A clock gene, *period*, plays a key role in long-term memory formation in *Drosophila*

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The cAMP-responsive transcription factor, CREB, is required for formation of long-term memory (LTM) in *Drosophila melanogaster* and regulates transcription of a circadian clock gene, *period* (*per*). Involvement of CREB both in LTM and circadian rhythm raises the possibility that *per* also plays a role in LTM. Assaying the experience-dependent courtship inhibition in male flies as a measure for LTM, we show here that *per* mutants are defective in LTM formation. This defect was rescued by induction of a wild-type *per* transgene in a *per*-null mutant, and overexpression of *per* enhanced LTM formation in the wild-type background. Furthermore, we found that synaptic transmission through *per*-expressing cells is most likely to be required during retrieval of LTM. In contrast, mutations in other clock genes (*timeless*, *dClock*, and *cycle*) did not affect LTM formation. Thus, independent of the core oscillator of circadian clock, *per* plays a key role in LTM formation.

The cAMP signaling cascade plays a central role in the formation of memory (1). In *Drosophila melanogaster*, the genetic analyses of olfactory conditioning that are based on electric shocks associated with olfactory cues have identified several genes participating in the cAMP signaling cascade as critical contributors to memory formation (2–6). These genes include *rutabaga* (*rut*; Ca^{2+}/calmodulin-responsive adenylyl cyclase), *dunce* (*dnc*; cAMP phosphodiesterase), *DC0* (a catalytic subunit of cAMP-dependent protein kinase A) and *amnesiac* (*amn*; an adenylyl cyclase-activating peptide). Mutant flies for these genes are defective in memory that lasts minutes (*rut* and *dnc*) or hours (*DC0* and *amn*) after conditioning. This type of short-lasting memory does not require protein synthesis. In contrast, long-term memory (LTM) that lasts more than several days requires protein synthesis (7). The cAMP-responsive element-binding protein (CREB) is critically involved solely in this process (8–11). Overexpression of a repressor isoform of *CREB* (*dCREB2-b*) in *Drosophila* selectively abolishes LTM formation induced by olfactory conditioning without affecting short-lasting memory (10), whereas overexpression of an activator isoform of *CREB* (*dCREB2-a*) greatly enhances LTM formation (11).

In addition to LTM formation, *CREB* also plays crucial roles in circadian rhythm (12, 13), the molecular mechanism of which has been studied most extensively in *Drosophila* (14–16). Clock genes *period* (*per*) and *timeless* (*tim*) are essential for establishing circadian rhythm. A mutation in *dCREB2* shortens the circadian locomotor rhythm and dampens the oscillation of PER protein level (12). Furthermore, the region 1.2–4 kbp upstream of the transcription start site of *per*, which includes the CREB binding sites, is required to maintain the normal expression level of PER (12). The involvement of *CREB* in both LTM formation and circadian rhythm, along with its role in regulation of *per* expression, has led us to the hypothesis that *per*, like *CREB*, is essential for LTM formation.

To determine the role of *per* in LTM, we studied the memory formation of *Drosophila* males engaged in courtship behaviors. The *Drosophila* male has characteristic courtship behaviors (17, 18), including orienting himself toward a female, following her movement, tapping her body with his forelegs, vibrating his wing unilaterally to generate courtship songs, licking her genitalia, and curling his abdomen in an attempt to copulate. A virgin male paired with a female that has mated earlier initially courts her but reduces his courtship activities after repeated rejections. With this experience, in addition to an exposure to courtship-inhibiting chemical cues that the fertilized female fly elicits (conditioning), the male fly reduces his courtship activities even toward a virgin female. This depression of courtship behaviors is experience-dependent (19–23).

Jackson *et al*. (24) reported that *per* mutant males having a lengthened circadian rhythm (per^{l1} and per^{l2}) were defective in short-lasting memory induced by courtship conditioning. It was subsequently shown, however, that per^{l2} males were normal in short-lasting memory and that per^{l1} males were defective in courtship-related chemosensory perception but not in memory formation *per se* (25). In any case, these previous studies have demonstrated that *per*-null mutations do not affect short-lasting memory (24, 25).

In this study, we tested whether circadian clock genes, including *per*, are involved in LTM formation and found that *per* is crucially involved in LTM formation but other clock genes are not. Furthermore, we found that disruption of dynamin function in *per*-expressing cells blocks the retrieval, but not the formation or storage, of LTM.

