Behavioral/Cognitive

MicroRNA-276a Functions in Ellipsoid Body and Mushroom Body Neurons for Naive and Conditioned Olfactory Avoidance in *Drosophila*

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MicroRNA (miRNA)-mediated gene regulation plays a key role in brain development and function. But there are few cases in which the roles of individual miRNAs have been elucidated in behaving animals. We report a miR-276a::DopR regulatory module in *Drosophila* that functions in distinct circuits for naive odor responses and conditioned odor memory. *Drosophila* olfactory aversive memory involves convergence of the odors (conditioned stimulus) and the electric shock (unconditioned stimulus) in mushroom body (MB) neurons. Dopamine receptor DopR mediates the unconditioned stimulus inputs onto MB. Distinct dopaminergic neurons also innervate ellipsoid body (EB), where DopR function modulates arousal to external stimuli. We demonstrate that miR-276a is required in MB neurons for memory formation and in EB for naive responses to odors. Both roles of miR-276a are mediated by tuning DopR expression. The dual role of this miR-276a::DopR genetic module in these two neural circuits highlights the importance of miRNA-mediated gene regulation within distinct circuits underlying both naive behavioral responses and memory.

Introduction

MicroRNAs (miRNAs) are small RNAs (~21–23 nt long) thought to regulate as many as 50% of genes at the post-transcriptional level by binding to complementary sequences in target mRNAs (Bartel, 2009). MiRNA-mediated regulation has emerged as a key mechanism governing synaptic plasticity (Schratt et al., 2007). We demonstrate a role for miR-276a in *Drosophila* for both naive responses to odors and for olfactory memories. We focused on this particular miRNA gene because it maps nearby to one of the mutations identified from a forward mutagenesis screen for memory defects (Dubnau et al., 2003). By manipulating spatial and temporal function of this miRNA, we uncovered a complex role in both naive and conditioned odor responses. We also demonstrate that DopR, a type-one dopamine receptor, is a functional downstream effector of miR-276a.

Pavlovian olfactory conditioning in *Drosophila* has provided a powerful system to investigate genetic and circuit mechanisms of memory (Margulies et al., 2005; Keene and Waddell, 2007; Busto et al., 2010). A model has emerged in which mushroom body (MB) neurons integrate odor conditioned stimulus (CS) inputs with neuromodulatory unconditioned stimulus inputs. For aversive learning, the unconditioned stimulus information is mediated by several characterized dopaminergic neurons (Schwaerzel et al., 2003; Claridge-Chang et al., 2009; Krashes et al., 2009; Aso et al., 2010) projecting onto MB neurons. Formation of all stages of aversive olfactory memory (short, middle, and long-term) requires DopR expression in MB (Kim et al., 2007a; Qin et al., 2012). However, long-term memory (LTM) involves a broader neural circuit because CREB-mediated gene expression is required outside MB, in dorsal-anterior-lateral neurons that send inputs to MB (Chen et al., 2012), and NMDA-receptor function is required for LTM in R4 subtypes of ellipsoid body (EB) neurons (Wu et al., 2007).

Outside MB, DopR function also is required in modulating various forms of arousal to external stimuli, including ethanol, cocaine, and caffeine (Andretic et al., 2008; Kong et al., 2010; Van Swinderen and Andretic, 2011), as well as startle-induced arousal caused by repetitive air puffs (Lebestky et al., 2009). For certain forms of arousal, DopR expression is required within R2/R4m subsets of EB neurons (Lebestky et al., 2009; Kong et al., 2010), which include the cell type(s) where NMDA-receptor function is needed for olfactory LTM (Wu et al., 2007). Thus naive responses to external stimuli and long-lived conditioned responses to odors share a role for EB neurons and also share a role for dopamine signaling onto DopR.

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In this study, we show that miR-276a is required within MB for conditioned olfactory LTM and in R2/R4m subtypes of EB neurons for naive olfactory avoidance. Furthermore, we are able to fully suppress both the LTM and naive olfactory response defects of miR-276a disruptions simply by reducing the *DopR* copy number. Overexpression of DopR within MB also is sufficient to phenocopy LTM defects of the *miR-276a* mutants. We propose a model in which miR-276a fine-tunes the levels of DopR within MB for conditioned olfactory LTM and within EB for naive odor responses.

Materials and Methods

Fly stocks. Fly stocks were cultured in standard fly food and room temperature (22.5°C). The wild-type flies used in this study were w^{1118} (*iso1CJ*). All other mutant strains and transgenic strains were backcrossed to this wild-type strain for at least five generations. The $miR\text{-}276^{Rosa}$ mutant was originally generated in the Tully laboratory from a forward mutagenesis screen by inserting a P-element p{lacW} (Dubnau et al., 2003). The null allele $miR\text{-}276a^{D8}$ was generated by providing transposase Δ *2-3* in trans and mobilizing the P-element imprecisely. To detect the molecular lesion in $miR\text{-}276a^{D8}$, genomic DNA from the $miR\text{-}276a^{D8/Rosa}$ animals was purified and PCR-amplified by primers priming flanking regions (forward primer: 5′-AATAGAGTTGACAAAGCG TTCGGCGCCCACG-3′; reverse primer: 5′-GCGGAGGAAGGGAATC TGGCACTCGAATCG-3′) with the Roche Expand Long Range dNTP pack (Cat. No. 04829034001, Roche). The PCR product was sequenced and it was determined that in the $miR\text{-}276a^{D8}$ allele, a ~3.6 kb genomic region to the right of the P-element insertion site was deleted and a ~2.8 kb residual sequence of the P-element was left in the genome. Animals homozygous for $miR\text{-}276a^{D8}$ are semilethal and few animals survive during late pupal stage. Similarly, the precise excision alleles $miR\text{-}276a^{A6}$ and $miR\text{-}276a^{D2.2}$ were also generated by providing transposase Δ *2-3* in trans and mobilizing the P-element precisely. PCRs (forward primer: 5′-AA TAGAGTTGACAAAGCGTTCGGCGCCCACG-3′; reverse primer: 5′-TGAACGTAGGAACTCTATACCTCGCTGATGG-3′) were used to verify that the P-elements were removed and genomic structures were restored in these alleles.

The bacterial artificial chromosome (BAC) rescue genomic constructs were obtained from BACPAC Resources Center. These BAC clones flank the *miR-276a* precursor regions, but were selected carefully to avoid any sequence flanking upstream or downstream genes. The names and approximate sizes of the constructs are as follows: CH322-133G18 (~20 kb), CH322-151H13 (~19 kb), and CH321-46B15 (~75 kb). These BAC clones were engineered into the attB-P[acman]-Cm^R-BW vector (Venken et al., 2009) and these transgene constructs were directly injected and permanently integrated at specific docking sites (engineered genomic loci containing *attP* sequence) in the genome using ΦC31 transposase (Venken et al., 2006, 2009). We selected the 9736 (53B2) docking site (P[acman] Resources, www.pacmanfly.org). This docking site is farther away from annotated genes and presumably will not affect animal behavior. The transgenic fly injection services were provided by BestGene. Because the docking site stock itself contains a $yellow^1$ mutant allele on the X chromosomes and this mutation could potentially impair animal behavior, we crossed male transformants with wild-type flies and select male progenies before starting subsequent backcrossing to remove $yellow^1$.

The *UAS::miR-276a-4.7Kb* rescue construct was made by cloning a ~4.7 kb genomic region from wild-type flies into the pUAST vector. Forward primer 5′-GTTTGTGCCTCAAGTGGCAGTCATAAATTTGA G-3′ and reverse primer 5′-AACCGCACCTCAATCGCCCTTTACTT GG-3′ were used to PCR-amplify a ~4.7 kb genomic region containing the *miR-276a* precursor and largely upstream regions. The PCR product was cloned with a Zero Blunt TOPO PCR Cloning Kit (Invitrogen) and then subcloned into the pUAST vector. The resulting pUAST-miR-276a-4.7Kb constructs were injected at BestGene by standard P-transposase-mediated integration.

The *UAS::EGFP::miR-276aSPONGE* and *UAS::EGFP::SCRAMBLED* transgenic flies were generated as previously described (Loya et al., 2009). The "sponge" sequence used for *UAS::EGFP::miR-276aSPONGE* is as follows:5′-TCTAGAAGAGCACGGTACTAGTTCCTACCGTAGAGCACGG TACTAGTTCCTACCGTAGAGCACGGTACTAGTTCCTACCGTAGAG CACGGTACTAGTTCCTACCGTAGAGCACGGTACTAGTTCCTACCG TAGAGCACGGTACTAGTTCCTACCGTAGAGCACGGTACTAGTTCC TACCGTAGAGCACGGTACTAGTTCCTACCGTAGAGCACGGTACTA GTTCCTACCGTAGAGCACGGTACTAGTTCCTACTCGAG-3′. And the "sponge" sequence used for *UAS::EGFP::SCRAMBLED* is as follows: 5′-CT CGAGTTAGAATTTAAACCTCACCATGATGCATTAGAATTTAAACCT CACCATGAGCGGTTAGAATTTAAACCTCACCATGAAGGCTTAGAA TTTAAACCTCACCATGAGTCCTTAGAATTTAAACCTCACCATGAT GGCTTAGAATTTAAACCTCACCATGAACTTTTAGAATTTAAACCTC ACCATGAGGCATTAGAATTTAAACCTCACCATGATAGATTAGAATT TAAACCTCACCATGAGCCTTTAGAATTTAAACCTCACCATGATCTA GA-3′. Two transformant lines (presumably different insertion sites) of each transgene were randomly selected for the behavior study. For clarity, these transformant lines were named as follows: *UAS::EGFP::SPONGE#1*, *UAS::EGFP::SPONGE#2*, *UAS::EGFP::SCRAMBLED#1*, and *UAS::EGFP::SCRAMBLED#4*.

The GAL4 stocks used in this study included the following: pan-neuronal driver *elav,* olfactory sensory neuron driver *Or83b*, antenna lobe projection neuron driver *GH146*, antenna lobe local neuron driver *GH298*, MB drivers *OK107* and *c747*, EB drivers *c232* and *c547*, and heat-shock GAL4 (*hs-GAL4*). Two GAL4 stocks combined with *tubulin* promoter-driven temperature-sensitive GAL80 transgenes *elav; $GAL80^{ts}$* and *$GAL80^{ts}$;;OK107* were also used. *UAS::mCD8::GFP* (Bloomington Stock Center) was also used to virtualize GAL4 expression patterns.

Two strong alleles of *DopR* gene, $dumb^1$ and $dumb^2$ (Kim et al., 2007a), were used in this study.

