

The AKAP Yu is required for olfactory long-term memory formation in *Drosophila*

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Extensive neurogenetic analysis has shown that memory formation depends critically on cAMP-protein kinase A (PKA) signaling. Details of how this pathway is involved in memory formation, however, remain to be fully elucidated. From a large-scale behavioral screen in *Drosophila*, we identified the *yu* mutant to be defective in one-day memory after spaced training. The *yu* mutation disrupts a gene encoding an A-kinase anchoring protein (AKAP). AKAPs comprise a family of proteins, which determine the subcellular localization of PKAs and thereby critically restrict cAMP signaling within a cell. Further behavioral characterizations revealed that long-term memory (LTM) was disrupted specifically in the *yu* mutant, whereas learning, short-term memory and anesthesia-resistant memory all appeared normal. Another independently isolated mutation of the *yu* gene failed to complement the LTM defect associated with the *yu* mutation, and this phenotypic defect could be rescued by induced acute expression of a *yu*⁺ transgene, suggesting that *yu* functions physiologically during memory formation. AKAP Yu is expressed preferentially in the mushroom body (MB) neuroanatomical structure, and expression of a *yu*⁺ transgene to the MB, but not to other brain regions, is sufficient to rescue the LTM defect of the *yu* mutant. These observations lead us to conclude that proper localization of PKA by Yu AKAP in MB neurons is required for the formation of LTM.

A-kinase anchoring protein | cAMP pathway | mushroom body | olfactory memory

Extensive studies have shown that memory formation depends critically on the cAMP-protein kinase A (PKA) signaling pathway in vertebrates and invertebrates (1, 2). The subcellular location of PKA is determined by a family of A-kinase anchoring proteins (AKAPs), which are defined by their ability to copurify with PKA activity (3). These molecules are thought to serve as signal-organizing molecules and play a critical role in specifying functions of the cAMP pathway by compartmentalizing PKA with other signaling molecules. AKAPs have been reported to be involved in synaptic plasticity by the interaction with the regulatory subunits of PKA (4). For instance, in *Aplysia*, disruption of an AKAP::PKA-RII interaction by the inhibitory peptide, Ht31, prevents both the short- and long-term facilitation induced by serotonin (5-HT) (5). In rat, infusion into the lateral amygdale of inhibitory peptides that compete with PKA regulatory subunits for binding with AKAPs affects auditory fear memory (6). To date, however, no specific AKAP has been shown to be involved in memory formation.

Memory in *Drosophila* typically has been studied with a Pavlovian olfactory task in which an odor is temporally associated with foot-shock punishment (7) and has been extensively characterized at the molecular and anatomical levels (2, 8–10). Genetic dissection of the conditioned behavioral response has suggested several temporal phases, including acquisition (or learning, LRN), short-term memory (STM), middle-term memory (MTM), anesthesia-resistant memory (ARM), and protein synthesis-dependent long-term memory (LTM) (11). STM and MTM appear labile and short-lived, whereas ARM and LTM persist for several days. LTM is disrupted, but STM, MTM, and

ARM are normal, in flies fed the protein synthesis inhibitor, cycloheximide (CXM). One training session and ten massed training (no intersession rest interval) produce only a decremental and CXM-insensitive memory (STM, MTM, and ARM), but 10 spaced training sessions (a 15-min inter-session rest interval) produces STM, MTM, ARM and a protein synthesis-dependent LTM that persists for more than a week. We use one-day memory to determine ARM and LTM for massed training one-day memory contains only ARM, whereas for spaced training, one-day memory consists of ARM and LTM. STM and MTM have diminished 1 d after either massed or spaced training (11).

The cAMP signaling pathway is important for memory formation. The STM mutants, *rutabaga* (*rut*) (12) and *dunce* (*dnc*) (13), encodes adenylyl cyclase (AC) and cAMP-specific phosphodiesterase (PDE), respectively. Mutation of the *PKA-RI* gene disrupts both learning and memory (14). A temperature-sensitive mutant of *PKA-CI* (*DCO*) disrupts MTM (15). And, disruption of the transcription factor CREB (cAMP responsive element binding protein) specifically blocks LTM (16). Some of these memory-related functions of cAMP signaling have been mapped to the mushroom body (MB). Expression of a *rut*⁺ transgene in the MB is sufficient to rescue the STM defects of the *rut* mutant (17), and overexpression of CREB repressor in the MB blocks LTM formation (18).