Materials and Methods

Flies. The four wild-type strains used in this study were Canton-S (CS), Oregon-R (OR), OGS-4, and 1619. Four memory mutants (rut^{178}, dnc^{1}, $DC0^{B3}$, and amn^{28A}), five circadian clock mutants [per^{01}, per^{S}, tim^{01}, $dClock^{Jrk}$ ($dClk^{Jrk}$), and $cycle^{0}$ (cyc^{0})], and transgenic flies were also used. per^{01}(+) has a *per*-null mutation with autosomes of unknown origin. The second and third chromosomes of per^{01}(OR) and per^{01}(CS) are derived from OR and CS, respectively. *per-Gal4* (26) and UAS-$shibire^{ts1}$ (shi^{ts1}; UAS, upsteam activation sequence) (27) lines were outcrossed for five or six generations to *white* flies with CS genetic background. All flies were raised on glucose–yeast–cornmeal medium at 24.5 ± 0.5°C in a 12-h light/12-h dark cycle (lights on at 9:00 a.m.).

Virgin males and females were collected without anesthesia within 6 h after eclosion and maintained individually in vials until experiments. All experiments, except for heat-shock treatment, were carried out during daytime at 24.5 ± 0.5°C.

Courtship Conditioning. For this study, we used two paradigms to measure memory induced by courtship conditioning. One is for LTM that lasts for days, and the other is for "1-h memory."

Mated CS females were prepared the night before they were

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Abbreviations: LTM, long-term memory; *per*, *period*; *tim*, *timeless*; *rut*, *rutabaga*; *dnc*, *dunce*; *amn*, *amnesiac*; *shi*, *shibire*; *dClk*, *dClock*; *cyc*, *cycle*; *hsp*, *heat-shock promoter*; CREB, cAMP-responsive element-binding protein; CI, courtship index; MB, mushroom body; UAS, upstream activation sequence; CX, central complex; CS, Canton-S; OR, Oregon-R.

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used for conditioning the males. For LTM, a 3-d-old virgin male was placed with a mated female (3, 4, or 5 d old) in a conditioning chamber (15 mm in diameter × 5 mm in depth) containing food for 4, 5, or 7 h, and kept in a food vial until tested 3 or 5 d later. For 1-h memory, a virgin male was conditioned for 30 min in a chamber (15 mm in diameter × 3 mm in depth) without food and tested 1 h after the end of conditioning. As a control, a naive male was introduced into another conditioning chamber without a mated female. In the test, we used an ether-immobilized or a freeze-killed virgin female as a target. We observed male courtship behaviors of individual flies and, as an indicator of male courtship activities, calculated a courtship index (CI), defined as the percentage of time spent in courtship behaviors during a given observation period. To measure the CI, we observed for 10 min or until occurrence of copulation or until initial movement of the ether-immobilized female due to recovery from anesthesia. We compared the courtship activities of a conditioned male with those of a naive male. Conditioning and test chambers were made of transparent acrylic plastic and covered with a rectangular acrylic plastic at the top.

Temperature-Shift Experiment. Three paradigms of temperature-shift were used in the experiments. (*i*) Males were conditioned and subsequently maintained at 25°C (permissive temperature) until 10 min before testing, at which point they were shifted to 30°C (restrictive temperature) until the end of test (see 25-25-30 in Fig. 5*D*). (*ii*) Males were conditioned and tested at 25°C and shifted to 30°C immediately after conditioning and shifted back to 25°C 10 min before testing (see 25-30-25 in Fig. 5*D*). (*iii*) Males were conditioned at 30°C and subsequently shifted to 25°C (see 30-25-25 in Fig. 5*D*).

Statistics. In all cases, CI values were not distributed normally. Even after arcsin transformation, the data were not normally distributed. Hence, the data were analyzed nonparametrically by using the Mann–Whitney *U* test or Kruskal–Wallis test.

Supporting Information for Methods. For details of the methods used, see *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site.

Results and Discussion

Mutants for the cAMP Signaling Cascade Are Defective in LTM Induced by Courtship Conditioning. To assay the relationship between courtship behaviors and memory in *Drosophila*, we measured a CI, defined as the percentage of time that a male fly spent for courtship behaviors during a given observation period. We found that on the third and fifth days after 7-h conditioning, the CI was significantly lower in conditioned wild-type CS males compared with naive (unconditioned) males (Fig. 1*A*), whereas no significant difference was found after 4-h (data not shown) or 5-h (Fig. 1*B*) conditioning under the experimental conditions we used. The experience-dependent courtship inhibition shown in Fig. 1*A* did not depend on wild-type strains. The CIs in conditioned males of three other wild-type strains (OR, OGS-4, and 1619) were significantly lower than those in naive males (Fig. 1*D*), although the CI values in naive males varied considerably among the four strains (Fig. 1*D*, open columns; Kruskal–Wallis test, $P < 0.01$).

