Crossmodal Interactions Between Olfactory and Visual Learning in *Drosophila*

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Different modalities of sensation interact in a synergistic or antagonistic manner during sensory perception, but whether there is also interaction during memory acquisition is largely unknown. In *Drosophila* reinforcement learning, we found that conditioning with concurrent visual and olfactory cues reduced the threshold for unimodal memory retrieval. Furthermore, bimodal preconditioning followed by unimodal conditioning with either a visual or olfactory cue led to crossmodal memory transfer. Crossmodal memory acquisition in *Drosophila* may contribute significantly to learning in a natural environment.

Information received by the nervous system through multiple sensory modalities must be processed in an integrated manner. Extensive crossmodal interaction during sensory processing and perception has been found in psychophysical studies, functional imaging of the human brain, and electrophysiological recordings from monkeys (*1*). Odor localization in *Drosophila* requires visual feedback during free flight (*2*), which suggests dynamic crossmodal sensory processing. Crossmodal interaction during memory acquisition has also been suggested by the finding that honeybees exposed to sequential color and scent conditioning can recall a specific color when they encounter a particular scent (*3*). In the present study, we aimed to explore possible crossmodal interactions during learning in *Drosophila*. Previous studies of *Drosophila* learning using unimodal sensory cues have shown robust visual learning of colors, patterns, and textures (*4*–*7*), and olfactory learning of distinct odors (*8*–*11*). Using a modified version of the classical visual flight simulator (*12*), we simultaneously applied visual and olfactory cues during reinforcement learning (*13*).

We first examined reinforcement learning (Fig. 1A) using an olfactory learning paradigm analogous to visual operant conditioning (*4*–*7*). Individual flies were trained to associate a particular odor-related flight orientation in the dark with a punishment (noxious heat stimulus). As shown in Fig. 1B, the fly frequently shifted its orientation from one 90° sector to another during the pretraining period (6 min), with no preference between 3-octanol (OCT, 1.96%) and 4-methylcyclohexano1 (MCH, 1.96%). The flies were then exposed to a training session (two 4-min periods separated by a 2-min test period) during which a noxious heat stimulus was switched on whenever OCT-related flight orientation enters the frontal 90° sector of fly's visual field. Posttraining test (8-min session in the absence of heat application) showed that the flies exhibited persistent preference for MCH (Fig. 1B). Course-control maneuvers of the tethered flight were evaluated quantitatively using the "preference index" (PI) defined as $PI = (t_c - t_h)/(t_c + t_h)$, where t_c is the time the fly was oriented toward a sector not associated with heat, and t_h is the time the fly was oriented toward a sector that was associated with heat during training (*13*). As shown in Fig. 1C, PI before the training (blocks 1 to 3) was close to zero, whereas PI during training (blocks 4, 5, 7, and 8) and during the test after training (blocks 9 to 12, "test PI") were significantly positive. A similar