From a large-scale behavioral screen for mutants with defects in one-day memory after spaced training, we have identified the *yu* mutant (*yu* means “foolish” in Chinese). LRN, STM, MTM, and ARM are normal, but LTM specifically is defective, in the *yu* mutant. The *yu* mutation disrupts a gene encoding an AKAP. Using anti-Yu antibody, we show that this gene is expressed preferentially in the MB. With a *yu*⁺ transgene, we also show that expression of Yu in the MB is sufficient to rescue the LTM defect of the *yu* mutant.

Results

Disruption of *yu* Specifically Impairs LTM. We conducted a P-element mutagenesis to screen behaviorally for X-linked mutants with defective one-day memory after spaced training. Eleven mutants were identified from 2,021 homozygous-viable transposant lines. By means of plasmid rescue and DNA se-

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Abbreviations: AKAP, A-kinase anchoring protein; ARM, anesthesia-resistant memory; LTM, long-term memory; MB, mushroom body; PI, performance index; PKA, protein kinase A; MTM, middle-term memory; RT, room temperature; STM, short-term memory.

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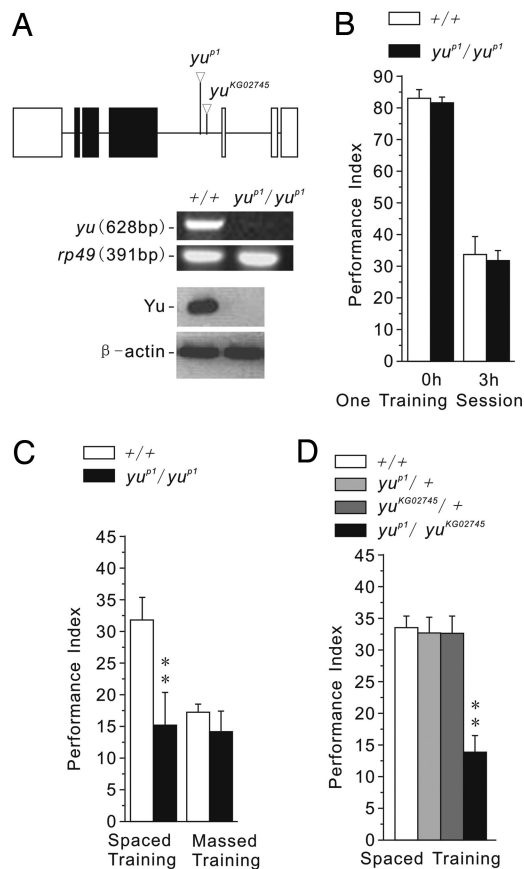


Fig. 1. LTM is specifically impaired in *yu* mutants. (A) Molecular-genetic characterization of *yu^{P1}* mutant. (Top) Plasmid rescue and sequencing of genomic DNA flanking the P-element insertion site indicated the P-element was inserted in the intron of *CG3249*, which encodes a novel putative AKAP. Perusal of the Bloomington Stock Center identified a second P-element insertion, *yu^{KG02745}*, near the insertion site of *yu^{P1}*. Blank box represents an untranslated region, the black box represents a protein coding region, and the line represents an intron. The transcription direction is from right to left. (Middle) RT-PCR experiments showed that the *yu* transcript was greatly reduced in *yu^{P1}/yu^{P1}* mutants. *Ribosomal protein 49* (*rp49*) was used as a loading control. (Bottom) Western blot analysis showed that Yu protein also was reduced greatly in *yu^{P1}/yu^{P1}* mutants. β -Actin was used as a loading control. Left blots are control flies (+/+); right blots are *yu^{P1}/yu^{P1}* mutants. (B) Memory retention, measured immediately or 3 h after one training session, was normal in *yu^{P1}/yu^{P1}* mutants ($n = 8$ PIs per group). All PIs of these and the following behavioral experiments are means and SEM. Statistical significances are determined from *t* test. Asterisks indicates critical values of *, $P < 0.05$ and **, $P < 0.01$. (C) One-day memory after spaced training was significantly impaired, but that after massed training was normal, in *yu^{P1}/yu^{P1}* mutants ($n = 8$ PIs per group). (D) Genetic complementation analysis between *yu^{P1}* and *yu^{KG02745}* for one-day memory after spaced training revealed the two mutations to be recessive to wild-type (+) and allelic (noncomplementing). Thus, the *yu* gene was involved in LTM formation ($n = 8$ PIs per group).