Quantitative real-time PCR. TaqMan MicroRNA Assays (Applied Biosystems) were used to quantitate the expression level of miR-276a in wild-type, mutant, and rescue animals. TaqMan miRNA assays for *miR-276a* (assay ID 000297) and the endogenous control *2s rRNA* (assay ID 001766), MultiScribe reverse transcription kit (4366596), and TaqMan Universal PCR Master Mix No AmpErase UNG (4324018) were purchased from Applied Biosystems. The QPCR was performed according to the assay manual. In brief, massive numbers of fly heads were collected for each genotype and total RNA was purified with Trizol (Invitrogen) and treated by DNaseI (Promega). Following reverse transcription (RT) reaction with miRNA-specific stem-loop RT primers, QPCRs were performed with TaqMan MicroRNA probes in an Applied Biosystems 7900HT Fast Real-Time PCR System. Threshold cycle (Ct) values obtained from the QPCRs were further converted to relative fold changes with a ΔΔCt method (Schmittgen, 2001).

TaqMan Gene Expression Assays (Applied Biosystems) were used to quantitate the expression levels of *Zfh2*, *DopR*, *Pino*, *Nf1*, and *dpr* genes for validating potential miR-276a targets. TaqMan Gene Expression Assays for *Zfh2* (assay ID Dm01825551_m1), *DopR* (assay ID Dm02134813_m1), *Pino* (assay ID Dm01845906_m1), *Nf1* (assay ID Dm02151064_g1), *dpr* (assay ID Dm01836227_m1); the endogenous control *RpII140* (assay ID Dm02134593_g1); High Capacity RNA-to-cDNA kit (4387406); and TaqMan Gene Expression Master Mix (4369016) were purchased from Applied Biosystems. The QPCR was performed according to the assay manual. In brief, massive numbers of fly heads were collected for each genotype and total RNA was purified with Trizol (Invitrogen) and treated by DNaseI (Promega). Following RT reaction, QPCRs were performed with TaqMan Gene Expression Assay probes in an Applied Biosystems 7900HT Fast Real-Time PCR System. Ct values obtained from the QPCRs were further converted to relative fold changes with a ΔΔCt method (Schmittgen, 2001).

Behavior assays. Olfactory associative memory was tested by training 2–3-d-old flies in a T-maze apparatus using a Pavlovian conditioning paradigm (Tully and Quinn, 1985; Tully et al., 1994). Approximately 100 flies were loaded into an electrifiable training grid. For a single training session, flies were exposed sequentially to one odor [the conditioned stimulus, (CS+)], which was paired with a 60 V electric shock and then a second odor [the unconditioned stimulus (CS−)] without shock. Three minutes after this training session, the flies were tested and allowed to choose between the two odors. A half performance index was calculated by subtracting the number of flies that chose incorrectly from the num-

ber of flies that chose correctly, and then dividing that number by the total number of flies in the experiment. The same protocol was then performed with another group of 100 flies and reciprocal odor presentation. The final performance index was calculated by averaging both reciprocal half performance indices. The LTM experiment was an adaptation of this training protocol. Flies were subjected to 10 such training sessions in robotic trainers spaced out with a 15 min rest interval between each. Flies then were transferred into food vials and incubated at 18°C until being tested 24 h after the training. A massed training protocol was also performed (10 consecutive single training sessions in robotic trainers without rest intervals). All genotypes were trained and tested in parallel, and rotated between all the robotic trainers to ensure a balanced experiment. Odor pairs and concentrations used for these behavior paradigms are as follows: 3-octanol (OCT, 1.5×10^{-3} v/v) and 4-methylcyclohexanol (MCH; 1×10^{-3} v/v), or OCT (1.5×10^{-3} v/v) and benzaldehyde (BA; 0.5×10^{-3} v/v). Pure odors were purchased from Sigma-Aldrich and delivered as the stated concentrations with air flow at 750 ml/min.

Olfactory avoidance was quantified by exposing naive flies to each odor versus air in the T-maze (i.e., odor from the left and air from the right). After 2 min, flies in each arm of the T-maze were counted. Subsequently the directions of odor and air were switched (i.e., odor from the right and air from the left) and another group of naive flies was tested. A half performance index was calculated by subtracting the number of flies that chose the odor from the number of flies that chose air, and then dividing that number by the total number of flies in the experiment. The final performance index was calculated by averaging both reciprocal half performance indices. The odor concentrations used for olfactory acuity tests were indicated in each figure. Pure odors were purchased from Sigma-Aldrich and delivered as the stated concentrations with air flow at 750 ml/min.

Shock reactivity was quantified by exposing naive flies to two electrifiable grids in the T-maze, while delivering a 60 V electric shock to one of the grids. Flies were allowed 2 min to choose. A performance index was calculated by subtracting the number of flies that chose the shock from the number of flies that chose to avoid the shock, and dividing that number by the total number of flies in the experiment.

In all cases, behavior experiments within a figure were performed in parallel.

Quantification of 24 h embryo-hatching rate. Crosses with corresponding genotypes were made and maintained in cages on agar plates containing fruit juice for a few days (3–5 d). Early in the morning, the agar plates were replaced with fresh new ones and yeast paste to make sure female flies start to lay fertilized eggs in large quantities. Then a 4 h egg collection was performed. Twenty-four hours after the end of egg collection, hatched embryos from each cross were counted and hatching rates were calculated by dividing the number of hatched embryos by the total number of embryos.

GAL80^{ts} temperature-shift experiment. The female virgin flies with genotypes of *elav;GAL80^{ts}* or *GAL80^{ts};;OK107* were collected and crossed to WT, *UAS::EGFP::miR-276aSPONGE*, and *UAS::EGFP::SCRAMBLED* transgenic flies. All the crosses were raised at the permissive temperature (29°C). Upon eclosion, we separated the progenies of each cross into two groups: one was continuously incubated at the permissive temperature (29°C) and the other one was incubated at the restrictive temperature (18°C). Both groups were incubated for an additional 72 h before testing for the avoidance behavior and olfactory memory. The avoidance behavior test was described as above except for the use of MCH that was three times more concentrated (3×10^{-3} v/v). Permissive temperature incubation during the entire process of development affected the animal behavior. We raised the testing odor concentration to ensure that a similar level of avoidance index in WT flies with animals raised at 22.5°C can be obtained with animals raised at 29°C. Following temperature-shift treatment, olfactory avoidance tests were conducted at 25°C in an environment-controlled room with 70% humidity. LTM experiments were conducted by performing 10× spaced training sessions at 25°C in an environment-controlled room with 70% humidity. The trained flies were kept at 18°C before testing for LTM at 25°C 24 h later.

Imaging. Expression levels of GAL4 lines were virtualized by *UAS::mCD8::GFP* with confocal microscopy as described previously (Blum et al., 2009). DopR antibody staining was performed with a method and an anti-DopR antibody described previously (Lebestky et al., 2009).

Statistics. Statistical analyses were performed using JMP software. Student's *t* test (2-tailed) was used for comparisons between two groups. One-way ANOVA followed by *post hoc* analysis was used for comparisons of multiple groups. Behavioral data from the Pavlovian memory task are normally distributed and are shown in all figures as means ± SEM (Tully et al., 1994). QPCR data are presented as means ± SEM of fold changes.

Results

Genetic reagents to manipulate the *miR-276a* gene locus

The *miR-276a^{Rosa}* mutant isolated from a forward mutagenesis screen (Dubnau et al., 2003) has a p{lacW} element inserted 1.2 kb upstream of the gene region coding for the predicted *dme-mir-276a* precursor. *dme-mir-276a* and the p{lacW} element insertion site fall within a large intergenetic region of ~100 kb, where there are no known or predicted protein coding genes within ~50 kb either upstream or downstream of the miRNA sequence (Fig. 1*A*). *dme-mir-276b*, which also belongs to the *dme-mir-276* gene family, sits ~45 kb upstream. Both of these miRNA loci produce RNA precursors that can contribute to the expression of a miRNA passenger sequence, miR-276*, with identical sequence from the two loci (Fig. 1*B*,*C*). The mature miRNAs, miR-276a and miR-276b, differ from each other by only 1 nt. Expression profiling of miRNAs from cultured *Drosophila* S2 cells or various tissues indicates that the abundance of miR-276a is 10-fold higher than miR-276b and most miR-276* arises from the *dme-mir-276a* precursor locus (Czech et al., 2008).

To investigate the function of *miR-276a* locus in behavior, we first generated a suite of reagents to manipulate miR-276a expression with both temporal and cell-type specificity. We generated both precise and imprecise excisions of the p{lacW} element insertion (Fig. 1*A*; see Materials and Methods). In the *miR-276a^{D8}* allele, a ~3.6 kb genomic region to the right of the p{lacW} element insertion site was deleted and a ~2.8 kb residual sequence of the P-element was left in the genome. *miR-276a^{D8}* therefore removes the entire *mir-276a* precursor and can be considered a null allele. In the *miR-276a^{A6}* and the *miR-276a$^{D2.2}$* alleles, the P-element is almost completely removed and no flanking deletions were detected. These excision alleles therefore are predicted to restore the normal function of this locus (Fig. 1*A*). In addition to these mutant alleles, we generated transgenic rescue animals containing genomic BAC clones, CH322-133G18 (~20 kb), CH322-151H13 (~19 kb) and CH321-46B15 (~75 kb), which were carried in p[acman] vectors (Venken et al., 2006, 2009). These BAC clones cover the *mir-276a* precursor region and do not include any nearby protein coding genes or the *mir-276b* precursor region (Fig. 1*A*).

To characterize the expression of miR-276a in the above mutants and BAC rescue transgenes, we used QPCR to detect miR-276a levels in fly heads (Figs. 1*D*, 2*C*). In the *miR-276a^{Rosa}* homozygous mutant animal heads, miR-276a expression level was reduced by ~40% compared with wild-type animals. In the *miR-276a^{D8}* homozygous mutant animal heads, miR-276a expression (Fig. 1*D*; $F_{(3,18)} = 25.09$, $p < 0.05$) was nearly eliminated (the low residual expression presumably derives from *miR-276b* locus, which differs from *miR-276a* by only 1 nt). This is consistent with the conclusion that *miR-276a^{D8}* is a null allele of *miR-276a*, while *miR-276a^{Rosa}* is a hypomorphic allele. Animals transheterozygous for the two mutant alleles *miR-276a^{D8}* and *miR-276a^{Rosa}* (*miR-276a$^{D8/Rosa}$*) yield defective miR-276a expression that is intermediate between that of *miR-276a^{Rosa}* and *miR-276a^{D8}* homozygous animals (Fig. 1*D*; $F_{(3,18)} = 25.09$, $p < 0.05$).