Because movements of female flies hinder an accurate measurement of the CI (19), we used ether-immobilized virgin females as a target of male courtship behaviors. In this situation, however, residual ether in the female body might affect experience-dependent courtship inhibition. To address this possibility, in one set of experiments we used freeze-killed females that had been kept at −80°C for 10 min and thawed. A significant difference in the CI was also detected between naive and conditioned males toward freeze-killed females (Fig. 1*C*). In CS

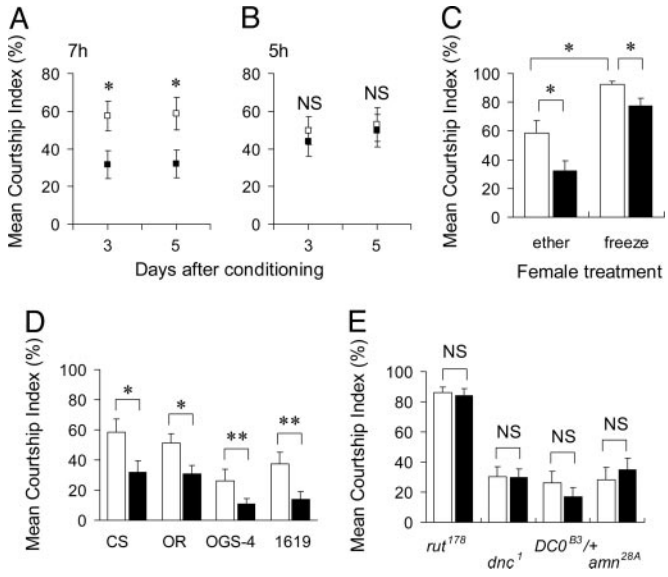

Fig. 1. Courtship conditioning in wild-type and memory mutants. In all figures, open columns and open squares represent the mean CI of naive males, and filled columns and filled squares represent the mean CI of conditioned males. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. Error bars show SEM. (*A* and *B*) Comparisons of the mean CI between naive and conditioned males of CS on the third and fifth day after 7 (*A*) or 5 (*B*) h of conditioning (n = 22–30 per point). (*C*) Ether-immobilized (ether) and freeze-killed (freeze) females were used in the tests. The test was carried out on the fifth day after 7-h conditioning by using CS (n = 30 for each column). (*D*) Comparisons of the mean CI between naive and conditioned males in four wild-type strains on the fifth day after 7-h conditioning (n = 26–30 for each column). (*E*) Comparisons of the mean CI between naive and conditioned males in memory mutants on the fifth day after 7-h conditioning. Because homozygous mutations of $DC0^{B3}$ are lethal, we used F_1 hybrid males between $DC0^{B3}/CyO$ males and CS females ($DC0^{B3}/+$) (n = 21–26 for each column).

naive males, the CI toward ether-immobilized virgin females was significantly lower than that toward freeze-killed females (Fig. 1*C*, open columns), suggesting a direct inhibitory effect of residual ether on male courtship behaviors. However, because ether anesthesia is easier to manipulate than freeze treatment, we used it routinely in this study unless otherwise stated.

In contrast to the results with wild-type strains, none of the four memory mutants (rut^{178}, dnc^{1}, $DC0^{B3}$, and amn^{28A}), which have defects in the cAMP cascade, displayed a significant difference in the CI between naive and conditioned males on the fifth day after 7-h conditioning (Fig. 1*E*). Thus, our results indicate that (*i*) 7-h conditioning yields memory lasting at least 5 d in wild-type flies, regardless of their genetic background (Fig. 1*D*), and (*ii*) mutations in genes that are involved in the cAMP signaling cascade block LTM (Fig. 1*E*). Our results were consistent with a previous report (7) that *amn* mutant flies are defective in LTM when assayed by olfactory conditioning. Taken together, our results suggest that the cAMP signaling cascade is involved in LTM induced by courtship conditioning.

Overexpression of a Repressor Isoform of *CREB*, *dCREB2-b*, Blocks LTM Formation. Given the involvement of the cAMP signaling cascade in LTM formation induced by courtship conditioning, we next asked whether *CREB* is required in its process. We used two transgenic lines, *hs-dCREB2-b* S30 and S64, both of which express a repressor isoform of *CREB* (*dCREB2-b*) under the control of a heat-shock promoter (refs. 10 and 28; see also *Supporting Materials and Methods*). When either S30 or S64 males were heat-shocked 3 h before conditioning (Fig. 2*A Upper*), LTM was not detected [Fig. 2 *B* and *C*, HS+(1)],