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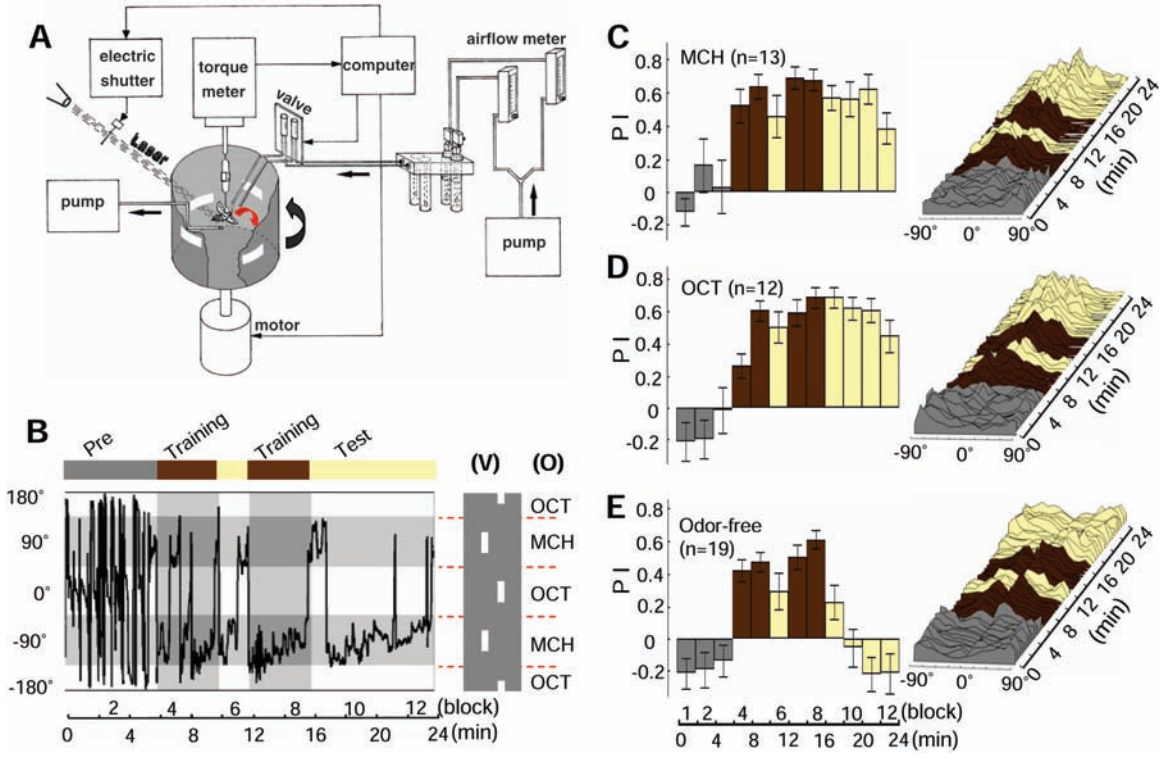

Fig. 1. Olfactory operant conditioning in the flight simulator. (**A**) The flight simulator and the operant conditioning procedure have been described previously (*12*). A description of the modifications for using them in olfactory conditioning is provided in the SOM (*13*). (**B**) A representative trace of flight path of a single fly with time in arena position (–180° to 180°) during preconditioning ("pre"), training, and test period, for unimodal olfactory conditioning in the dark. Shown on the right are corresponding visual (V) and olfactory (O) cue locations in the arena. (**C**) Summary of olfactory operant conditioning. Single *CS* flies were trained to prefer OCT to MCH. The test PI (average of PIs for block 9 to 12) was significantly positive ($P < 0.001$). (Right) The diagram of the relative amount of time spent by all tested flies in different directions between –90° and +90°. (**D**) Similar to (C), but *CS* flies were trained to prefer MCH to OCT. (**E**) Similar to (C), but the airflow was odor-free. Test PI was not significantly different from zero ($P > 0.05$). All *P* values were based on one-sample *t* test. n, the total number of flies examined.

preference for OCT rather than MCH was found when the flies were trained to avoid MCH (Fig. 1D). Also shown in Fig. 1, C and D, are angular histograms of flight path at 0.5-min intervals, indicating the relative amount of time spent by all flies in different directions between –90° and +90° relative to the preferred odor (the responses over four quadrants from –180° to +180° were pooled into two quadrants from –90° to +90° relative to the preferred direction). In control experiments using odor-free airflow, flies failed to learn to associate the airflow with the punishment, with test PIs not significantly different from zero (Fig. 1D).

