) The *GH146-GAL4>UAS-miR-980SP* flies avoided ben and electric shock pulses at similar avoidance indices as the *GH146-GAL4>UAS-scrambled* flies. They showed a higher oct avoidance index compared to the control. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6. $p<0.01$ for oct avoidance.

(D) *MiR-980* inhibition using MB driver, *OK107-GAL4* produced increased odor and shock sensitivity. The *OK107-GAL4>UAS-miR-980SP* flies exhibited increased ben, oct and shock sensitivity compared to the scrambled control. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with $n\geq6$. $p<0.001$ for *OK107-GAL4>UAS-miR-980SP* ben avoidance; $p<0.01$ for *OK107-GAL4>UAS-miR-980SP* electric shock avoidance; $p<0.05$ for *OK107-GAL4>UAS-miR-980SP* oct avoidance.

(E) *MiR-980* inhibition using MB driver, *238Y-GAL4,* exhibited ben and electric shock pulses at similar avoidance indices as the scrambled flies. However, avoidance of oct was increased compared to the control. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6. $p<0.01$ for *238Y>UAS-miR-980SP* oct avoidance.

(F) *R13F02-GAL4>UAS-miR-980SP* flies avoided ben, oct and electric shock pulses at similar levels to *R13F02-GAL4>UAS-scrambled* flies using both working and 10-fold diluted odor concentrations,

and 90V and 30V shock intensities. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6.
(G) *R13F02-GAL4>UAS-miR-980* flies avoided ben, oct and electric shock pulses at similar levels to either *UAS*-only and *GAL4*-only controls. *Statistics:* Scores were analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* tests. Data are the mean ± SEM with n=6. $p<0.01$ for ben avoidance of *UAS-miR-980/+* control and shock avoidance of *R13F02-GAL4/+* control. Increased ben or shock sensitivity compared to *UAS/+* or *GAL-4/+* controls does not explain the impaired memory of *miR-980* overexpression flies.

Figure S2. Three-Hour Memory of *miR-980SP* Flies Driven by *Gad-GAL4* and *R31F10-GAL4* *A*fter Three Shock Training, Related to Figure 2
(A) *Gad-GAL4>UAS-miR980SP* flies were trained with 3 shocks to avoid ceiling level scores and failed to show a 3h memory phenotype compared to the scrambled control. *Statistics:* PIs were analyzed by two-tailed, two-sample Student's t-test for each condition. Data are the mean ± SEM with n=6.
(B) *R31F10-GAL4>UAS-miR980SP* (optic lobe driver) flies were trained with 3 shocks to avoid ceiling level scores and failed to show a 3h memory phenotype compared to the scrambled control. *Statistics:* PIs were analyzed by two-tailed, two-sample Student's t-test for each condition. Data are the mean ± SEM with n=6.

Figure S3. A2bp1 Expression in Fly Heads, Related to Figure 4, Figure 5 and Figure 6
(A) Ant*i*-A2bp1 western blotting from *nSyb-GAL4>UAS-dcr-2* heads, which served as control for experiments with *A2bp1-RNAi* flies (Figure 5B). The protein band showing a reduction in signal with RNAi-expression (Figure 5B) is identified by an arrow and corresponds to an apparent mass of 105kD. The protein migrating at ~38kD did not exhibit abundance changes with expression of the *A2bp1-RNAi*.
(B) *A2bp1* RT-PCR analyses from whole heads using primers from the first and last exons of the annotated largest isoforms (isoforms RL and RH). The resulting PCR product of ~2.9kb was cloned into *pUAST* and 19 independent clones were isolated and sequenced. Six different splice variants were detected as indicated by the chart. The *A2bp1* gene has 20 annotated exons with 6 different splice variants detected by our analyses. The 6 splice variants we identified are novel. The number of independent clones identified for each variant is listed below the chart. The first splice variant indicated with a red arrow was used to construct *UAS-A2bp1 RN* flies. Although not detected in our

analysis, two annotated largest isoforms, RL and RH are shown for comparison. The exon-intron structure of isoform RE, which improved memory when overexpressed, is also depicted.
(C) *R13F02-GAL4>UAS-A2bp1RNAi; UAS-dcr-2* flies avoided ben, oct and electric shock pulses at similar levels as *R13F02-GAL4>UAS-dcr-2* flies. *Statistics:* Scores were analyzed by two-tailed, two-sample Student's t-tests for each condition. Data are the mean ± SEM with n=6.
(D) *R13F02-GAL4;GAL80^{ts}>UAS-A2bp1* animals avoided ben, oct and electric shock at similar levels to GAL4-only and UAS-only controls when A2bp1 is overexpressed in adults. *Statistics:* Scores were analyzed by one-way ANOVA followed by Bonferroni's *post-hoc* tests. Data are the mean ± SEM with n=6.