quencing, we determined the mutant, *yu^{P1}*, to carry a P-element insertion in an intron of the *CG3249* gene, which encodes a putative AKAP (Fig. 1A Top) (19). RT-PCR and Western blot analysis revealed that *yu* transcript and protein were undetectable in *yu^{P1}* mutants (Fig. 1A Middle and Bottom).

Memory retention in mutant *yu^{P1}* flies was normal when measured immediately or 3 h after one training session (Fig. 1B). Similarly, one-day memory after massed training (ARM) was normal, but one-day memory after spaced training (ARM and LTM) was impaired (Fig. 1C). One-day memory after spaced training, in fact, was similar to that after massed training in the *yu* mutant. Sensorimotor responses to the odors and foot-shock

Table 1. Sensorimotor responses of different genotypes to the odors and foot-shock used for Pavlovian learning

Genotype	OA (OCT)	OA (MCH)	SR
<i>W¹¹¹⁸ (isoCJ1)</i>	76 \pm 1.4	76 \pm 2.7	83 \pm 2.1
<i>W¹¹¹⁸ (isoCJ1) (+HS)</i>	71 \pm 2.3	75 \pm 1.2	83 \pm 2.3
<i>yu^{P1}/yu^{P1}</i>	77 \pm 2.9	76 \pm 2.4	80 \pm 1.3
<i>yu^{KG02745}/yu^{KG02745}</i>	74 \pm 3.1	78 \pm 2.3	82 \pm 1.9
<i>yu^{P1}/yu^{P1}; hs-yu⁺/hs-yu⁺</i>	71 \pm 3.3	74 \pm 2.6	84 \pm 2.6
<i>yu^{P1}/yu^{P1}; hs-yu⁺/hs-yu⁺ (+HS)</i>	73 \pm 1.0	73 \pm 2.9	81 \pm 0.7

No significant differences in "task-relevant" sensorimotor responses were detected for comparisons between control flies and mutants or other experimental genotypes (in each case, $n = 8$ for all the genotypes). All experiments were conducted at RT. OA, olfactory acuity; OCT, octanol; MCH, methylcyclohexanol; SR, shock reactivity; HS, a 30-min heat shock (37°C), followed by a 3-h recovery period.

used for the Pavlovian experiments also were normal in *yu* mutants (Table 1). Together, these results indicate that STM, MTM, and ARM are normal, but LTM specifically is impaired, in the *yu^{P1}* mutant.

To confirm that this memory defect results from disruption of *CG3249*, we conducted a genetic complementation experiment using *yu^{P1}* and another existing P-element insertion allele, *yu^{KG02745}* (Fig. 1A Top) (19). Although *yu^{P1}/+* and *yu^{KG02745}/+* heterozygotes showed normal one-day memory after spaced training, such memory was defective in *yu^{P1}/yu^{KG02745}* heteroallelic flies (Fig. 1D). We also constructed transgenic *yu^{P1}* mutants carrying an inducible *hs-yu⁺* construct. Acute expression of this transgene 3 h before training was sufficient to induce high levels of *yu* transcript and protein (Fig. 2A) and to rescue the memory defect in *yu^{P1}* mutants (Fig. 2B). These latter observations