Crossmodal facilitation of visual and olfactory perception has been found in human studies (*14*). There is the "principle of inverse effectiveness," in which crossmodal enhancement of perception is more pronounced when individual sensory stimuli are minimally effective (*15*). Moreover, flies' motor response to simultaneously presented visual and olfactory cues closely approximates the linear superposition of the response to each stimulus presented in isolation (*16*). We thus inquired whether *Drosophila* learning exhibits crossmodal facilitation of memory acquisition by testing visual or olfactory memory near the threshold level of conditioning stimuli. The threshold level of visual or olfactory learning in the flight simulator was defined by the magnitude of the conditioning cue at which the test PI becomes statistically not different from zero. For unimodal visual (V) learning, we trained the fly to associate punishment with either one of two white horizontal bars (on a dark background) located at different vertical positions defined by their center of gravity (COG) (*7*), alternatively presented in one of four quadrants in the visual field. The test PIs dropped to zero between 5° and 7° COG separation for both *WTB* (*wild-type Berlin*) and CS (*Canton-S*) wild-type flies (Fig. 2A, top). For determining the threshold of unimodal olfactory (O) learning in the flight simulator, the percentage concentration of the two odors (OCT and MCH) applied was varied from 0.99% to 0%. Flies failed to associate odor with punishment when the odor concentration was reduced from 0.59 to 0.39% for both *WTB* and *CS* flies (Fig. 2A, bottom).

To examine crossmodal facilitation, we applied bimodal conditioning by training single flies to associate the punishment with simultaneously presented visual and olfactory cues, which consisted of a fixed pairing of odor application and a particular flight orientation associated with the visual cue. When the subthreshold cue intensity for unimodal learning (5° for COG separation and 0.39% for odor concentration) was used for bimodal conditioning, highly significant test PI values for both *CS* and *WTB* flies were found (Fig. 2B). This apparent facilitation of learning via bimodal conditioning was not simply due to the addition of airflow in visual learning or of visual stimulation during olfactory learning, because the flies failed to exhibit significant learning when odor-free airflow (0%) or zero visual cue (0°) was used (Fig. 2C). Finally, to address whether acquisition of unimodal memory can be enhanced by the presence of simultaneous conditioning with a different sensory stimulus, which would imply crossmodal interaction during memory acquisition, we examined flies' memory with the unimodal cue below its threshold level for unimodal learning after the bimodal conditioning described above. We found significant memory retrieval during the test period (block 10 to 12) with either the subthreshold visual cue (5°) or the olfactory cue (0.39%) after bimodal conditioning with these cues (Fig. 2D). These results extend the notion of synergism in memory acquisition from unimodal to bimodal learning.

Previous flight simulator studies have shown that robust "sensory preconditioning" of flies by exposure to simultaneous color and shape visual cues without reinforcement led to the transfer of memory from the color to the shape cue, or vice versa, after pairing of the single (color or shape) cue with the punishment (*17*). In the present study, we examined this concept of memory transfer from unimodal to bimodal memory acquisition, using a similar sensory preconditioning paradigm. Single flies were