Figure S4. A2bp1 Overexpression Detected by Western Blotting and Immunohistochemistry, Related to Figure 6
(A) Representative full size anti-A2bp1 western blots. This blot illustrates the decrease/increase in expression of proteins of ~105 and ~125 kD with expression of *A2bp1 RNAi* or the *miR-980SP,* respectively. Proteins that migrate at ~37 and ~105 kD appeared routinely on all blots. In addition, proteins that migrated at ~74 and ~45 kD irregularly (see also Figure S3A) exhibited immunoreactivity, sometimes robustly as illustrated by the ~74kD protein shown in some lanes on this blot. The ~74kD protein immunoreactivity tended to follow the *nSyb-GAL4* driver for reasons that are unknown.
(B) Representative anti-A2bp1 and anti-α-tubulin western blots from *R13F02-GAL4;tub-GAL80ts>UAS-A2bp1-RN* fly heads raised at 18°C during development and at 30°C during adulthood. Shown below is the quantification of the A2bp1 protein change compared to GAL4-only and UAS-only controls. Each lane was normalized to the α-tubulin signal. Overexpressing A2bp1-RN in the MB with *R13F02-GAL4* produced a 4-fold increase over endogenous A2bp1 levels. *Statistics:* Protein levels were analyzed using one-way ANOVA followed by Bonferroni's *post-hoc* tests. PIs are the mean ± SEM with n=4. $p<0.0001$.
(C) Representative images of A2bp1 overexpression in adult MB. Single section images of the central brain stained with anti-A2bp1 (green) and anti-Dmef2 (magenta) antibodies from *R13F02-GAL4; tub-GAL80ts>UAS-A2bp1-RN* and *R13F02-GAL4; tub-GAL80ts>UAS-A2bp1-RE* flies raised at 18°C during development and at 30°C during adulthood. *R13F02-GAL4; tub-GAL80ts /+* and *UAS-A2bp1/+* brains were used as controls. MBn are outlined with a yellow dotted line based on the Dmef2 signal and mean signal intensity for the region of interest was measured. The ratio of A2b*p*1 to Dmef2 signal intensity was calculated and shown on the histogram below. Overexpressing A2bp1 in MBn increased the A2bp1/Dmef2 ratio ~2-3 fold for the RE and RN isoforms. *Statistics:* The data were analyzed

using one-way ANOVA followed by Bonferroni's *post-hoc* tests. Data are the mean ± SEM with n=5. $p<0.001$.