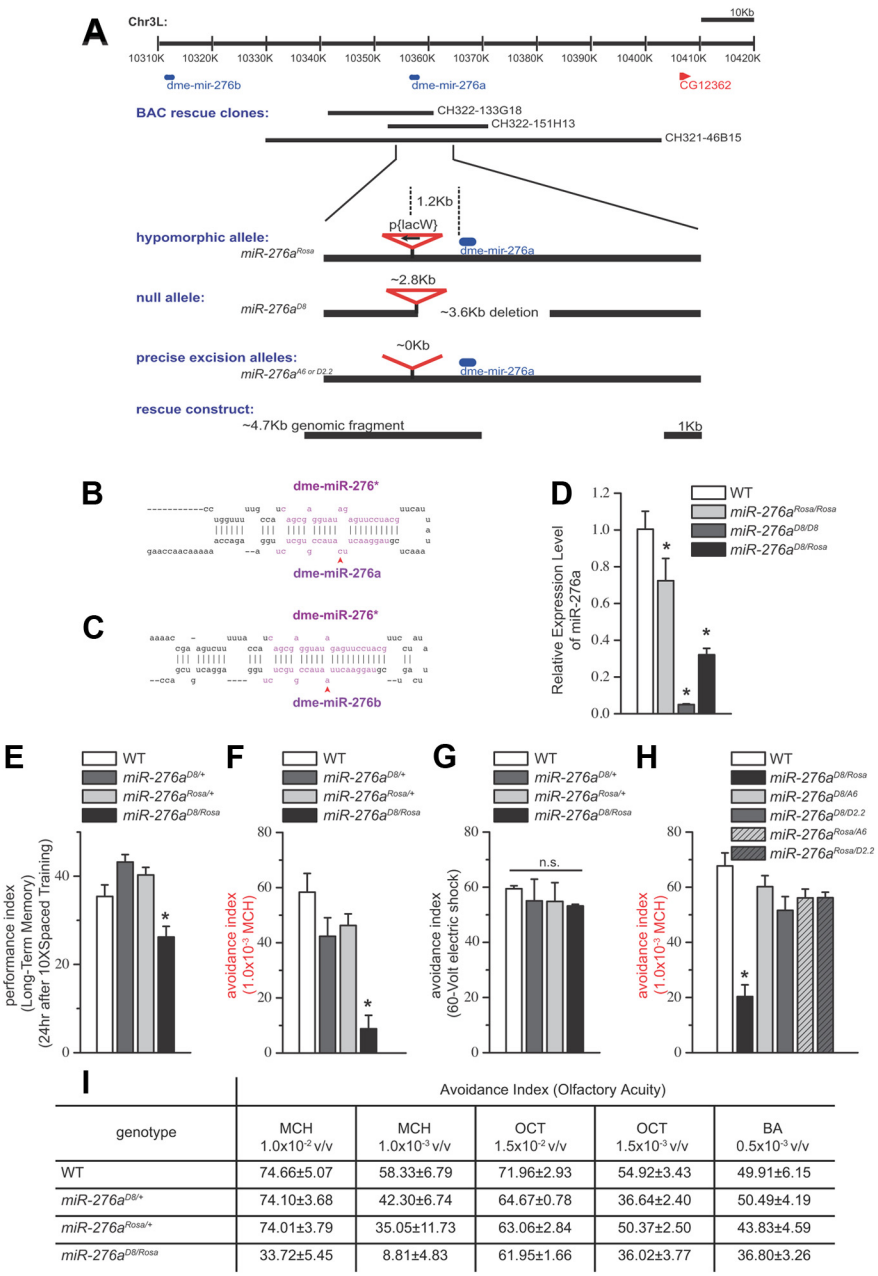
Because the homozygous *miR-276a*^{D8} mutant is semilethal (few survive to adulthood), we used *miR-276a*$^{D8/Rosa}$ as a viable but strong allele combination for behavioral experiments. For the BAC rescue transgenes, only CH321-46B15, the largest of the three constructs (~75 kb genomic fragment) restored miR-276a expression (Fig. 2*C*; $F_{(3,19)} = 16.06$, $p < 0.05$).

miR-276a$^{D8/Rosa}$ mutant animals exhibit defective long-term olfactory memory and naive olfactory avoidance

An LTM defect originally was reported for the *miR-276a*^{Rosa} hypomorphic allele (Dubnau et al., 2003). We tested short-term and long-term olfactory memory as well as task-relevant sensorimotor responses to the odors and electric shock using the strong *miR-276a*$^{D8/Rosa}$ allele combination. We found that the *miR-276a*$^{D8/Rosa}$ animals exhibited significantly defective performance for LTM measured 24 h after 10 spaced training sessions, while animals heterozygous for *miR-276a*^{D8} or *miR-276a*^{Rosa} allele performed normally compared with WT (Fig. 1*E*; $F_{(3,60)} = 11.77$, $p < 0.05$). However, the *miR-276a*$^{D8/Rosa}$ animals also exhibited significantly defective naive avoidance responses (Fig. 1*F*,*I*) to MCH (Fig. 1*F*,*I*; $F_{(3,18)} = 5.88$, $p < 0.05$) and OCT (Fig. 1*I*; $F_{(3,34)} = 9.51$, $p < 0.05$), but not BA (Fig. 1*I*; $F_{(3,20)} = 1.88$, not significant) at the concentrations used for our standard memory assay (1×10^{-3} v/v, 1.5×10^{-3} v/v, and 0.5×10^{-3} v/v respectively for MCH, OCT, and BA), compared with WT or heterozygous mutant controls. At 10-fold higher concentrations, responses appeared normal for OCT (Fig. 1*I*; $F_{(3,20)} = 4.06$, not significant), but not for MCH (Fig. 1*I*; $F_{(3,20)} = 19.74$, $p < 0.05$). These observations raised the possibility that the reduced naive odor response of strong allele combinations of *miR-276a* contributes to the olfactory memory defect (but see below). Avoidance of electric shock (60 V) appeared normal in all genotypes (Fig. 1*G*; $F_{(3,11)} = 0.20$, not significant).

miR-276a function underlies naive olfactory avoidance defects

In addition to the complementation tests shown in Figure 1*E*,*I*, we also tested whether the naive odor avoidance defect was reverted with the precise excision alleles in which the P-element was removed and the genomic structure was restored. Indeed, animals transheterozygous for *miR-276a* mutant alleles (hypomorphic allele *miR-276a*^{Rosa} or null allele *miR-276a*^{D8}) and either of the two precise excision alleles (*miR-276a*^{A6} or *miR-276a*$^{D2.2}$) exhibit normal naive olfactory avoidance responses to MCH (Fig. 1*H*; $F_{(5,30)} = 16.96$, $p < 0.05$). We next tested whether transgenes containing genomic BAC clones are sufficient to rescue the naive olfactory response defects (Fig. 2). All three BAC clones tested include the predicted *miR-276a* precursor region but exclude any other protein coding genes



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genotype	Avoidance Index (Olfactory Acuity) MCH 1.0x10^{-2} v/v	MCH 1.0x10^{-3} v/v	OCT 1.5x10^{-2} v/v	OCT 1.5x10^{-3} v/v	BA 0.5x10^{-3} v/v
WT	74.66±5.07	58.33±6.79	71.96±2.93	54.92±3.43	49.91±6.15
miR-276a$^{D8/+}$	74.10±3.68	42.30±6.74	64.67±0.78	36.64±2.40	50.49±4.19
miR-276a$^{Rosa/+}$	74.01±3.79	35.05±11.73	63.06±2.84	50.37±2.50	43.83±4.59
miR-276a$^{D8/Rosa}$	33.72±5.45	8.81±4.83	61.95±1.66	36.02±3.77	36.80±3.26

Figure 1. Genetic and behavioral characterization of *miR-276a* gene locus. ***A–C***, The *dme-miR-276a* gene falls into a large intergenic region (***A***). The p{lacW} element that causes the *miR-276a*^{Rosa} mutant phenotype is inserted 1.2 kb upstream of the *dme-mir-276a* precursor region. The structure of each mutant allele or transgene is illustrated, including hypomorphic allele *miR-276a*^{Rosa}, null allele *miR-276a*^{D8}, and precise excision alleles *miR-276a*^{A6} and *miR-276a*$^{D2.2}$. The structure and relative sizes of rescue constructs, including three BAC rescue clones and a 4.7 kb genomic fragment, also are shown (***A***). The *dme-miR-276* gene family produces two miRNA precursors, which give rise to three miRNAs (miRBase: http://www.mirbase.org): *dme-miR-276a*, *dme-miR-276b*, and *dme-miR-276** (***B***, ***C***). ***D***, The relative expression level of miR-276a in the heads of WT, *miR-276a*$^{Rosa/Rosa}$, *miR-276a*$^{D8/D8}$, and *miR-276a*$^{D8/Rosa}$ animals, measured by QPCR. *$p < 0.05$; $N = \sim 3–8$. ***E***, *miR-276a*^{D8} and *miR-276a*^{Rosa} alleles failed to complement each other for LTM measured 24 h after 10 spaced training sessions. *$p < 0.05$; $N = 16$. ***F***, *miR-276a*^{D8} and *miR-276a*^{Rosa} alleles failed to complement each other for olfactory avoidance test with MCH (1.0×10^{-3} v/v). *$p < 0.05$; $N = \sim 4–6$. ***G***, Shock avoidance (60 V) appeared normal in WT, *miR-276a*$^{Rosa/+}$, *miR-276a*$^{D8/+}$, and *miR-276a*$^{D8/Rosa}$ animals. n.s., not significant; $N = 4$. ***H***, Two precise excision alleles, *miR-276a*^{A6} and *miR-276a*$^{D2.2}$, can reverse the olfactory avoidance defect seen in *miR-276a*$^{Rosa/D8}$ mutant animals. *$p < 0.05$; $N = 6$. ***I***, Olfactory avoidance tests with MCH, OCT, and BA at various concentrations for WT, *miR-276a*$^{Rosa/+}$, *miR-276a*$^{D8/+}$, and *miR-276a*$^{D8/Rosa}$ animals. $N = \sim 6–10$.