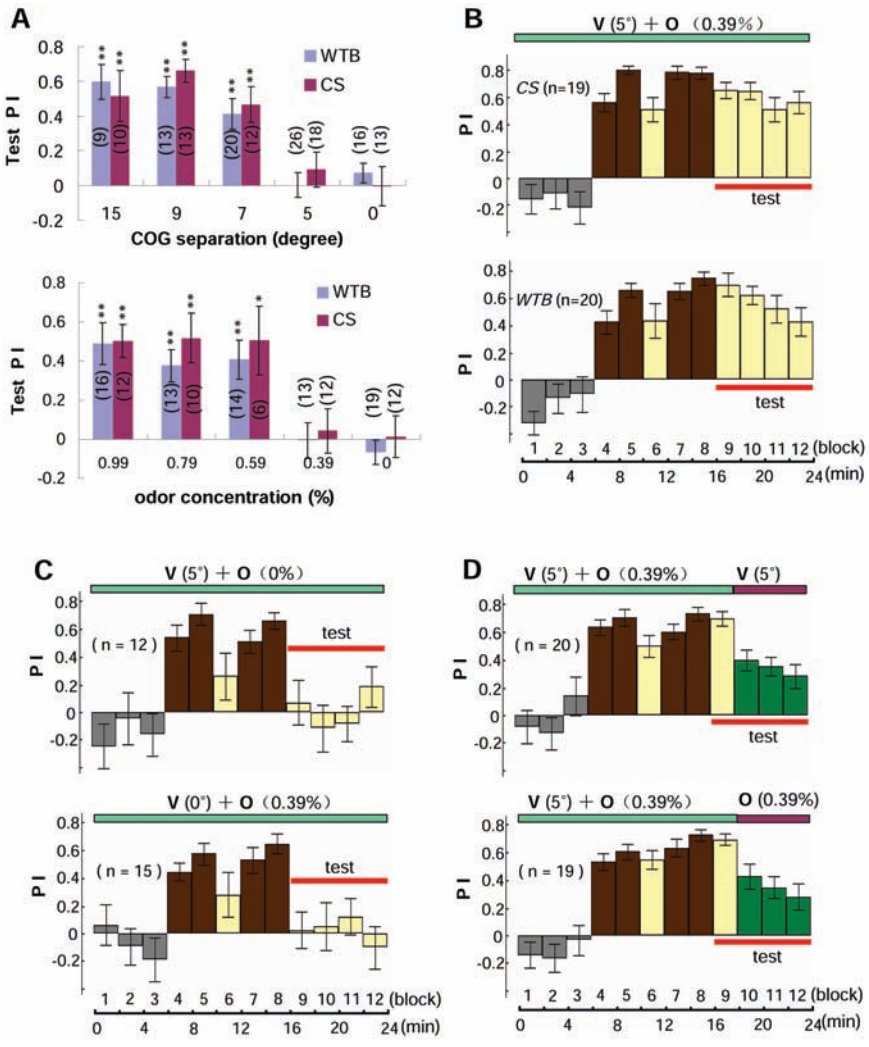

Fig. 2. Crossmodal enhancement in memory acquisition. (**A**) (Top) The threshold of visual learning. Five visual cue separations (ΔCOG from 0° to 15°) were tested. The average PI values during the test period (test PI) for 5° and 0° were not significantly different from zero ($P > 0.05$). $^{**}P < 0.01$; $^{*}P < 0.05$. (Bottom) The threshold of olfactory learning. Five concentrations of MCH/OCT (from 0 to 0.99%) were examined. Test PI for 0.39% and 0% was not significantly different ($P > 0.05$). The number in parentheses refers to the total number of flies examined. (**B**) Flies were conditioned with paired visual (5°) and olfactory (0.39%) cues. The test PI for *CS* (top) and *WTB* (bottom) was significant ($P < 0.001$). (**C**) Control experiments with the visual cue (5°), together with odor-free airflow (top), and with olfactory cue (0.39%), together with visual cue separation of 0° ΔCOG (bottom). The test PI was not significant ($P > 0.05$). (**D**) The same as in (B), except that the PI was examined during the test period for unimodal memory below its threshold. Test PI (blocks 10 to 12) for visual (V, 5°, top) or olfactory (0.39%, bottom) cue alone were significantly positive ($P < 0.001$).

allowed to fly without reinforcement (unconditioned stimulus, US^-) for eight 2-min blocks with the simultaneous presentation of fixed pairs of visual and olfactory cues (COG separation of 30°, OCT or MCH concentration at 1.96%). The flies were then conditioned (US^+) by pairing the heat punishment with the same visual cue, followed by memory acquisition test (US^-, 3 blocks) with the olfactory cue that was associated with the visual cue during preconditioning. Alternatively, the flies were conditioned by the olfactory cue after bimodal cue preconditioning and tested for memory acquisition with the associated visual cue used in preconditioning. We observed significant crossmodal transfer of memory between vision and olfaction (Fig. 3A). Note that it is necessary to present visual (V) and olfactory (O) cues concurrently during sensory preconditioning, because sequential presentation of the visual and olfactory cues (V/O, each for 50 ms) without interruption during preconditioning resulted in no transfer of memory after the single-cue conditioning (Fig. 3B).