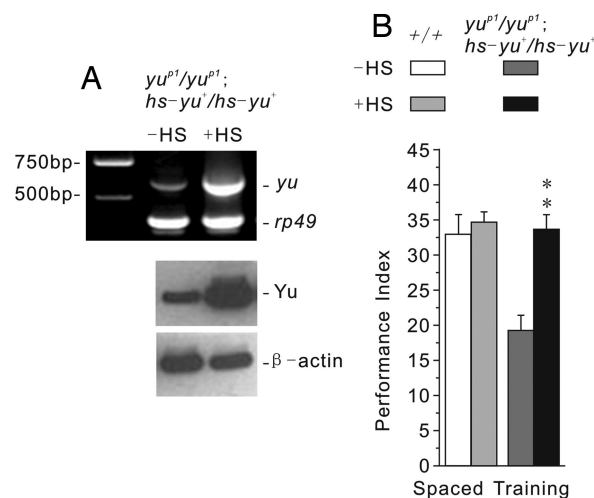


Fig. 2. Acute expression of *yu⁺* transgene in the mutant background can rescue the LTM defect. (A) mRNA and protein expression levels of *yu⁺* in *yu^{P1}/yu^{P1}; hs-yu⁺/hs-yu⁺* transgenic mutants immediately or 3 h after a 30-min heat shock treatment (37°C), respectively. (Upper) RT-PCR revealed strong overexpression of *yu⁺* after a heat shock treatment. Left column, DNA makers; center column, transgenic mutants with no heat shock treatment (-HS); right column, transgenic mutants after heat shock treatment (+HS). *Rp49* was used as a loading control. (Lower) Western blot analysis showed strong overexpression of Yu after heat shock treatment. Left column, transgenic mutants with no heat shock treatment (-HS); right column, transgenic mutants after heat shock treatment (+HS). β -actin was used as a loading control. (B) The defect in one-day memory after spaced training was rescued in *yu^{P1}/yu^{P1}; hs-yu⁺/hs-yu⁺* transgenic mutants when animals were trained 3 h after a 30-min heat shock treatment (37°C) ($n = 8$ PIs per group).

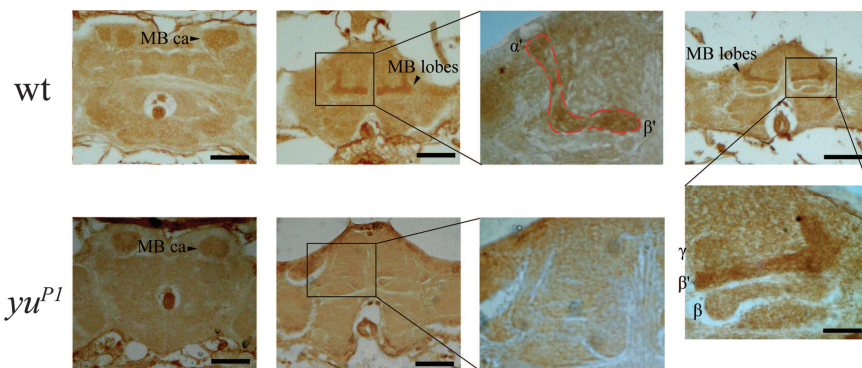


Fig. 3. Yu is expressed preferentially in the MB. (*Upper*) Frontal sections of wild-type adult fly brains after immunostaining (dark brown) with an affinity-purified antiserum against a peptide fragment of Yu. Mild signal was detected in sections through the MB calyx regions (CA). Stronger signal was apparent in the MB, especially in regions of α' / β' lobes (MB). (*Lower*) Frontal sections of yu^{P1} mutant adults after immunostaining with the Yu antibody. Yu expression in the MB was greatly reduced. (Scale bar, 50 μm .)

demonstrate that the memory defect of the yu mutant does not result from abnormal development. Instead, Yu appears to be involved physiologically in the adult brain during the formation of LTM.

Yu is expressed preferentially in the MB. To gain insights into the neural basis for this genetic dissection of LTM formation by Yu AKAP, we performed immunohistochemistry analysis. Yu appears preferentially expressed in the MB, α' and β' lobes in particular (Fig. 3), and the staining in the rest of the brain is minimal and similar that seen for the mutant where Western blot analysis suggests no expression (Fig. 1*A Bottom*). These observations suggested the hypothesis that Yu functions in the MB during the formation of LTM.