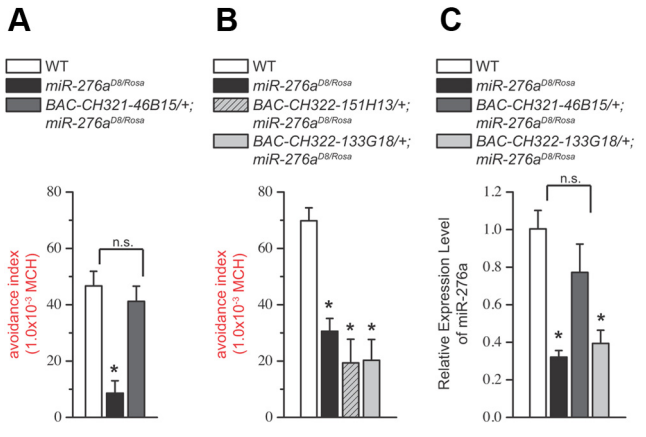


Figure 2. Transgene rescue of *miR-276a* mutants. ***A***, A copy of ~75 kb BAC rescue clone CH321-46B15 fully rescues the olfactory avoidance behavior defect in *miR-276a*$^{D8/Rosa}$ mutant animals. *$p < 0.05$; $N = \sim 12–16$. ***B***, The two ~20 kb BAC rescue clones, CH322-151H13 and CH322-133G18, failed to rescue the olfactory avoidance defect in *miR-276a*$^{D8/Rosa}$ mutant animals. *$p < 0.05$; $N = 4$. ***C***, Expression levels of miR-276 are restored with the ~75 kb BAC CH321-46B15 but not with the ~20 kb BAC CH322-133G18. *$p < 0.05$; n.s., not significant; $N = 4–8$.

or *miR-276b* coding region (Fig. 1*A*). We found that the expression levels and behavioral defect of *miR-276a*$^{D8/Rosa}$ can be fully rescued by providing a transgenic copy of a ~75 kb BAC clone CH321-46B15 (Fig. 2*A*, $F_{(2,35)} = 5.49$, $p < 0.05$). In contrast, smaller BAC clones (~20 kb CH322-133G18 and ~19 kb CH322-151H13) (Fig. 1*A*) failed to rescue *miR-276a*$^{D8/Rosa}$ expression or olfactory behavior (Fig. 2*B*; $F_{(3,12)} = 13.42$, $p < 0.05$). Together, the above findings provide convergent evidence that miR-276a is responsible for the defect in naive odor responses.

Dominant negative miRNA "sponge" phenocopies naive olfactory response defect of *miR-276a* mutants

As a complementary and independent approach, we made use of the miRNA "sponge" system (Ebert et al., 2007), which was recently adapted to the *Drosophila* model (Loya et al., 2009). The "sponge" transgenes include 10 repetitive sequences complementary to miR-276a with mismatches at positions 9–12 for enhanced stability. When *UAS::EGFP::miR-276aSPONGE* expression is induced by GAL4, endogenous miR-276a should be "soaked up" and its normal function should be interfered with (Fig. 3*A*). As controls, we used *UAS::EGFP::SCRAMBLED* flies in which 10 repetitive complementary sequences are replaced by a scrambled sequence not recognized by any miRNA in *Drosophila*. We demonstrated the efficiency and specificity of this approach with an *in vivo* assay in which expression of *UAS::EGFP::miR-276aSPONGE* can suppress the developmental lethality from pan-neuronal overexpression of miR-276a (Fig. 3*B*). We next tested naive olfactory avoidance behavior in animals expressing *UAS::EGFP::miR-276aSPONGE* or *UAS::EGFP::SCRAMBLED* under the pan-neuronal *elav*-GAL4 driver (Fig. 3*E*). We used two independent transgenic lines each for *UAS::EGFP::miR-276aSPONGE* and *UAS:: EGFP::miR-SCRAMBLED*. We found that expression of the *UAS::EGFP::miR-276aSPONGE* in neurons impaired the animals' performance in the naive olfactory avoidance assay, while animals that express the *UAS::EGFP::SCRAMBLED* transgenes performed normally (Fig. 3*E*; $F_{(5,42)} = 24.48$, $p < 0.05$). The control animals that contained the UAS-transgenes but not the Gal4 driver performed normally as well (Fig. 3*F*; $F_{(4,25)} = 0.75$, not significant).

In addition to recapitulating the mutant phenotype, the "sponge" system also provided an indirect observation of miR-276a expression pattern. Because endogenous miR-276a can bind to the 3′UTR of the *UAS::EGFP::miR-276aSPONGE* transgene, expression of EGFP driven from the pan-neuronal *elav*-GAL4 was dramatically reduced when compared with that of *UAS::EGFP::SCRAMBLED*. This is consistent with the idea that miR-276a is broadly expressed in adult fly heads (Fig. 3*C*,*D*).

Postdevelopment function of miR-276a is sufficient for naive olfactory responses

To define the temporal requirements for miR-276a function, we combined a *tubulin* promoter driven GAL80 temperature-sensitive (*GAL80*^{ts}) transgene (McGuire et al., 2004) with *elav*-GAL4. GAL80^{ts} is a suppressor of GAL4 and, at the permissive temperature (18°C), GAL80^{ts} is active and suppresses GAL4-controlled *UAS::EGFP::miR-276aSPONGE* transgene expression. At the restrictive temperature (29°C), GAL80^{ts} is inactivated, and the SPONGE or SCRAMBLED transgenes are expressed. We crossed either *UAS::EGFP::miR-276aSPONGE* or *UAS::EGFP::SCRAMBLED* transgenic flies to *elav;GAL80*^{ts} animals. Progeny from these crosses were kept at the restrictive temperature (29°C). Hence transgene expression was kept on, and miR-276a function was blocked during development. After eclosion, we separated the progeny of each cross into two groups: one was continuously incubated at the restrictive temperature (29°C), where miR-276a function is disrupted, and the other one was shifted to the permissive temperature (18°C), allowing miR-276a function to be turned back on. Both groups were incubated for an additional 72 h before being tested for avoidance behavior (Fig. 3*G*). We found that when miR-276a function was kept off after eclosion (no shift to permissive temperature), the flies that contained *UAS::EGFP::miR-276aSPONGE* transgenes exhibited reduced naive odor avoidance compared with *UAS::EGFP::SCRAMBLED* and *elav/+; GAL80*^{ts}*/+* control animals. This was true for each of the two independent "sponges" versus "scrambled" transgenes. In contrast, when the *UAS::EGFP::miR-276aSPONGE* transgene was turned off after development (permitting a recovery of miR-276a function), we observed a significant restoration of naive olfactory avoidance in the temperature-shifted group (Fig. 3*G*; *SPONGE#1*, $t_{(22)} = 4.65$, $p < 0.05$; *SPONGE#2*, $t_{(22)} = 2.71$, $p < 0.05$). In control crosses with the *UAS::EGFP::SCRAMBLED* transgenes, there was no significant difference between temperature-shifted and temperature-unshifted groups (Fig. 3*G*; *SCRAMBLED#1*, $t_{(6)} = 0.73$, not significant; *SCRAMBLED#4*, $t_{(6)} = 0.68$, not significant). These findings demonstrate that acute (postdevelopmental) function of miR-276a is sufficient for normal naive odor avoidance. Thus this behavioral effect is unlikely to derive from defects in neural development.

miR-276a is required in EB neurons for normal naive olfactory responses to MCH

To map the neural cell types in which miR-276a function is required, we conducted a small-scale screen in which the *UAS::EGFP::miR-276aSPONGE* was tested in combination with a set of GAL4 lines that each interrogate distinct subsets of the known circuits that underlie either olfaction or olfactory memory. Because some of these GAL4 lines may drive modest levels of expression, we combined the two *UAS::EGFP::miR-276aSPONGE* transformant lines to increase the levels of transgene expression (Loya et al., 2009). We selected GAL4 lines that express in olfactory sensory neurons (*Or83b*), antenna lobe projection neurons (*GH146*), antenna lobe local interneurons (*GH298*), MBs (*OK107* and *c747*), and two different sets of EB neurons (*c232* and *c547*) (Fig. 3*H*,*I*). In each case, we tested naive olfactory responses to MCH in animals that contained both the GAL4 driver and two *UAS::EGFP::miR-276aSPONGE* trans-