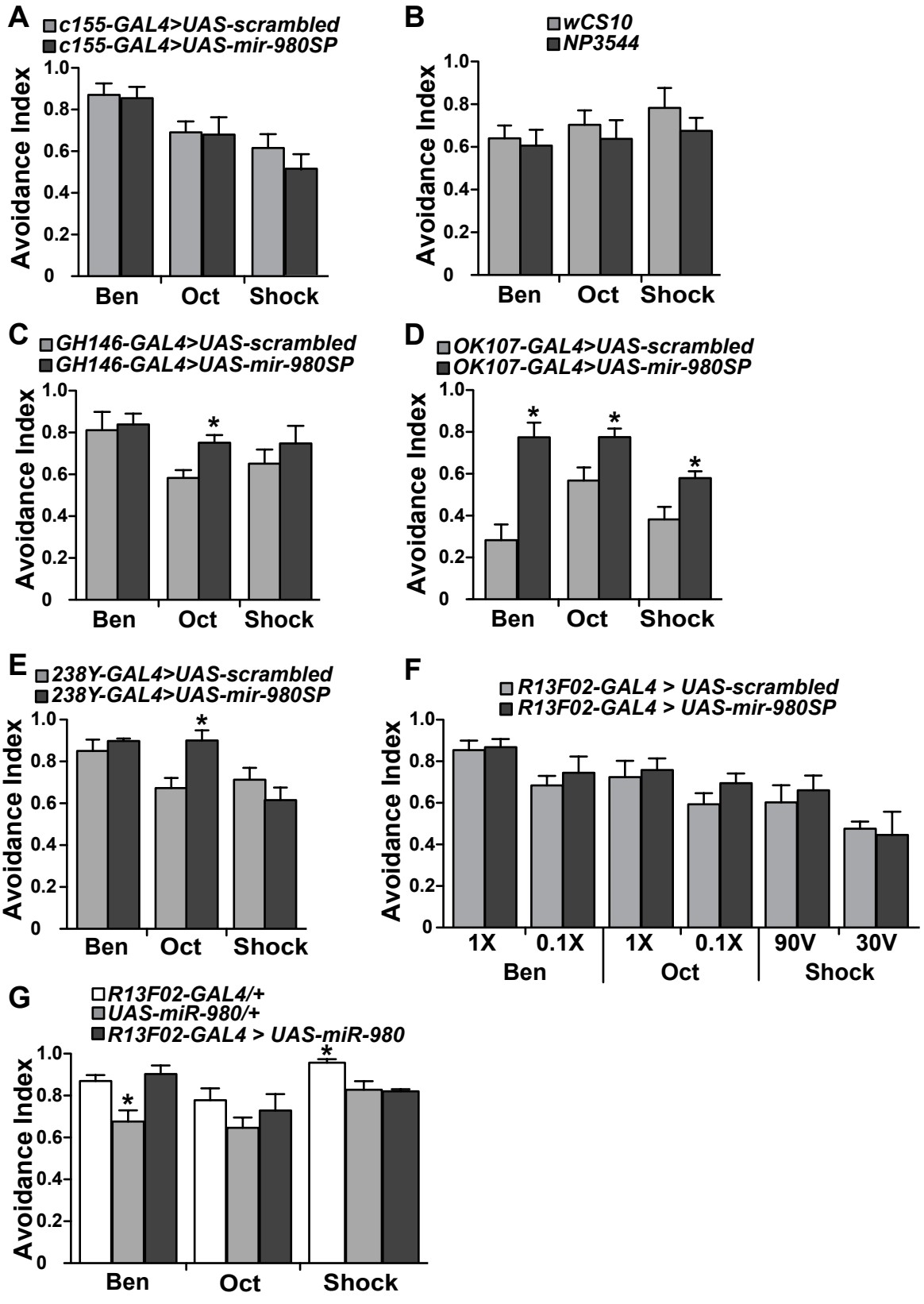

Figure S1
Guven-Ozkan et al

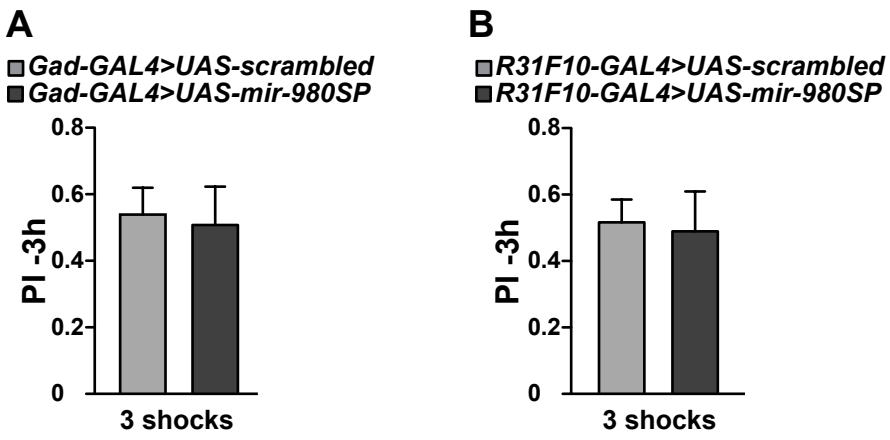

Figure S2
Guven-Ozkan et al

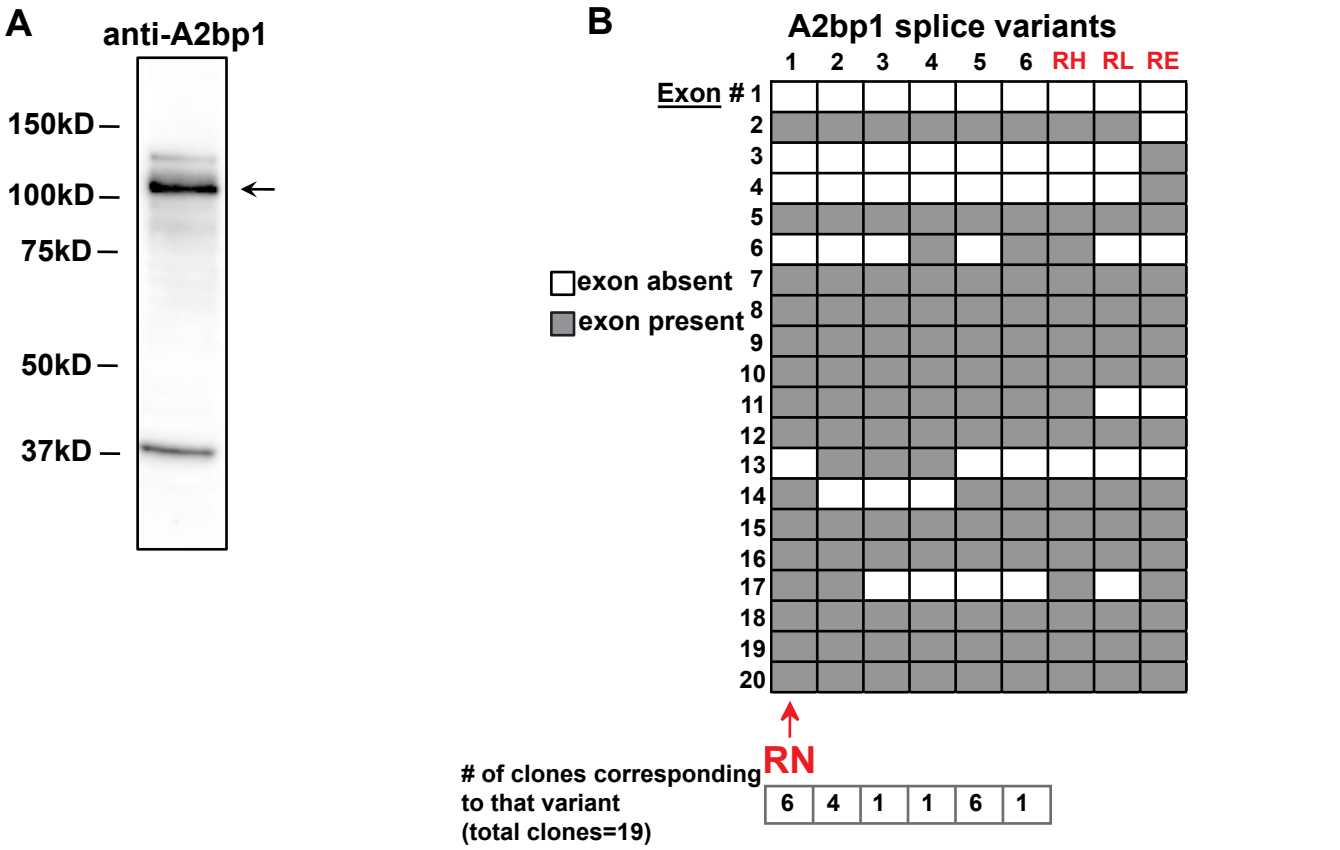

Figure S3
Guven-Ozkan et al

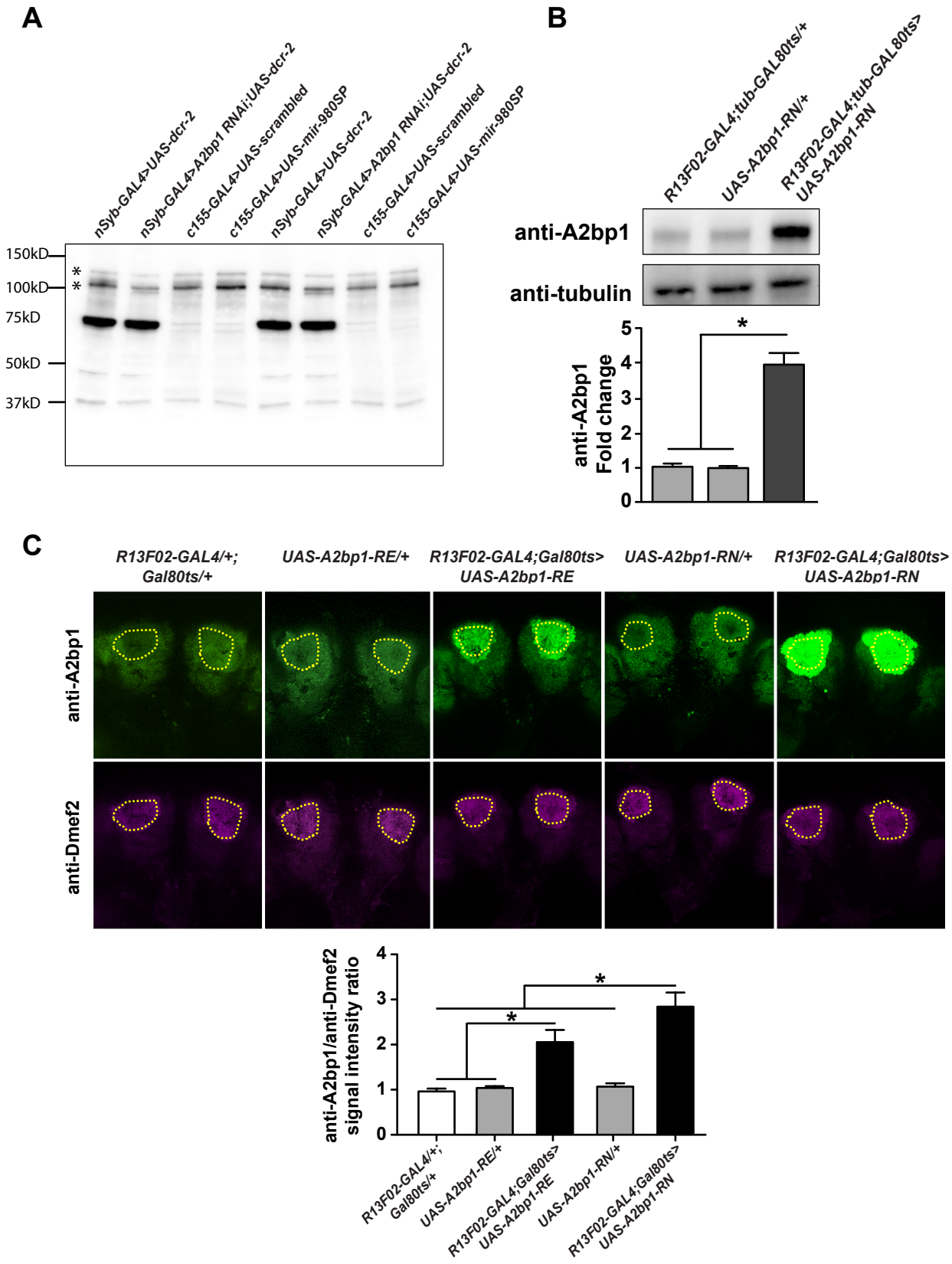

Figure S4
Guven-Ozkan et al

Table S1. Putative *miR-980* Target Genes, Related to Figure 4

Symbol	ID	KK line
CG34135	CG34135	103154
A2bp1	CG32062	110518
Cp19	CG6524	110305