Expression of yu^+ in the MB rescues the LTM defect of yu mutants. To map Yu-related functional brain regions, we used several Gal4 lines to target expression of $UAS-yu^+$ in specific neuroanatomical regions associated with memory (20). *OK107-Gal4* (*OK107*) and *c747-Gal4* (*c747*) is highly specific to MB with strong expression in a majority of MB neurons (21, 22), whereas *OK66-Gal4* (*OK66*) is mainly expressed in antennal lobe (AL) local neurons with weak expression in a small population of MB neurons (23), and *c232* shows rather exquisite expression restricted to the central complex (24). *OK107*- or *c747*-driven expression of $UAS-yu^+$ in the MB of the yu^{P1} mutant was sufficient to restore normal LTM (Fig. 4). In contrast, the LTM defect of the yu^{P1} mutant remained when $UAS-yu^+$ transgenic expression was driven by *OK66* in antennal lobe or by *c232* in central complex (Fig. 4). These observations lead us to conclude that the function of Yu AKAP in the MB is essential for the formation of LTM.

Yu in the MB Interacts with cAMP-Dependent Signaling During the Formation of LTM. Expression of Rut in the MB is known to be critical for STM (17), whereas PKA-C1 is expressed highly in the MB and its mutations thereof have been shown to affect both learning and MTM (25). We show here that one-day memory after spaced training is also disrupted in *rut* mutants (Fig. 5*A*). More pertinently, we also have established an epistatic interaction between Yu and PKA-C1. Flies heterozygous for either gene (*PKA-C1^{H2}/+* or $yu^{P1}/+$) displayed normal one-day memory after spaced training, but such memory was impaired in the double-heterozygous flies ($yu^{P1}/+$; *PKA-C1^{H2}/+*) (Figs. 1*D* and 5*B*). This genetic observation implies that Yu and PKA-C1 function together in the same cAMP pathway during the formation of LTM.

Discussion

Studies in several species have revealed a time-dependent process of memory consolidation with distinct temporal phases of memory (26–28). After *Drosophila* olfactory learning, behavioral, pharmacological, and genetic manipulations have “dissected” memory formation into four distinct but interdependent phases: STM, MTM, ARM, and LTM (11). Our current study focuses on the role of a specific AKAP gene, yu , in formation of LTM. We have shown that (*i*) LTM specifically was abolished in yu mutants, whereas other memory phases appeared normal (Fig. 1*B* and *C*), (*ii*) this LTM defect was produced by (independent) mutations in the yu transcription unit, *CG3249* (Fig.

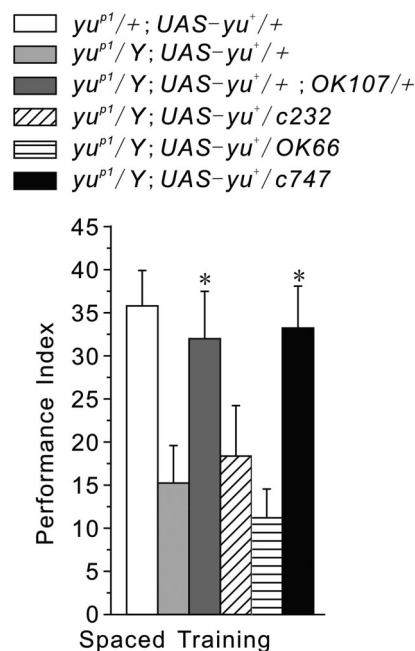


Fig. 4. LTM formation depends on yu expression in the MB. One-day memory after spaced training was evaluated in transgenic yu^{P1} mutants with various Gal4 drivers expressing a $UAS-yu^+$ transgene. The LTM defect of yu mutants was rescued when $UAS-yu^+$ was expressed in the MB (yu^{P1}/Y ; $UAS-yu^+/+$; *OK107/+* and yu^{P1}/Y ; $UAS-yu^+/+$; *c747/+*). The LTM defect was not rescued, however, when $UAS-yu^+$ was expressed in the AL (yu^{P1}/Y ; $UAS-yu^+/+$; *OK66/+*) or in CC (yu^{P1}/Y ; $UAS-yu^+/+$; *c232/+*). Transgenic $yu^{P1}/+$; $UAS-yu^+/+$ flies were controls for genetic background and leaky UAS -driven transgene effects ($n = 8$ PIs per group).