Sensory preconditioning without reinforcement may represent a form of "incidental learning" (*18*), in which two equally salient stimuli are presented simultaneously in an equivalent manner, in contrast to the temporally asymmetric presentation of conditioned and unconditioned stimuli. We thus inquired whether crossmodal transfer of memory to unconditioned cue (either V, US^- or O, US^-; Fig. 3A) requires the presence of the memory for the conditioned cue (either O, US^+ or V, US^+). We performed reversal conditioning experiments in which the conditioned flies were punished during a brief postconditioning period by noxious heat stimulus whenever they assumed flight orientations that previously had been "safe." Flies were conditioned to prefer one of the two horizontal bars in standard training sessions and then subjected to a single 1-min reverse conditioning (RC) session (16 to 17 min) (Fig. 3C, top). Subsequent tests of memory acquisition showed that preference for the previously conditioned cue was completely abolished. Similar experiments for olfactory conditioning also showed the effectiveness of 1-min RC in abolishing the memory for the conditioned olfactory cue. Control experiments using no visual stimulation (dark, D) or no odor (stopping odor delivery, S) during the 1-min postconditioning block showed the persistence of memory for the conditioned cue (Fig. 3C, bottom). The test PIs for all RC experiments on unimodal visual or olfactory learning are summarized in Fig. 3D.

Next, we examined whether memory transfer still occurs in flies subjected to RC experiments. Flies preconditioned with simultaneous visual and olfactory cues (0 to 16 min) were then subjected to the visual cue conditioning (16 to 26 min) (Fig. 3E). Subsequent 1-min RC (26 to 27 min) on the visual cue resulted in a complete absence of memory for the olfactory cue that was paired with the conditioned visual cue during preconditioning, suggesting no memory transfer from vision to olfaction. Control experiments using no visual stimulation (dark, D) during the 1-min postconditioning block (26 to 27 min) in the absence of RC punishment showed the persistence of memory