Cpr76Bb	CG9290	106797
mthl6	CG16992	108048
slmb	CG3412	107825
CG32834	CG32834	100964
jeb	CG30040	103047
Krn	CG32179	104299
CG13407	CG13407	108195
CG2269	CG2269	100231
CG14443	CG14443	105254
otk	CG8967	104688
Cks30A	CG3738	108401
Cpr35B	CG3474	102492
Pdp1	CG17888	110551
trn	CG11280	107883
vvl	CG10037	110723
CG15262	CG15262	104163
CG3630	CG3630	110512
CG9392	CG9392	108024
Ate1	CG9204	104360
Chd1	CG3733	103640
Cp38	CG11213	106008
enc	CG10847	101500
hts	CG9325	103631
Osi21	CG14925	100032
ppa	CG9952	100298
sens	CG32120	106028
Syx17	CG7452	108825
tey	CG8780	106065
CG12054	CG12054	104777
CG13685	CG13685	107228
CG15756	CG15756	105695
CG2811	CG2811	107018
CG31221	CG31221	103017
CG3884	CG3884	109135
CG4793	CG4793	109919
CG8668	CG8668	110703
CG9426	CG9426	103746
CG1832	CG1832	108039
fruitless	CG14307	105005
Mical	CG33208	105837
CG7766	CG7766	110184