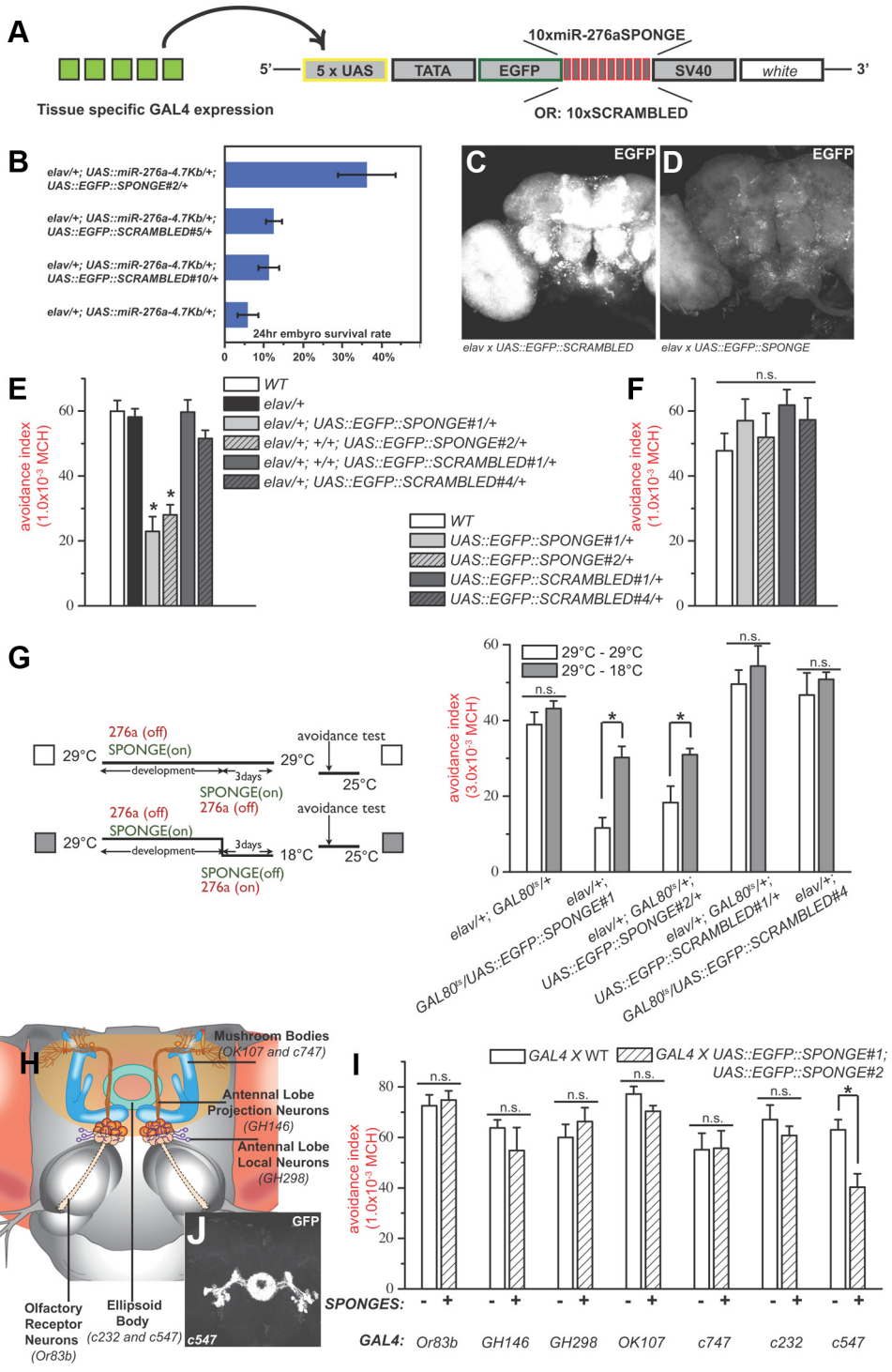


Figure 3. Postdevelopment miR-276a function in EB neurons for naive olfactory responses. ***A–D***, The inducible miRNA "sponge" system in *Drosophila* is illustrated (Loya et al., 2009) (***A***). Quantification of early embryo hatching rate from crossing *UAS::miR-276a-4.7Kb* transgene to *elav* with or without *UAS::EGFP::miR-276aSPONGE* indicated that the miR-276a "sponge" can efficiently "soak up" excessive miR-276a (***B***). "Sponge" as a sensor to report endogenous brain expression of miR-276a. EGFP expression in a representative brain from the progeny of crossing *elav* to *UAS::EGFP::SCRAMBLED* (***C***) and EGFP expression in a representative brain from the progeny of crossing *elav* to *UAS::EGFP::miR-276aSPONGE* (***D***). ***E***, ***F***, Neuronal expression of *UAS::EGFP::miR-276aSPONGE* with *elav* GAL4 causes a similar behavior defect, as observed in *miR-276a* mutant animal, and expression of control *UAS::EGFP::SCRAMBLED* transgenes does not affect olfactory avoidance (***E***; $*p < 0.05$; $N = 8$). Animals heterozygous for each transgene exhibit normal olfactory avoidance behavior (***F***; n.s., not significant; $N = 6$). ***G***, Postdevelopment expression of miR-276a is sufficient to restore normal naive olfactory avoidance. Temperature-shift induction scheme is illustrated (left; for detail, see Results and Materials and Methods). Olfactory avoidance was partially rescued when miR-276a function was turned on postdevelopmentally (right; $*p < 0.05$; n.s., not significant; $N = \sim 4–12$). ***H–J***, To map the circuitry where miR-276a is required for naive olfactory avoidance, *UAS::EGFP::miR-276aSPONGE* transgenes were expressed with a collection of GAL4 lines (*Or83b*, *GH146*, *GH298*, *OK107*, *c747*, *c232*, and *c547*), each of which label defined cell types that make up the *Drosophila* olfaction or olfactory memory circuit (***H***). Expression of *UAS::EGFP::miR276aSPONGE* transgenes with *c547* GAL4, which labels the R2/4m neurons in EB, phenocopies the naive olfactory avoidance defect observed in *miR-276a* mutant animals (***I***; $*p < 0.05$; $N = 8$). In contrast, other cell types did not affect this olfactory behavior (***I***; n.s., not significant; $N = 4$). *c547* expression pattern is demonstrated in ***J***.

A

gene	Pictar Rank#	TargetScanFly6.0 # of conserved site	ElMMO Rank#	miRanda Rank#	expression pattern in adult head	function
zfh2	1	3	1	1	Unknown	nervous system development
dpr	3	not predicted	2	>50	Gustatory Organ	salt aversion response
DopR	5	1	14	>50	Mushroom Body Central Complex	olfactory learning and memory various forms of arousal
Pino	11	1	>50	2	Antenna	olfactory avoidance
Nf1	17	1	22	>50	Broad	olfactory learning and memory

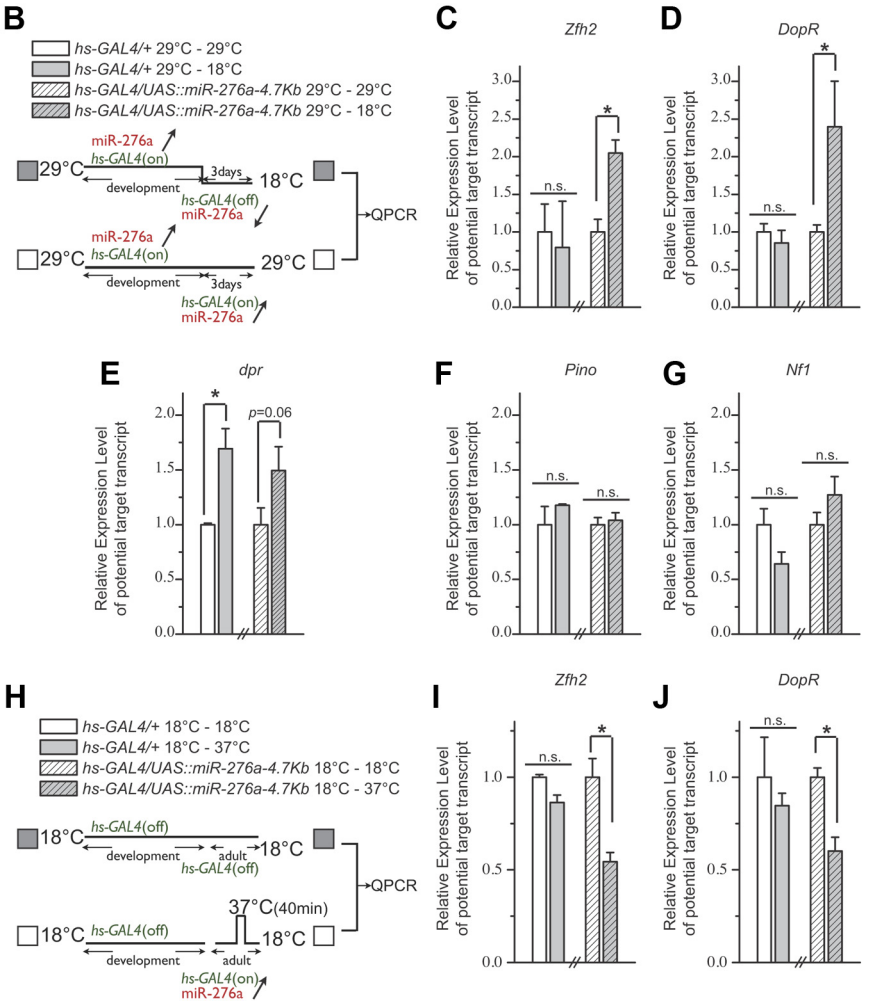


Figure 4. miR-276a regulates *DopR* expression level. ***A***, *miR-276a* target prediction was obtained from four published methods: Pictar, TargetScan, ElMMo, and miRanda (Pictar: http://pictar.mdc-berlin.de/; TargetScanFly6.0: http://www.targetscan.org/fly/; ElMMo: http://www.mirz.unibas.ch/ElMMo2/; and miRanda: http://www.microrna.org/microrna/home.do) (Enright et al., 2003; Grün et al., 2005; Gaidatzis et al., 2007; Ruby et al., 2007; Betel et al., 2010). We selected several targets with known neuronal or olfactory functions, including *zfh2*, *dpr*, *DopR*, *Pino*, and *Nf1* (Nakamura et al., 2002; Rollmann et al., 2005; Kim et al., 2007b; Andretic et al., 2008; Lebestky et al., 2009; Kong et al., 2010; Qin et al., 2012). ***B–G***, To test whether the predicted target gene expression is regulated by miR-276a, we used a heat-inducible *hs-GAL4* line in combination with a *UAS::miR-276a-4.7Kb* transgene to overexpress miR-276a. Because high level of neuronal expression through development is lethal (Fig. 3*B*), we used a mild-temperature regime (***B***; see Results and Materials and Methods) to provide a less severe heat induction during development. We then tested the effects on target gene transcript levels by shifting animals to lower temperature postdevelopment. In the temperature-shifted groups (29 to 18°C) we see significantly induced levels of target transcript levels for *Zfh2* and *DopR*, compared with groups maintained at 29°C (***C*** and ***D***; $*p < 0.05$; $N = \sim 2–3$). Transcript levels of *Zfh2* and *DopR* were not affected by the temperature shift in control *hs-GAL4/+* animals (***C*** and ***D***; n.s., not significant; $N = \sim 2–3$). With *dpr*, we observed induction in temperature-shifted groups for both *hs-GAL4/UAS::miR-276a-4.7Kb* and *hs-GAL4/+* animals, indicating temperature shift, but not miR-276a, can alter *dpr* expression levels (***E***; $*p < 0.05$; $N = \sim 2–3$). For *Pino* and *Nf1*, no change in transcript levels was observed for any genotype or treatment (***F*** and ***G***; n.s., not significant; $N = \sim 2–3$). ***H–J***, Another temperature-shift regimen was also used to test whether miR-276a regulates *Zfh2* and *DopR* gene expression (***H***). Forty-minute heat-shock induction of miR-276a expression at 37°C in adult *hs-GAL4/UAS::miR-276a-4.7Kb* animals caused a significant downregulation of transcript levels of *Zfh2* and *DopR*, which are predicted to be targets of miR-276a (***I*** and ***J***; $*p < 0.05$; n.s., not significant; $N = 3$).

genes in comparison with controls heterozygous for the GAL4 drivers. Surprisingly, the collection of GAL4 lines (*Or83b*, *GH146*, *GH298*, *OK107*, and *c747*) that query the main olfactory system from olfactory receptor neurons to MBs yielded normal naive olfactory avoidance behavior (Fig. 3*I*; *Or83b*, $t_{(6)} = 0.39$, not significant; *GH146*, $t_{(6)} = 0.93$, not significant; *GH298*, $t_{(6)} = 0.84$, not significant; *OK107*, $t_{(6)} = 1.81$, not significant; *c747*, $t_{(6)} = 0.06$, not significant). In contrast, only *c547*, which labels the R2/R4m subset of neurons in the EB (Fig. 3*J*), showed significantly reduced naive olfactory avoidance of MCH (Fig. 3*I*; $t_{(14)} = 3.37$, $p < 0.05$). *c232*, which labels R3/R4d EB neurons, did not affect olfactory avoidance (Fig. 3*I*; *c232*, $t_{(6)} = 0.92$, not significant). The finding with *c547*-labeled R2/R4m neurons was unexpected because EB neurons are several synapses downstream of the primary circuits thought to code for olfactory stimuli (Chiang et al., 2011) (FlyCircuit Database: http://www.flycircuit.tw). Several studies, however, have linked EB neurons to long-term olfactory memory (Wu et al., 2007) and arousal to external stimuli (Lebestky et al., 2009; Kong et al., 2010). The arousal phenotypes of mutations in a *DopR* gene are of particular relevance because they can be fully rescued by DopR expression using the *c547* GAL4 but not *c232* GAL4 (Lebestky et al., 2009; Kong et al., 2010). The requirement for miR-276a function in R2/R4m EB neurons thus guided our search for functional targets of miR-276a.