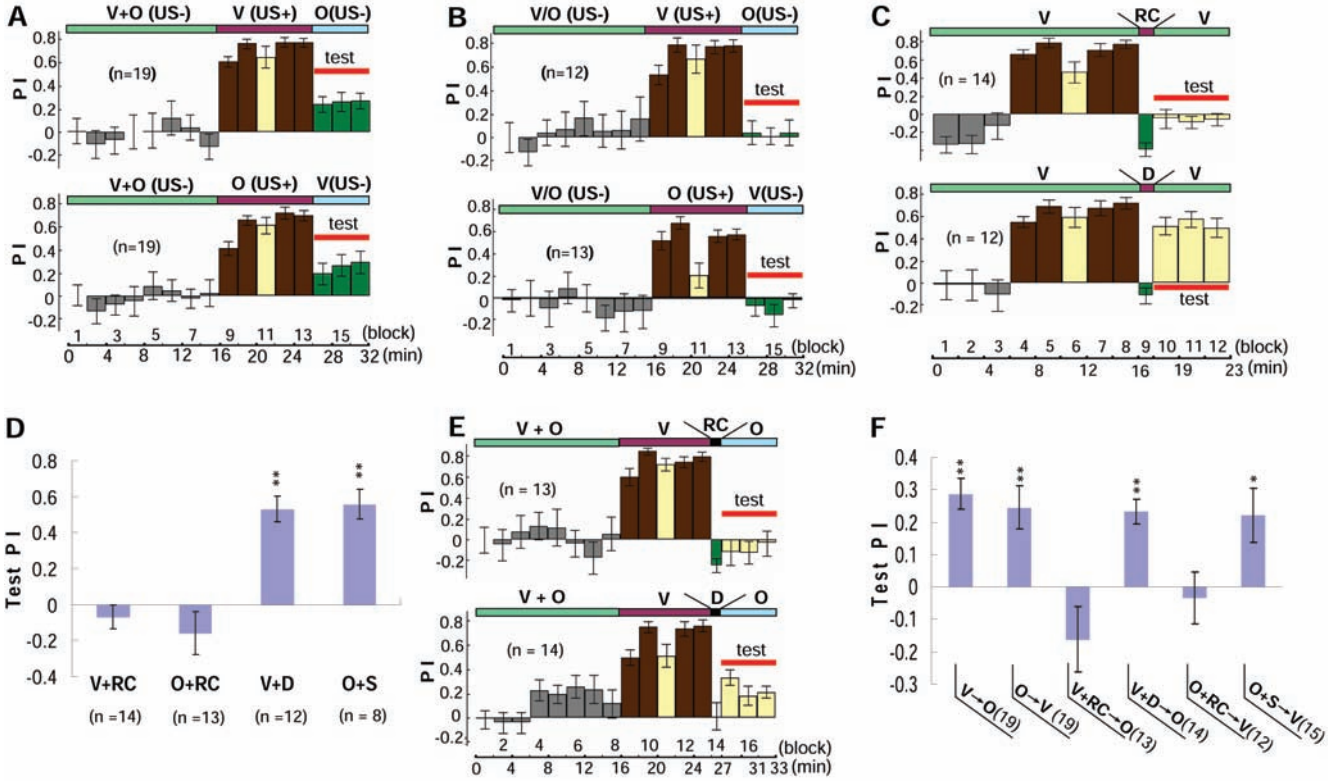

Fig. 3. Crossmodal memory transfer. (**A**) (Top) Individual *CS* flies were subjected to bimodal stimuli of ΔCOG = 30° and OCT/MCH (1.96%) for 16 min without reinforcement (O + V, US^-), followed by unimodal visual training (V, US^+), with an intervening 2-min test period. The test PI for retrieval with the olfactory cue (O, US^-) was significantly positive ($P < 0.001$). (Bottom) The test PI (V, US^-) was significantly positive ($P < 0.01$) after olfactory unimodal training. Similar results were obtained from *WTB* flies (not shown in figure). (**B**) Similar to (A), except that the visual and olfactory cues were presented alternately during preconditioning (50 ms each). The mean test PI (top: O, US^-; bottom: V, US^-) was not significant ($P > 0.05$). (**C**) (Top) RC after visual conditioning caused memory erasure; the test PI was not significant ($P > 0.05$). (Bottom) One-min darkness (D) did not impair memory, with positive test PI ($P < 0.001$). (**D**) Summary of all results showing that RC caused memory erasure (test PIs not significant, $P > 0.05$), stopping of the odor delivery for 1 min (at 16 to 17 min, O + S) did not cause memory erasure, with positive test PI ($P < 0.001$). (**E**) (Top) RC after visual conditioning (V + RC) caused failure of memory transfer from V to O (V→O), with insignificant test PI ($P > 0.05$). (Bottom) One minute of darkness after visual conditioning (at 26 to 27 min, V + D) caused no failure of memory transfer; the test PI was significant ($P < 0.001$). (**F**) Summary of all experiments similar to that in (A) and (E), together with data on the effects of the RC on olfactory training (O + RC), which caused failure of memory transfer (O→V), with insignificant test PI ($P > 0.05$). Stopping the odor delivery (26 to 27 min, O + S) caused no failure of memory transfer; the test PI was significant ($P < 0.05$). $^{**}P < 0.01$, $^{*}P < 0.05$ (One-sample *t* test).

transfer from vision to olfaction (Fig. 3E, bottom). Data for all memory transfer experiments are summarized in Fig. 3F. Thus, persistent memory for the conditioned cue is essential for crossmodal memory transfer.

The neural circuits and cellular mechanisms underlying the crossmodal enhancement and transfer of memory are unknown. Further understanding requires the elucidation of visual and olfactory circuits and their interconnection, as well as the locus for storage of visual and olfactory memory. It is possible that "multisensory integrative neuron" may also exist in the *Drosophila* brain and that crossmodal interaction between different sensory modalities may also be achieved through synchronized activity between modality-specific brain regions (*19*). Crossmodal interaction between sensory systems can enhance the detection and discrimination of external objects and can provide information about the environment that is unobtainable by a single modality in isolation. Our findings indicate that individual flies make use of crossmodal interactions between two sensory systems during operant conditioning, which further suggests that crossmodal interactions using multiple sensory systems may also facilitate learning in the natural environment. These results provide a basis for further studies of the circuit mechanisms underlying crossmodal interactions during memory acquisition.