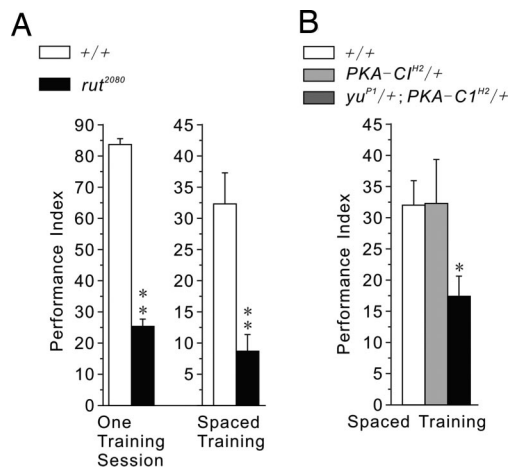


Fig. 5. Yu-mediated cAMP signaling in the MB contributes to the formation of LTM. (A) Both immediate memory after one training session and one-day memory after spaced training were impaired in *rut*²⁰⁸⁰ mutants ($n = 6$ or 8 PIs per group for experiments using one training session or spaced training, respectively). (B) One-day memory after spaced training was normal in heterozygotes carrying mutations of PKA (*PKA-C1*^{H2/+}) or of *yu* AKAP (*yu*^{P1/+}) but significantly disrupted in double heterozygotes (*yu*^{P1/+}; *PKA-C1*^{H2/+}) ($n = 6$ PIs per group).

ID), and (iii) the LTM defect of the *yu* mutant could be rescued via acute induction of a *yu*⁺ transgene (Fig. 2B). These data clearly define a specific, physiological function for Yu AKAP during memory formation.

Immunostaining of adult brain has revealed that Yu is expressed preferentially in the MB anatomical region (Fig. 3), which many studies have established plays a key role in olfactory memory formation (2, 8, 29, 30). Using multiple “enhancer trap” GAL4-drivers, with overlapping patterns of expression in MB or with preferential expression in other potentially critical anatomical regions [i.e., antennal lobes (31, 32) and central complex (33)], we have spatially restricted the expression of a UAS-*yu*⁺ transgene in the *yu* mutant. LTM was normal only when Yu was expressed in the MB (Fig. 4). Finally, a classically designed genetic epistasis experiment has shown that Yu AKAP interacts with PKA-C1 during LTM formation (Fig. 5B). This synergistic effect supports the idea that Yu functions as an AKAP in mediating LTM formation.

Although genetic dissection has revealed distinct memory phases and gene disruption experiments have identified several components of cAMP signaling to be important for this process, few studies have determined whether these molecules actually function together during memory formation. Rut-AC and PKA-C1, for instance, are critical not only for learning and STM or MTM (12, 15, 25, 34) but also for LTM (Fig. 5A and B). But, do they function together in the same place during each memory phase? When considering AKAPs, more specifically, proteins of this family bind regulatory subunits of PKA, and thereby may serve to localize PKA to different subcellular compartments (35). But specifically which AKAP is involved in which cellular function in *Drosophila* (19, 36–38)? Our work on the Yu AKAP presents the hypothesis that localization of Yu helps to define a function of cAMP signaling specific to LTM formation. As a corollary, we speculate that other AKAPs (in the MB) may introduce specificities for cAMP signaling during earlier memory phases by means of differential localization of PKA to other subcellular compartments.

Although both STM and LTM are localized in the MB, these two memory components may not reside in the same population of MB neurons. There are evidences that the horizontal lobe is

especially important for Rut-dependent STM formation (24, 39), whereas vertical (α and α') lobes are critical for LTM formation (40). Previous studies have shown that *Drosophila* α/β neurons not only function in the memory retrieval (41, 42) but also form a branch-specific LTM trace after spaced training (18). Functional analysis of subsets of MB neurons show that α'/β' neurons are required to acquire and stabilize an olfactory memory (42). To that end, our study also shows that Yu is expressed preferentially in α' and β' lobes.