miR-276a impact on naive avoidance of MCH is mediated by *DopR*

We used four published methods (Pictar: http://pictar.mdc-berlin.de/; TargetScan Fly6.0: http://www.targetscan.org/fly/; ElMMo: http://www.mirz.unibas.ch/ElMMo2/; and miRanda: http://www.microrna.org/microrna/home.do) (Enright et al., 2003; Grün et al., 2005; Ruby et al., 2007; Gaidatzis et al., 2007; Betel et al., 2010) to predict mRNA targets of miR-276a and obtained a list of predicted target genes that we prioritized based on prediction scores from each method, known nervous system expression patterns, and neuronal or behavioral functions (Fig. 4*A*). We focused on the following genes: *Zn finger homeodomain 2* (*zfh2*), *defective proboscis extension response* (*dpr*), *DopR*, *Pinocchio* (*Pino*; also known as *smi21F, smell impaired 21F*). and *Neurofibromin 1* (*Nf1*). These predicted target genes have high ranking scores with all prediction methods and have established functions related to nervous system development (Lundell and Hirsh, 1992), regulating arousal (Andretic et al., 2008; Lebestky et al.,

2009; Kong et al., 2010), mediating olfactory responses (Nakamura et al., 2002; Rollmann et al., 2005), and olfactory learning and memory (Kim et al., 2007a; Qin et al., 2012). We tested whether reducing miR-276a expression can acutely alter predicted target gene expression. We crossed a heatshock GAL4 (*hs-GAL4*) driver to a *UAS::miR-276a-4.7Kb* transgene to overexpress miR-276a through development at 29°C, at which temperature *hs-GAL4* has leaky expression (Xia et al., 2005). After eclosion, we separated the progeny into two groups: one was continuously incubated at 29°C, and the other was incubated at 18°C to reduce heatshock-driven expression (Fig. 4*B*). *hs-GAL4/+* heterozygous animals were used as a control. We used QPCR to compare the expression levels of each candidate target gene in heads from animals that had been kept at 29°C with those that had been shifted to 18°C to reduce the transgenic expression of miR-276a. In the case of *Zfh2* and *DopR*, we observed a significant increase in expression levels when the *miR-276a* transgene was silenced (Fig. 4*C,D*; *C*, $t_{(4)} = 4.32$, $p < 0.05$; *D*, $t_{(4)} = 2.27$, $p < 0.05$). With *Pino* and *Nf1*, no change in expression was detected (Fig. 4*F,G*; not significant). With *dpr*, we saw a trend of increased expression in both the *hs-GAL4/UAS::miR-276a-4.7Kb* and the *hs-GAL4/+* control genotypes, indicating that temperature shift on its own can affect *dpr* expression levels (Fig. 4*E*; $t_{(3)} = 5.03$, $p = 0.02$; $t_{(4)} = 1.85$, $p = 0.06$). Thus, two of five tested candidates showed miR-276a-dependent changes in transcript levels (see below for protein level). The increased expression of *Zfh2* and *DopR* when the *miR-276a* transgene is turned off supports the idea that the miRNA normally represses these two genes. As a second test of this regulatory relationship, we used an acute induction protocol in which the heatshock-driven *miR-276a* transgene was kept off (18°C) during development and then acutely induced with a 40 min heat shift (37°C) followed by 4 h recovery. With this acute induction protocol (Fig. 4*H*), both *Zfh2* and *DopR* levels were decreased (Fig. 4*I,J*; *I*, $t_{(4)} = 4.07$, $p < 0.05$; *J*, $t_{(4)} = 4.46$, $p < 0.05$).

The *DopR* 3′UTR contains at least one putative miR-276a binding site, which is highly conserved across *Drosophila* species (Fig. 5*A*). While we cannot be certain that the effects on DopR are direct and mediated by the putative target motif, we do observe a negative regulatory effect of miR-276a on DopR expression as predicted for a direct target. In either case, the negative sign of interaction predicts that mutation of *miR-276a* would cause increased DopR expression. This idea was of particular interest because of our finding that miR-276a functions within R2/R4m EB neurons labeled by *c547*. DopR function within these neurons has an established role in two different forms of arousal (Lebestky et al., 2009; Kong et al., 2010) and the *DopR* mutations exhibit a dominant increase in arousal, suggesting dosage sensitivity (Lebestky et al., 2009). In our case, mutations of *miR-276a* would lead to increased *DopR* expression, which should cause decreased arousal. We therefore tested whether reducing the copy number of *DopR* can suppress miR-276a mutations. We introduced a copy of the $DopR^{dumb2}$ allele into the $miR\text{-}276a^{D8/Rosa}$ mutant and tested naive olfactory avoidance. Remarkably, the $DopR^{dumb2/+}$, $miR\text{-}276a^{D8/Rosa}$ animals exhibited normal avoidance (Fig. 5*B*; $F_{(2,29)} = 23.55$, $p < 0.05$). Together with the expression studies described above, this experiment provides strong evidence that *DopR* is a functional downstream effector of miR-276a within R2/R4m EB neurons and that this regulatory relationship affects naive responses to this olfactory stimulus.

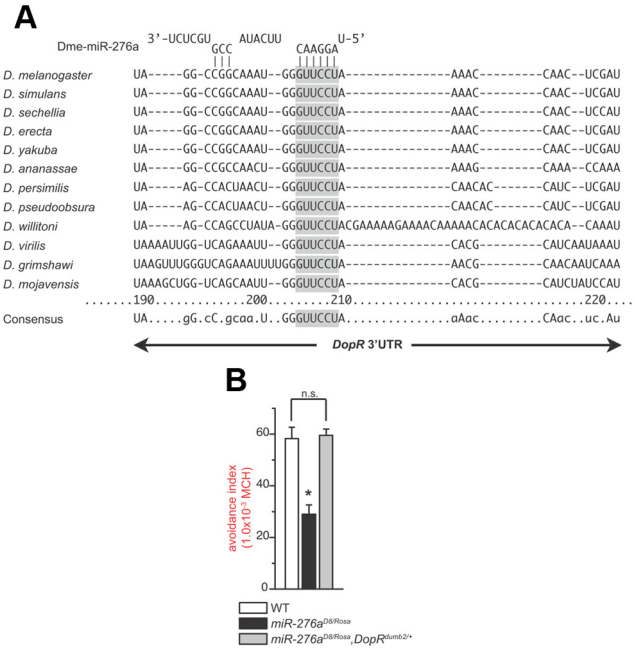
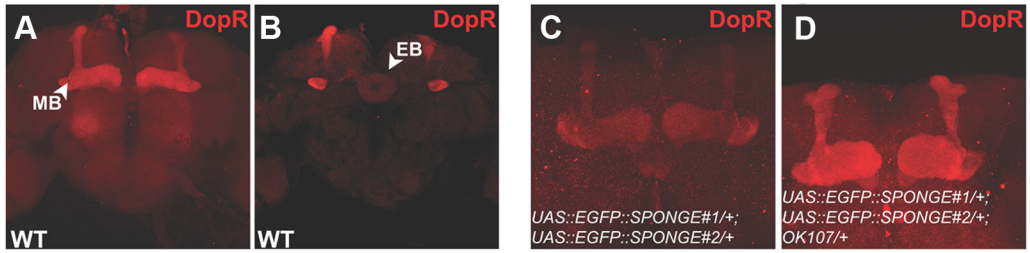


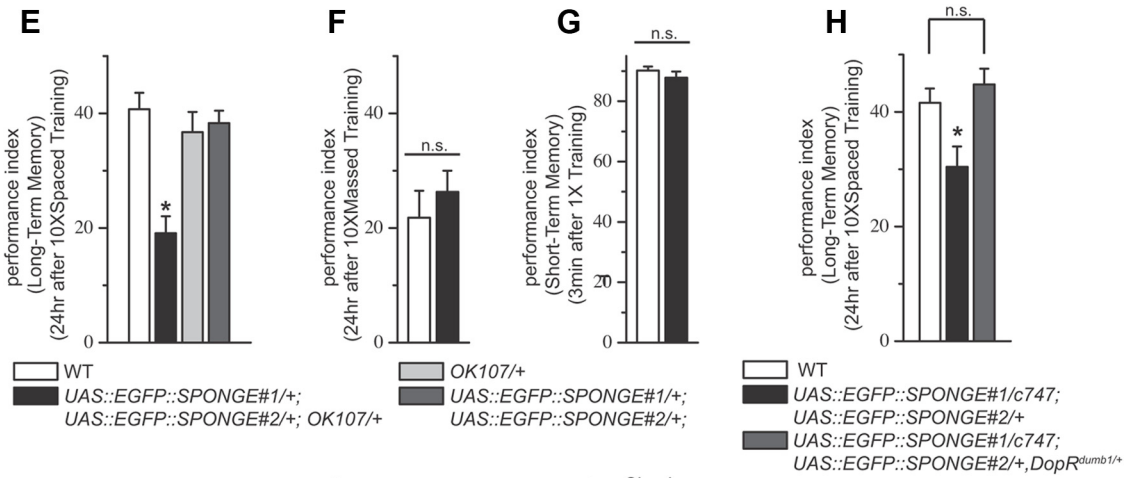
Figure 5. *DopR* is a downstream target of miR-276a for naive olfactory responses. ***A***, miR-276a seed sequence recognizes a highly conserved 6 nt target site in the 3′UTR of the *DopR* transcript. The predicted miR-276a-binding site (shaded boxes) is conserved within the 3′UTR sequences of *DopR* transcripts from 12 different *Drosophila* species (TargetScanFly6.0). ***B***, Removing a copy of *DopR* gene by introducing a strong hypomorphic allele of *DopR* (the $DopR^{dumb2}$ allele) was sufficient to fully suppress the naive olfactory avoidance defect in $miR\text{-}276a^{D8/Rosa}$ mutant animals. $^*p < 0.05$; n.s., not significant; $N = \sim 8–16$.

miR-276a impacts olfactory LTM via effects on DopR expression in MBs

In addition to EB, DopR also is expressed in MB (Kim et al., 2007a; Lebestky et al., 2009; Fig. 6*A,B*), the main anatomical structure underlying olfactory memory and learning in *Drosophila*. Mutations in the *DopR* gene can abolish short-term memory and LTM and restoring DopR expression in the γ-neuron subset of MB is sufficient to rescue both short-term memory and LTM (Kim et al., 2007a; Lebestky et al., 2009; Qin et al., 2012). We therefore wondered whether the same *miR-276a::DopR* regulatory relationship in EB also occurs in MB to modulate DopR expression levels. We examined DopR expression levels by immunohistochemistry in brains where *UAS::EGFP::miR-276aSPONGE* is expressed in *OK107*-labeled MB neurons. We found that there is indeed a substantial elevation of DopR expression in MB when we drive the "sponge" transgene in MB compared with control animals (*UAS::EGFP::miR-276aSPONGE/+* heterozygous animals) (Fig. 6*C,D*). These results are consistent with the effects on DopR expression observed with QPCR (Fig. 4).