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Supporting Online Material

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Materials and Methods

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MicroRNA Expression in Zebrafish Embryonic Development

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MicroRNAs (miRNAs) are small noncoding RNAs, about 21 nucleotides in length, that can regulate gene expression by base-pairing to partially complementary mRNAs. Regulation by miRNAs can play essential roles in embryonic development. We determined the temporal and spatial expression patterns of 115 conserved vertebrate miRNAs in zebrafish embryos by microarrays and by in situ hybridizations, using locked-nucleic acid–modified oligonucleotide probes. Most miRNAs were expressed in a highly tissue-specific manner during segmentation and later stages, but not early in development, which suggests that their role is not in tissue fate establishment but in differentiation or maintenance of tissue identity.

Current estimates of miRNA gene numbers in vertebrates are as high as 500 (*1*), of which many are conserved, and miRNAs may regulate up to 30% of genes (*2*). The miRNA first discovered, *lin-4*, is involved in developmental timing in the nematode *Caenorhabditis elegans* (*3*). In mammals, miRNAs have been implicated in hematopoietic lineage differentiation (*4*) and homeobox gene regulation (*5*). Zebrafish that are defective in miRNA processing arrest in development (*6*). Recently, miRNAs were shown to be dispensable for cell fate determination, axis formation, and cell differentiation but are required for brain morphogenesis in zebrafish embryos (*7*). Together, these findings indicate that miRNAs can play essential roles in development. However, little is known about the individual roles of most miRNAs. To focus future miRNA studies, we determined the spatial and temporal expression patterns of 115 conserved vertebrate miRNAs (see online Material and Methods; table S1; table S2) in zebrafish embryos.

First, we determined the temporal expression of miRNAs during embryonic development by microarray analysis (Fig. 1A and fig. S1A). Up to segmentation [12 hours post fertilization (hpf)], most miRNAs could not be detected. Most miRNAs became visible 1 to 2 days after fertilization and showed strong expression when organogenesis is virtually completed (96 hpf). In adults, the majority of miRNAs remained expressed (Fig. 1A). In addition we determined the expression of miRNAs in dissected organs of adult fish. For some miRNAs, a high degree of tissue specificity was observed (figs. S1B and S2, and table S3).

In situ hybridization of miRNAs had thus far not been possible in animals. Recently LNA (locked-nucleic acid)–modified DNA oligonucleotide probes have been shown to increase the sensitivity for the detection of miRNAs by Northern blots (*8*). By Northern blots analysis and in situ hybridization, using LNA probes, we detected predominantly mature miRNAs, which were reduced in dicer knockout zebrafish (fig. S3). We used these LNA probes for the whole-mount in situ detection of the conserved vertebrate miRNAs in zebrafish embryos and made a catalog of miRNA expression patterns (fig. S4 and database S1).

Most miRNAs (68%) were expressed in a highly tissue-specific manner. For example, miR-140 was specifically expressed in the cartilage of the jaw, head, and fins, and its presence was entirely restricted to those regions (Fig. 1B and database S1). Representative examples are shown (Fig. 1C) of six miRNAs that were expressed in different organ systems: nervous system, digestive system, muscles, circulatory system, sensory organs, and excretory system. Even within organs, there is specificity, as exemplified in Fig. 1D, where miR-217 can be seen to be expressed in the exocrine pancreas, and miR-7 in the endocrine pancreas (Langerhans islets). More than half of the miRNAs (43) were expressed in (specific regions of) the central nervous system (fig. S4). Many miRNA genes are clustered in the genome and, therefore, are probably expressed as one primary transcript, and indeed, we observed that many such clustered genes showed identical or overlapping expression patterns (figs. S4 and S5). We compared the in situ data with microarray

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