calypso	CG8445	107757
Cad96Cb	CG13664	103296
CG7603	CG7603	100911
bru-2	CG43065	104949
CG14402	CG14402	104749
CG13602	CG13602	100439
CG4334	CG4334	106785
CG7386	CG7386	100364
Rbp9	CG3151	101412
shutdown	CG4735	105832
Tsp26A	CG9093	101473
CG18266	CG18266	102675
Socs44A	CG2160	102764
Cdep	CG31536	104438

Candidate mRNA targets of *miR-980* were screened for a role in *Drosophila* memory formation using an RNAi approach (Walkinshaw et al., 2015). RNAi lines for 63 genes predicted by TARGETSCAN and *microrna.org* were crossed to the *nSyb-GAL4* driver and screened by four experimenters as part of large, RNAi screening project with 3h memory as an endpoint (Walkinshaw et al., 2015). The 58 lines that were tested are shown. The KK line number is from the Vienna *Drosophila* RNAi Center (http://stockcenter.vdrc.at/control/main).

Table S2. Three-Hour Memory Screen for Memory Specific *miR-980* Targets, Related to Figure 4

Gene Name	Flybase ID	KK line	PI for *R13F02-GAL4* screen	*R13F02-GAL4* retest	p value
A2bp1	CG32062	110518	0.42 ± 0.04	0.36 ± 0.04	0.001
CG32834	CG32834	100964	0.53 ± 0.04		
jeb	CG30040	103047	0.52 ± 0.08		
CG13407	CG13407	108195	0.56 ± 0.04		
otk	CG8967	104688	0.60 ± 0.05		
Cks30A	CG3738	108401	0.55 ± 0.04		
CG3630	CG3630	110512	0.38 ± 0.04	0.36 ± 0.04	0.01
Cp38	CG11213	106008	0.49 ± 0.05	0.44 ± 0.04	0.01
Osi21	CG14925	100032	0.56 ± 0.05		
sens	CG32120	106028	0.43 ± 0.06	0.49 ± 0.05	
CG2811	CG2811	107018	0.66 ± 0.07		
fruitless	CG14307	105005	0.57 ± 0.06		
calypso	CG8445	107757	0.60 ± 0.03		
CG13602	CG13602	100439	0.56 ± 0.02		
CG7386	CG7386	100364	0.61 ± 0.02		
CG18266	CG18266	102675	0.43 ± 0.10		
Gug	CG6964	107413	0.65 ± 0.10		
Lar	CG10443	107996	0.70 ± 0.07		
CG14402	CG14402	104749	0.40 ± 0.04		
CG1832	CG1832	108039	0.60 ± 0.06		

Potential *miR-980* candidate target genes from the *nSyb-GAL4* RNAi screen were re-screened using the restrictive mushroom body driver, *R13F02-GAL4*. Eighteen lines from the primary screen plus two additional lines that failed to produce any progeny with *nSyb-GAL4* were first tested using an n=4 replicates. Each individual RNAi line was compared to a daily *R13F02>UAS-dcr-2* control. The average performance index for the *R13F02-GAL4>UAS-dcr-2* control was 0.57 across the screen (n=42, SEM=0.02). Four lines with a significant difference or a strong trend compared to daily control were re-tested with 6 replicates. Three of the 20 initial RNAi lines had PIs significantly lower than the daily control and are highlighted with yellow (n=10 after combining primary screen scores with re-test). *Statistics:* Two-tailed, two-sample Student's t-tests. Results shown are the mean PIs with ± SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Fly stocks used in the current study