The finding that miR-276a regulates DopR in MB suggests a role in olfactory memory. In the case of the $miR\text{-}276a^{D8/Rosa}$ mutant animals, the defect in naive odor responses precluded drawing conclusions about memory performance per se because the conditioning process involves responses to these same odors. But the differing effects on naive odor avoidance of cell type-specific "sponge" expression in EB versus MB suggested that we might be able to separate effects on memory from those on naive odor responses. Expression of the *UAS::EGFP::miR-276aSPONGE* in MB using GAL4 driver line *OK107* in fact did not cause defects in naive olfactory avoidance to any of the three odors tested or to shock avoidance (Figs. 3*I*, 6*I*; BA, $F_{(2,9)} = 2.13$, not significant; OCT, $F_{(2,9)} = 0.47$, not significant; shock, $F_{(2,19)} = 0.39$, not significant). Because odor avoidance and





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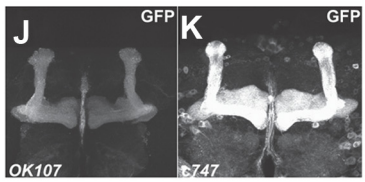
genotype	Olfactory Acuity		Shock Reactivity
	BA $0.5x10^{-3}$ v/v	OCT $1.5x10^{-3}$ v/v	60 volts
UAS::EGFP::SPONGE#1/+; UAS::EGFP::SPONGE#2/+	28.61±4.17	50.85±2.58	54.07±5.05
OK107/+	18.36±3.36	45.64±9.11	47.06±7.39
UAS::EGFP::SPONGE#1/+; UAS::EGFP::SPONGE#2/+;OK107/+	20.57±3.51	55.15±7.38	45.26±7.50



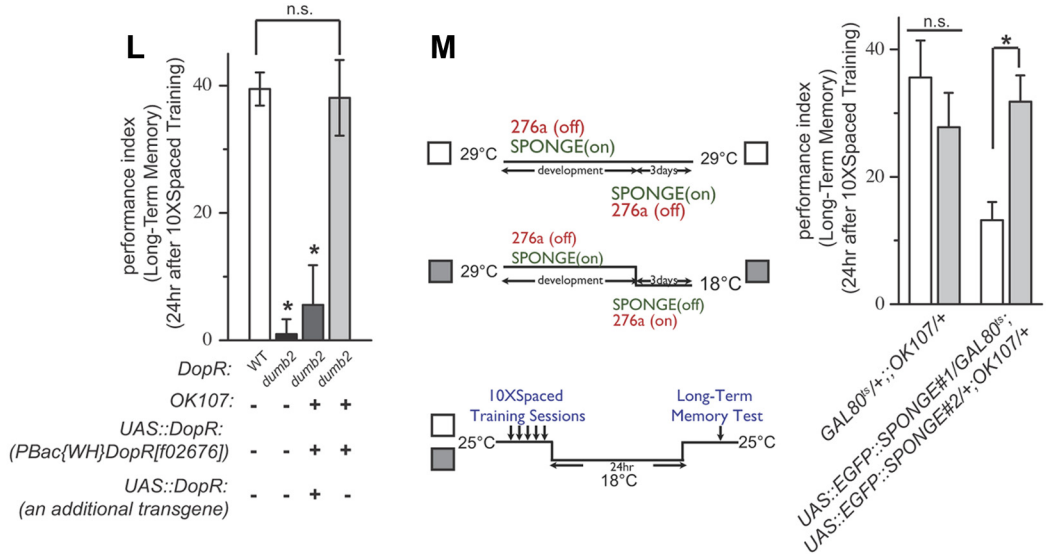


Figure 6. miR-276a regulates DopR in MB for LTM. ***A***, ***B***, DopR expresses in MBs and EB of the fly brain. ***C***, ***D***, Expression of *UAS::EGFP::miR276aSPONGE* transgenes with MB GAL4 line *OK107* results in elevated DopR expression in MB (***D*** compared with ***C***). Immunohistochemistry for control (***C***) and experimental (***D***) brains were performed in parallel and images were taken at identical confocal settings. ***E–J***, Expression of *UAS::EGFP::miR276aSPONGE* transgenes with MB GAL4 line *OK107* causes impaired LTM performance compared with WT and heterozygous controls (***E***; $*p < 0.05$; $N = 8$). In contrast, memory measured 24 h after 10 massed training sessions (***F***) or short-term memory measured 3 min after a single training session (***G***) each appeared normal (***F***; n.s., not significant; $N = 7$; ***G***, n.s., not significant; $N = 8$). As with the *OK107* GAL4 line, expression of *UAS::EGFP::miR276aSPONGE* with *c747*, a second MB-expressing GAL4 line resulted in significantly impaired LTM performance. The LTM defect can be fully suppressed in the presence of one copy of $DopR^{dumb1}$ strong mutant allele (***H***; $*p < 0.05$; n.s., not significant; $N = 14$). Olfactory acuity and shock reactivity appeared normal when *UAS::EGFP::miR276aSPONGE* transgenes were expressed with *OK107* (***I***; n.s. not significant; $N = 4–8$). *OK107* and *c747* expression patterns are shown in ***J*** and ***K***. ***L***, A PiggyBac (PBac) insertion within *DopR* produces the $DopR^{dumb2}$ allele, which is defective in forming LTM ($*p < 0.05$; $N = 8$). This PBac insertion contains a UAS-enhancer element (*Figure legend continues.*)

shock reactivity were normal with miR-276a "sponge" expression in MB, it provided a means to test the role in memory in a meaningful way without the caveats that come with defects in task-relevant sensorimotor responses. We therefore tested the effects on memory and learning of expressing the sponge in MB with two GAL4 lines (Fig. 6*E–H,J,K*). Although responses to all three odors tested appear normal with MB-driven "sponge" expression, we selected OCT and BA for these discriminative olfactory conditioning experiments because the naive response defects to these odors are mild compared with MCH even for the case where miR-276a function is compromised in the whole animal (Fig. 1*I*). With the *OK107* GAL4 line, we observed a significantly reduced memory performance measured 24 h after spaced repetitive training (LTM) (Fig. 6*E*; $F_{(3,28)} = 11.49$, $p < 0.05$) but not after repetitive massed training or immediately after one training session (Fig. 6*F,G*; *F*, $t_{(12)} = 0.08$, not significant; *G*, $t_{(16)} = 2.67$, not significant). Thus, the defect appears specific to LTM, which requires new protein synthesis (Tully et al., 1994). LTM is similarly reduced with GAL4 line *c747*, which also labels MB neurons (Fig. 6*H*; $F_{(2,39)} = 6.60$, $p < 0.05$).

We wondered whether miR-276a function in MB for LTM is mediated by regulation of *DopR* expression, as was the case in EB for naive olfactory responses to MCH. We introduced a copy of a strong *DopR* allele into animals that also express *UAS::EGFP::miR-276aSPONGE* in MB. Because this experiment made use of GAL4 to drive the sponge transgene, we used the $DopR^{dumb1}$ allele rather than $DopR^{dumb2}$ because the latter allele contains a GAL4-responsive UAS element upstream of the *DopR* coding region (see below). We found that removing one copy of the *DopR* gene was sufficient to fully suppress the effects on olfactory memory caused by expressing the dominant negative miR-276a "sponge." The defective LTM observed in *UAS::EGFP::SPONGE#1/c747; UAS::EGFP::SPONGE#2/+* animals is fully reversed in *UAS::EGFP::SPONGE#1/c747; UAS::EGFP::SPONGE#2/+*, $DopR^{dumb1/+}$ animals (Fig. 6*H*). This experiment is consistent with the idea that miR-276a normally holds DopR levels in check within MB. When the miRNA function is reduced, DopR levels increase (Figs. 4, 5, 6*C,D*), and removing one copy of the *DopR* gene suppresses the effect. A prediction of this dosage sensitivity hypothesis is that overexpression of DopR in MB above and beyond the levels normally seen also should compromise LTM. To test this idea, we compared the effects on LTM of expressing three different levels of DopR in MB. First, the $DopR^{dumb2}$ homozygous mutation has very little expression of DopR and results in profoundly deficient LTM performance (Qin et al., 2012). The $DopR^{dumb2}$ allele is caused by insertion of a P-element in the upstream region (Kim et al., 2007b). Because this P-element contains a GAL4 responsive UAS enhancer, the memory defects can be rescued when combined with a strong MB-GAL4 line, such as OK107, which drives expression of the flanking DopR gene on each of the two $DopR^{dumb2}$ alleles in the homozygous mutant (Kim et al., 2007b; Qin et al., 2012). To drive even higher levels of DopR within MB neurons, we tested the effects of adding a third UAS-responsive transgene (*UAS::DopR*). This results in an LTM defect that is as severe as that seen with the strong loss-of-function homozygous mutant (Fig. 6*L*; $F_{(3,28)} = 19.66$, $p < 0.05$).

Acute function of miR-276a is sufficient for normal olfactory memory

Postdevelopment function of miR-276a is sufficient to restore the naive olfactory response defect of *miR-276a* mutant animals (Fig. 3*G*). To test whether acute expression of miR-276a also is sufficient to restore normal LTM, we again introduced a copy of temperature-sensitive $GAL80^{ts}$. Animals that contained the SPONGE transgenes, the *OK107* GAL4 line, and the $GAL80^{ts}$ (*UAS::EGFP::SPONGE#1/GAL80^{ts}; UAS::EGFP::SPONGE#2/+; OK107/+*), and control groups that contained the $GAL80^{ts}$ and the *OK107* GAL4 line ($GAL80^{ts}$*/+;;OK107/+*) were each raised at the restrictive temperature (29°C) to keep the "sponge" transgene induced and the miR-276a function blocked in *OK107*-labeled MB neurons during development. After eclosion, we separated the progeny from each cross into two groups: one was continuously incubated at the restrictive temperature (29°C) where miR-276a function remained off in MB, and the other one was incubated at the permissive temperature (18°C) allowing miR-276a function to be turned back on in MB. Both groups were incubated for an additional 72 h before being tested for LTM. We found that activation of miR-276a function in MB after development was sufficient to support fully normal LTM performance (Fig. 6*M*; $F_{(3,28)} = 4.35$, $p < 0.05$). In control groups there were no significant differences between temperature-shifted and temperature-unshifted groups. Thus, as with naive olfactory avoidance responses, postdevelopmental function of miR-276a also is sufficient to support LTM.

Together, our findings support the conclusion that naive and conditioned odor responses each require miR-276a function, but in distinct neural circuits. Moreover, in each case DopR is a functional downstream regulatory target. Our data support a model (Fig. 7) in which the levels of DopR are tuned by *miR-276a* within each of these two neural circuits.