Methods

Isolation of Memory-Defective Mutants. Transposon mutagenesis was carried out as described in previous works with minor modifications (43). A PlacW transposon, carried on the second chromosome, was used as a mutator to generate homo- or hemizygous-viable X-linked transposants [see [supporting information \(SI\) Fig. 6 and SI Methods](#)]. One-day memory after spaced training was quantified ($n = 1$) for 2,021 transposant strains. Two hundred fifty-nine mutants produced PIs that were <50% of the mean PI for *w*¹¹¹⁸ (*isoCJI*) control flies. PIs ($n = 4$) then were generated for these strains, and 63 of them yielded mean PIs <50% of the mean PI of control flies. The 63 low-scoring mutants were back-crossed for five generations to control flies to equilibrate genetic backgrounds. Out-crossed strains again were tested for one-day memory after spaced training and for olfactory acuity and shock reactivity. The *yu*^{P1} mutant was one such strain that retained poor one-day memory after spaced training and showed normal olfactory acuity and shock reactivity.

***Drosophila* Strains and Genetics.** The *KG02745* (19) were obtained from the Bloomington Stock Center (Bloomington, IN). Mutant *rut*²⁰⁸⁰ (33) flies were obtained from the laboratory of Li Liu at the Institute of Biophysics CAS (Beijing, China). *OK107* (MB-Gal4), *c747* (MB-Gal4), *c232* (central complex Gal4, CC-Gal4) were extant stocks (out-crossed) in our laboratory. To equilibrate genetics backgrounds, these stocks *KG02745* were out-crossed with “cantonized” FM7 flies carrying an X-chromosome and autosomes from the *w*¹¹¹⁸ (*isoCJI*) control flies. All other stocks including *OK66*, *hs-yu*⁺, and *UAS-yu*⁺ were equilibrated by five generations of out-cross to *w*¹¹¹⁸ (*isoCJI*).

Plasmid Rescue of P-Element Insertion. DNA sequences flanking the *yu*^{P1} insertion site were identified by digesting total genomic DNA from *yu*^{P1} mutants with either EcoRI or XbaI, ligating the resulting restriction fragments and then transforming bacteria to isolate ampicillin-resistant plasmids. Primers corresponding to P-element sequence then were used to direct DNA sequencing into flanking genomic DNA. The insertion site was confirmed by generating PCR primers on either side of the insertion, amplifying the intervening DNA and determining the size of the amplicon (43).

Generation of Transgenic *Drosophila* Lines. To create *hs-yu*⁺ and *UAS-yu*⁺ construct, the expected full-length *yu*⁺ cDNA was amplified by Pfu-polymerase from cDNA plasmid GM14014 (from *Drosophila* Genomics Resource Center, DGRC). The amplified fragment (≈ 3.3 kb) was subcloned into pGEM-T (Promega, Madison, WI) vector and sequenced to confirm the integrity of the amplified cDNA fragment. The subcloned *yu*⁺ fragment then was released from pGEM-T vector by restriction digest with NotI (NEB, Beverly, MA). The gel-purified fragment finally was subcloned into either a NotI-cut and calf intestinal alkaline phosphatase (CIP; NEB)-treated CaSpeR-hs (8.9 kb) or a pUAST (9 kb) germ-line transformation vectors. The direction of subcloning and sequence were confirmed. Germ-line transformation was carried out as described by Rubin and Spradling (44).

Pavlovian Olfactory Learning. The training and testing procedures were the same as described (7, 11, 16). Briefly, a group of 100 flies was sequentially exposed for 60 s to each of two odors (octanol (OCT) or methylcyclohexanol (MCH), with 45 seconds of fresh air in between). Flies were subjected to foot-shock (12 1.5-s pulses with 3.5-s intervals, 60 V) during exposure to the first odor (CS+: OCT) but not to the second (CS-; MCH). To measure "immediate memory," flies were transferred immediately after training to the choice point of a T-maze and forced to choose between the two odors for 2 min, at which time they were trapped in their respective T-maze arms, anesthetized, and counted. A reciprocal group of flies was trained and tested by using MCH as the CS+ and OCT as the CS-. PIs from these two groups finally were average for an $n = 1$. A PI of 0 represented a 50:50 distribution, whereas a PI of 100 represented 100% avoidance of the shock-paired odor (7). Spaced training consisted of 10 training sessions with a 15-min rest between each. Massed training consisted of 10 training sessions with no rest interval. To quantify one-day memory, flies were transferred after training to food vials and stored at 18°C for 24 h. At least 30 min before being tested, flies were returned to room temperature (RT).