Line	Source	Reference
UAS-MiR980SP	Van Vactor Lab (Harvard)	Loya et al., 2009; Fulga et al., 2015
UAS-scrambled	Van Vactor Lab (Harvard)	Loya et al., 2009; Fulga et al., 2015
P[GawB]CG3777^{NP3544} (backcrossed to wCS10 for 6 generations)	Kyoto Drosophila Genetic Resource Center	
UAS-miR-980	Lai Lab (Sloan Kettering)	Bejarano et al., 2012
UAS-A2bp1-RE	Shashidhara Lab (India)	Usha and Shashidhara, 2010
UAS-RNAi lines	VDRC	
UAS-dcr-2		Dietzl et al., 2007
UAS-GCaMP3		Tian et al., 2009
UAS-mCD8::GFP		Lee and Luo, 1999
tub-GAL80ts		McGuire et al., 2003
c155-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-Grelin et al., 2003
Or83b-GAL4		Kreher et al., 2005
NP2492-GAL4		Tanaka et al., 2008
Gad-GAL4		Ng et al., 2002
R13F02-GAL4		Pfeiffer et al., 2008
R31F10-GAL4		Jenett et al., 2012
nSyb-GAL4		Pauli et al., 2008

We generated the control line for the *A2bp1 RNAi; miR-980SP* epistasis experiment. *UAS-A2bp1 RNAi* and *UAS-miR-980SP* transgenes were combined with *UAS-scrambled* transgenes to normalize the number of *UAS* containing elements between experimental and control groups. To generate the *UAS-A2bp1RN* flies, we first prepared with reverse transcription a 2.9 kb cDNA fragment using head RNA. The primers used for this were: forward–GCCACGTCCTCGAGATGTCTGCGTCGGCAGTTGAAG, and reverse–CAGCTAGGTCTAGATTAATATGGCGTGAAACGATTG. The resulting product included *XhoI* and *XbaI* restriction sites for cloning into the pUAST vector (Brand and Perrimon, 1993). Nineteen clones were sequence verified and one representing the

A2bp1RN isoform was injected into embryos from the *wCS10* background. Fifteen transformants were obtained. Data presented in Figure 6A were generated using *UAS-A2bp1RN* line #3.

Behavior

Flies were exposed to 1min of CS+ odor paired with 12 electric shock pulses (90V, 1.25s) followed by 30s of air and 1min of the CS- odor. The odorants used were benzaldehyde (ben) and 3-octanol (oct). Memory was tested using a T-maze, which delivers CS+ from one arm, and CS- from the other. For TARGET experiments, flies were transferred to the 25°C behavioral room 30min prior to training and testing. For acquisition experiments, flies were exposed to 1, 2, 3, 4, 6 or 12 shock pulses evenly distributed during the 1min CS+. This was followed by 30s of fresh air and 1min of the CS- odor. Memory was tested immediately after training. Odor avoidance was tested by allowing naïve flies to choose between CS+ and CS- odors in T-maze for 2min. Shock avoidance was performed in a T-maze with one arm containing an electrified copper grid (one pulse every 5s) and one arm containing a non-electrified copper grid during 2min air exposure.

Immunohistochemistry

Two-to-five day old female fly brains were dissected in 1X PBS and transferred to 1% paraformaldehyde in PBS. We followed the protocol described by Fly Light Project (Jenett et al., 2012). Antibodies used were guinea pig anti-A2bp1 (1:5000, Tastan et al., 2010), rabbit anti-GFP (1:1000, Invitrogen), rabbit anti-Dmef2 (1:1000, gift from E. Olson), goat anti-guinea pig IgG (1:500, Alexa 633 or Alexa 488, Invitrogen) and goat anti-rabbit IgG (1:800, Alexa 488 or Alexa 633, Invitrogen). Images were obtained using Leica TCS SP5 or SP8 confocal microscopes. Regions of interest (ROI) were defined for *A2bp1 RNAi* and *miR-980SP* flies around the central brain by excluding the optic lobes. Mean signal intensities of maximum projection images were measured using ImageJ and compared to respective controls for statistical analyses. ROI for *UAS-miR-980, UAS-A2bp1RE* and *UAS-A2bp1RN* overexpression flies were defined around Kenyon cell bodies using Dmef2 co-labeling. Mean

signal intensities for the ROI of single slice images were measured for both anti-A2bp1 using ImageJ.