Discussion

MiRNAs have been proposed to provide robustness to gene regulatory networks (Herranz and Cohen, 2010; Peláez and Carthew, 2012), but they can also act as cell fate or developmental switches (Flynt and Lai, 2008). In the brain, perturbations of miRNA biogenesis have major impacts on development, neurodegeneration (Giraldez et al., 2005; Kim et al., 2007a; Schaefer et al., 2007; Berdnik et al., 2008; Cuellar et al., 2008; Davis et al., 2008), and behavior, such as circadian rhythms and memory. For example, disruption in mice of the miRNA-processing enzyme Dicer enhances synaptic plasticity and fear memory (Konopka et al., 2010). Cell type-specific disruption of *dicer-1* in *Drosophila* also impairs circadian behavior (Kadener et al., 2009). Because such disruptions of the miRNA biogenesis and effector machinery affect production or function of all or most miRNAs, it is not unexpected that phenotypic effects are pervasive and pleiotropic.

←

(*Figure legend continued.*) that can drive DopR expression when Gal4 is present. Expression of DopR in *OK107*-labeled MB neurons via the UAS element contained within this PBac is sufficient to restore both short-term memory and LTM (Qin et al., 2012). To investigate the effects of higher levels of overexpression of DopR in *OK107*-labeled MB neurons, we tested the effects of combined expression from an additional *UAS::DopR* transgene and the PBac insertion line. The addition of the *UAS::DopR* transgene results in LTM defect. $^*p < 0.05$; $N = 8$. ***M***, Postdevelopment induction of miR-276a function was accomplished with a $GAL80^{ts}$ transgene. Animals were grown at 29°C to allow expression of the *UAS::EGFP::miR276aSPONGE* under control of the *c747* GAL4 line. After development, animals were either maintained at 29°C or shifted to 18°C for 3 d to allow miR-276a function to return. Subsequent 10× spaced training sessions for LTM experiment were conducted at 25°C. Trained animals were kept at 18°C before testing for 24 h memory at 25°C (left; for details, see Results and Materials and Methods). The LTM defect resulting from expression of *UAS::EGFP::miR276aSPONGE* can be fully restored in the temperature-shift group in which miR-276a function was turned back on postdevelopmentally. LTM performance was normal in control $GAL80^{ts}$*/+; OK107/+* animals (right; $^*p < 0.05$; n.s., not significant; $N = 8$).

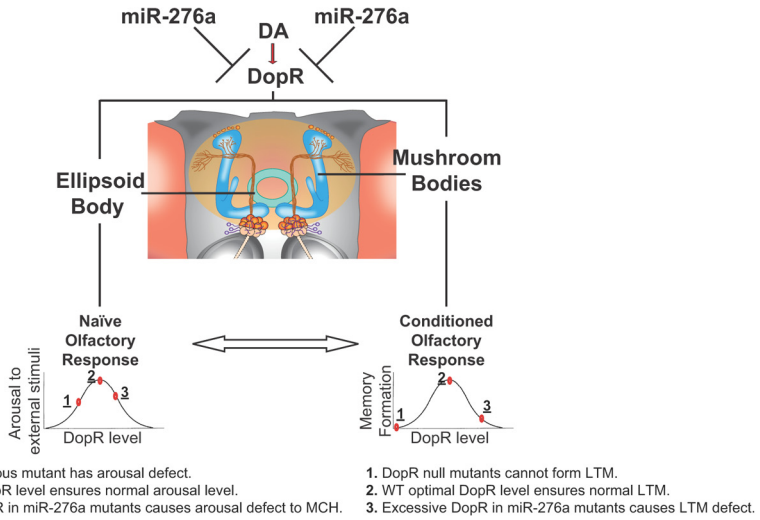


Figure 7. A model in which miR-276a tunes DopR levels in EB and MB for naive olfactory arousal response and LTM. Distinct dopaminergic fibers innervate EB and MB respectively. In EB, DA modulates arousal thresholds via activation of DopR. In MB γ neurons, DA release conveys the unconditioned stimulus reinforcement, which is mediated by DopR. Too much or too little DopR expression (red dots) within EB or MB is sufficient to disrupt the normal responses to external stimuli and the formation of LTM. DopR levels normally are tuned by miR-276a to provide an optimal balance. An intriguing hypothesis is that long-lasting plasticity in DopR levels within EB may affect retrieval of LTM by altering arousal to the CS+ odor, such as MCH.

MiRNA profiling studies in brain in fact demonstrate that different neuronal cell types express distinct populations of miRNAs and some of the neuronal miRNAs distribute to different subcellular localizations (Lagos-Quintana et al., 2002; Krichevsky et al., 2003; Kim et al., 2004, 2007a; Miska et al., 2004; Sempere et al., 2004; Kaern et al., 2005; Kye et al., 2007; Landgraf et al., 2007; Edbauer et al., 2010; Natera-Naranjo et al., 2010; Miñones-Moyano et al., 2011; He et al., 2012). Moreover, each miRNA gene in principle can regulate many different targets across multiple cell types, and each mRNA can in principle be targeted by multiple miRNAs. Thus cell-type-specific manipulations of individual miRNAs within an *in vivo* context are needed to decipher underlying mechanisms and functionally relevant targets. A series of recent studies have implicated individual miRNA genes in brain development, neurodegeneration, plasticity, and behavior (Cheng et al., 2007; Karres et al., 2007; Cayirlioglu et al., 2008; Chandrasekar and Dreyer, 2009; Kadener et al., 2009; Rajasethupathy et al., 2009; Gao et al., 2010; Hansen et al., 2010; Magill et al., 2010; Nudelman et al., 2010; Kye et al., 2011; Lin et al., 2011; Mellios et al., 2011; Liu et al., 2012; Luo and Sehgal, 2012). Several of these studies hint at the idea that, for a given phenotype, several miRNAs can converge on a common target, and there are a few cases where phenotypic effects may be mediated largely via one common target (Cayirlioglu et al., 2008; Yoon et al., 2011; Luo and Sehgal, 2012). In the case of memory and synaptic plasticity, there is some evidence for convergence of miRNAs (and Piwi-interacting RNAs) onto CREB as a target (Rajasethupathy et al., 2009, 2012; Gao et al., 2010; Yang et al., 2012). But there still are relatively few cases where miRNA gene function has been established within neural circuits for specific behaviors, and the architecture of downstream regulatory effects of miRNAs on brain function in general and on memory in particular are poorly understood.

We took advantage of both classic and modern *Drosophila* genetic approaches to manipulate *miR-276a* function in defined neural circuits with temporal specificity. Starting with a hypomorphic allele identified in a forward mutagenesis screen (Dubnau et al., 2003), we engineered a null mutation, precise excisions, BAC rescue transgenes, GAL4-responsive transgenes, and a GAL4-responsive dominant negative "sponge" transgene. The "sponge" method (Loya et al., 2009; Ebert and Sharp, 2010) in particular provided a means to manipulate *miR-276a* function *in vivo* with cell type and temporal specificity of the GAL4 transactivator system. Proof of principle experiments with "sponges" for miR-7, miR-8, and miR-9a in appropriate tissues produce comparable developmental phenotypes as classic loss-of-function mutant alleles (Loya et al., 2009).

Similarly, the miR-276a "sponge" used in this study was able to phenocopy the effects observed in *miR-276a* mutant animals. Combining this dominant negative "sponge" with GAL4/GAL80^{ts} reagents provided the means to dissect miR-276a postdevelopment function underlying two different behavioral phenotypes into distinct neural circuits. This provides the first example in which a miRNA gene's function is demonstrated in behaving animals with both cell-type and temporal specificity. By separately testing effects of miR-276a manipulation within two different neural cell types, we uncovered distinct effects on two related olfactory behaviors. When the sponge was used to interfere with miR-276a function within all neurons, we observed defective responses to odors with naive animals. This precluded a meaningful test of performance in the olfactory memory task. Surprisingly, sponge expression within each of the major cell types of the main olfactory system had no impact on olfactory responses, but when we used the sponge to block miR-276a function in EB neurons, we reproduced the defect in naive responses to MCH. In contrast, sponge expression in MB neurons did not affect naive responses, which provided an opportunity to test olfactory memory without the confounds that come from odor response defects. The cell-type specificity of miR-276a function in *c547*-labelled R2/R4m EB neurons for naive responses to MCH and in MB intrinsic neurons for LTM also pointed to a functionally relevant downstream target from among those suggested by bioinformatics predictions and QPCR validations.

We focused on *DopR* both because it contains a conserved miR-276a binding site and because, like miR-276a, DopR function has been mapped to MB for memory and to EB for naive responses (odors for miR-276a; ethanol and startle response for DopR). We were able to verify that DopR expression is regulated by miR-276a both at the transcript levels in response to transgenic miR-276a induction and at the protein level within MB in response to "sponge" expression. Although we cannot be certain that the regulation of DopR is direct, the sign of the effect is as predicted for a direct target. More importantly, the regulatory relationship is biologically relevant. Both behaviors are fully suppressed when one copy of *DopR* gene is removed. This supports the conclusion that overexpression of DopR contributes to both behavioral defects observed in miR-276a mutants. In fact, transgenic DopR overexpression in MB was sufficient to produce an LTM defect.

Together with evidence from the literature, these findings suggest a model in which DopR expression levels are dosage-sensitive both for LTM and for naive behavioral responses. We

propose that under physiological conditions, a miRNA::DopR regulatory module tunes the levels of DopR in neural circuits underlying naive olfactory responses and olfactory LTM (Fig. 5). In the case of LTM, loss-of-function mutations (Qin et al., 2012) or MB-driven overexpression of DopR (Fig. 4*E*) each yield decreased memory performance similar to that observed with MB expression of the miR-276a "sponge." Similarly the effects of EB-driven miR-276a "sponge" are rescued by reducing the copy number of *DopR*. This dosage sensitivity is consistent with the fact that loss of even one copy of *DopR* in a miR-276a $^{+/+}$ animal causes elevated startle-mediated arousal (Lebestky et al., 2009).

Dopaminergic signaling also has been demonstrated to set thresholds for multiple types of arousal (Van Swinderen and Andretic, 2011). While the role of DopR in these various forms of arousal is complex, effects on ethanol-induced and repetitive startle-induced arousal also have been mapped to *c547*-labeled R2/R4m EB neurons. We thus interpret the reduced naive odor responses with *c547*-driven miR-276a "sponge" to be a result of reduced olfactory arousal, although this remains to be tested. Also, it should be noted that we have tested the effects of c547-EB-driven sponge on naive responses to just one odor, MCH.

The potential connection between EB-mediated arousal and MB-mediated olfactory memory is an intriguing one. NMDA receptor function is in fact required in these same EB neurons for normal LTM formation (Wu et al., 2007). So the EB cell types in which miR276a functions for naive avoidance to MCH also are a part of the circuit for LTM. One attractive possibility is that behavioral experience modulates functional levels of DopR within MB and EB. In this case, the observed role of EB on olfactory LTM could derive from long-lasting changes in CS+ arousal mediated by a miR-276a::DopR regulatory mechanism.

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