Sensorimotor Responses. Odor-avoidance responses were quantified by exposing naïve flies to one odor (OCT or MCH) versus air in the T-maze (7). After 2 min, flies were trapped in their respective T-maze arms, anesthetized, and counted. A PI was calculated for each odor individually as reported (7). The ability to sense and escape from foot-shock (shock reactivity) was quantified in naïve flies by inserting electrifiable grids into both arms of the T-maze but delivering shock pulses only in one arm of the T-maze and allowing flies to choose between the two arms. After one minute, flies were trapped in their respective arms, anesthetized, and counted. Individual PIs were calculated as for olfactory acuity.

Heat Shock Protocol. Flies subjected to heat shock were placed in empty vials in an air incubator that was maintained at 37°C for 30 min (45). Flies then were transferred back to bottles with food and allowed to rest for 3 h at RT (20–24°C) before training. To minimize leaky expression of the transgene for groups not subjected to heat shock, flies were incubated at 18°C overnight before training. All training and testing was performed at RT.

RT-PCR of Heat Shock Induced *yu* Expression. Flies were raised at either 18 or 25°C. One group of flies was shifted from 18 to 37°C for 30 min and immediately frozen at –80°C. Total RNA was prepared from 50 frozen flies by using the classic TRIzol method. Three independent RNA preparations were made for each group of flies. First-strand cDNA was synthesized directly

from the mRNA by using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). PCR amplification with TaqDNA polymerase (Invitrogen) was carried out in Invitrogen 10× PCR buffer by using 1.5 mM MgCl₂ with 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min, followed by extension at 72°C for 10 min. A pair of *yu*-specific primers were designed against regions of the *Drosophila yu* cDNA (GenBank accession no. NM.131993). Primers *yus1* (5'-aatctagatccagagcaatggacgca-3') and *yus2* (5'-aatctagaacgctggccagggtcttg-3') amplify a 628-bp fragment. Lack of genomic DNA contamination was confirmed by the absence of any PCR products when RT-PCR was carried out in control reactions without reverse transcriptase. *Ribosomal protein 49* (*rp49*) primers (5'-atgaccatccgccagcctac-3' and 5'-gagaacgcagcgcagcgttgg-3') were designed to amplify a 391-bp fragment between bases 1 and 391 of the *rp49*-coding region (GenBank accession no. Y13939). The control *rp49* mRNA is expressed at equal levels in all cells at all stages (46).

Generation and Purification of Antisera and Western Blot Analysis. For generation of antisera, rabbits were injected with a purified polypeptide fragment containing *Drosophila yu* peptide fragments (TNYTNKECEQNNNCEPKKEEP). Anti-Yu antisera were affinity-purified by using Protein A spin chromatography kit (Pierce, Rockford, IL). For Western blot analyses, whole adult flies were frozen in liquid nitrogen and shaken through a sieve to separate the heads. The frozen heads (~1 ml) were crushed with a mortar and pestle and homogenized with an automatic homogenizer in 5 mM HEPES, 100 mM NaCl (pH 7.4) with protease inhibitors (Complete protease inhibitor mixture; Roche, Indianapolis, IN). Large cuticular debris was removed by centrifugation at 5,000 × *g* for 20 min at 4°C. Fly extracts (80 μg of protein) were electrophoresed on SDS/polyacrylamide gels and blotted onto NC membranes (Millipore, Bedford, MA). Blots were incubated with affinity-purified anti-Yu sera overnight at 4°C, HRP-conjugated goat-anti-rabbit IgG (Sigma, St. Louis, MO) for 1 h at RT and visualized with SuperSignal Chemiluminescent substrate (Pierce).

Immunohistochemistry. Immunohistochemistry on adult *Drosophila* heads was performed on paraffin sections. Details were available in *SI Methods*.

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