Western Blotting

Fifty fly heads were homogenized in 1% Triton-100 in Tris-buffered saline with protease inhibitors (Thermo Scientific) and mixed with 2X sample buffer. Western blotting was performed using standard procedures with the following antibodies: guinea pig anti-A2bp1 (1:5000, Tastan et al., 2010), mouse anti-α-tubulin (1:10000, Sigma, 1:1000, Abcam), HRP conjugated anti-guinea pig IgG (1:10000), and HRP conjugated anti-mouse IgG (1:10000). The intensity of the anti-A2bp1 signal was first normalized to the anti-tubulin signal, and then the experimental group was normalized to the control group using ImageJ for quantification.

SUPPLEMENTAL REFERENCES

Bejarano, F., Bortolamiol-Becet, D., Dai, Q., Sun, K., Saj, A., Chou, Y.T., Raleigh, D.R., Kim, K., Ni, J.Q., Duan, H.*, et al.* (2012). A genome-wide transgenic resource for conditional expression of *Drosophila* microRNAs. Development *139*, 2821-2831.

Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T., and O'Kane, C.J. (1996). Associative learning disrupted by impaired Gs signaling in *Drosophila* mushroom bodies. Science *274*, 2104-2107.

Dietzl, G., Chen, D., Schnorrer, F., Su, K.C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S.*, et al.* (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. Nature *448*, 151-156.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., and Birman, S. (2003). Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. J Neurobiol. *54*, 618-627.

Fulga, T.A., McNeill, E.M., Binari, R., Yelick, J., Blanche, A., Booker, M., Steinkraus, B.R., Schnall-Levin, M., Zhao, Y., DeLuca, T., Bejarano, F., Han, Z., Lai, E.C., Wall, D.P., Perrimon, N., and Van Vactor, D. (2015). A transgenic resource for conditional competitive inhibition of conserved *Drosophila* microRNAs. Nature communications. 6, 7279.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D., and Strausfeld, N.J. (1998). The organization of extrinsic neurons and their implications in the functional

roles of the mushroom bodies in *Drosophila melanogaster* Meigen. Learn Mem. *5*, 52-77.

Kreher, S.A., Kwon, J.Y., and Carlson, J.R. (2005). The molecular basis of odor coding in the *Drosophila* larva. Neuron *46*, 445-456.

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

Lin, D.M., and Goodman, C.S. (1994). Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron *13*, 507-523.

Loya, C.M., Lu, C.S., Van Vactor, D., and Fulga, T.A. (2009). Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Methods. *6*, 897-903.

Jenett, A., Rubin, G.M., Ngo, T.T., Shepherd, D., Murphy, C., Dionne, H., Pfeiffer, B.D., Cavallaro, A., Hall, D., Jeter, J.*, et al.* (2012). A GAL4-driver line resource for *Drosophila* neurobiology. Cell Rep. *2*, 991-1001.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in *Drosophila*. Science *302*, 1765-1768.

Ng, M., Roorda, R.D., Lima, S.Q., Zemelman, B.V., Morcillo, P., and Miesenböck, G. (2002). Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly. Neuron *36*, 463-474.

Pauli, A., Althoff, F., Oliveira, R.A., Heidmann, S., Schuldiner, O., Lehner, C.F., Dickson, B.J., and Nasmyth, K. (2008). Cell-type-specific TEV protease cleavage reveals cohesin functions in *Drosophila* neurons. Dev Cell. *14*, 239-251.

Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.T., Misra, S., Murphy, C., Scully, A., Carlson, J.W., Wan, K.H., Laverty, T.R.*, et al.* (2008). Tools for neuroanatomy and neurogenetics in *Drosophila*. Proc Natl Acad Sci U S A. *105*, 9715-9720.

Stocker, R.F., Heimbeck, G., Gendre, N., and de Belle, J.S. (1997). Neuroblast ablation in *Drosophila* P[GAL4] lines reveals origins of olfactory interneurons. J Neurobiol. *32*, 443-456.

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

Tian, L., Hires, S.A., Mao, T., Huber, D., Chiappe, M.E., Chalasani, S.H., Petreanu, L., Akerboom, J., McKinney, S.A., Schreiter, E.R.*, et al.* (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. Nat Methods. *6*, 875-881.

Waddell, S., Armstrong, J.D., Kitamoto, T., Kaiser, K., and Quinn, W.G. (2000). The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. Cell *103*, 805-813.

Usha, N., and Shashidhara, L.S. (2010). Interaction between Ataxin-2 Binding Protein 1 and Cubitus-interruptus during wing development in *Drosophila*. Dev Biol. *341*, 389-399.