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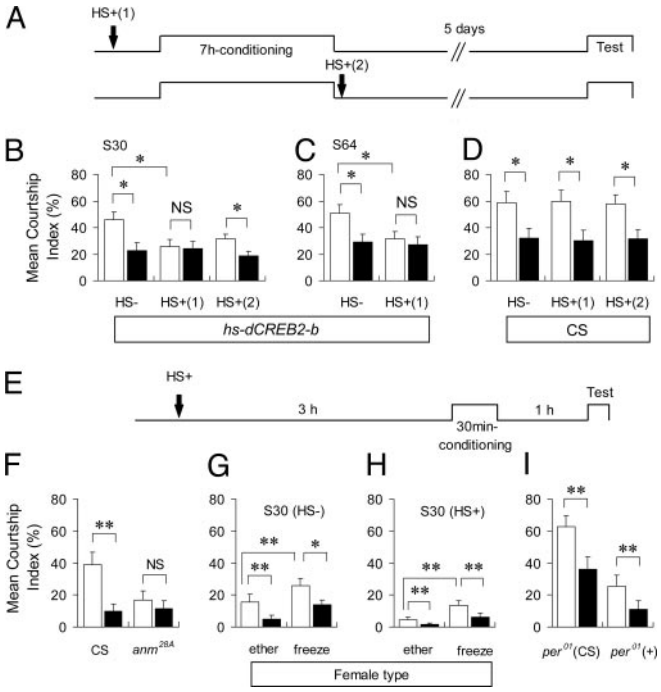

Fig. 2. Overexpression of *dCREB2-b* selectively blocks LTM formation. Experimental paradigms are indicated in *A* and *E*. HS−, nonheat-shocked males; HS+(1), heat-shocked males before conditioning; HS+(2), heat-shocked males after conditioning. Open columns represent the mean CI of naive males, and filled columns represent the mean CI of conditioned males. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (*B–D*) $n = 28–34$ for each column. (*F–I*) $n = 25–30$ for each column. (*B–D*, *F*, and *I*) Ether-immobilized females were used in the test. (*G* and *H*) Ether-immobilized and freeze-killed females were used in the test.

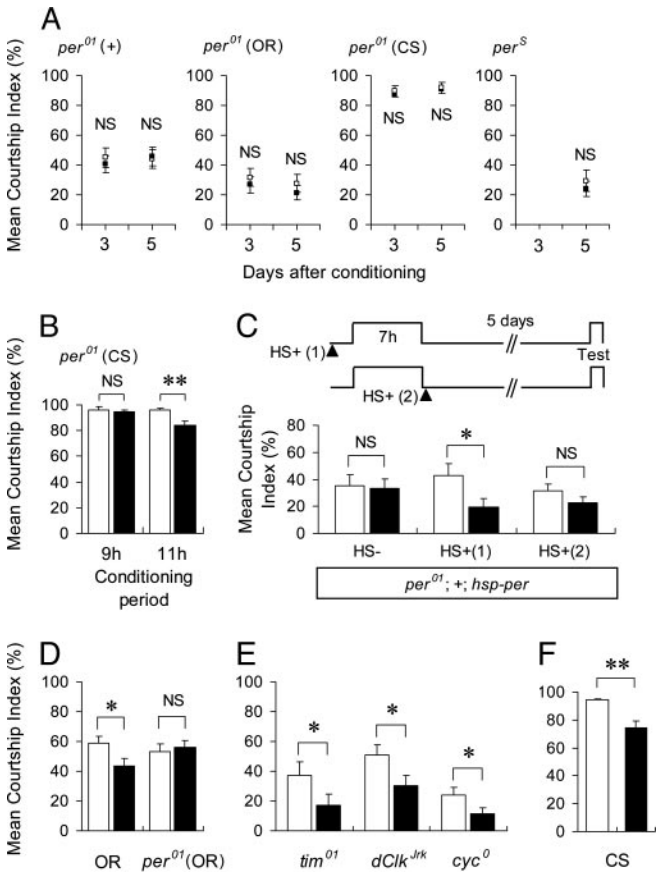

Fig. 3. LTM formation in mutant flies with defects in the circadian clock. Open columns and open squares represent the mean CI of naive males, and filled columns and filled squares represent the mean CI of conditioned males. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (*A*) LTM formation of *per*-null mutants [per^{01}(+), per^{01}(OR), and per^{01}(CS)] and a per^{S} mutant. The test was carried out on the third or fifth day after 7-h conditioning [$n = 30–32$ per point in per^{01}(+), per^{01}(OR), and per^{01}(CS); $n = 24$ in per^{S}]. (*B*) The test was carried out on the fifth day after 9- or 11-h conditioning using per^{01}(CS) males. (*C*) per^{01};+;*hsp-per*(s13) males were used. Experimental paradigms are indicated above the graphs ($n = 32–38$ for each column). (*D*) per^{01}(OR) or OR males were used in the experiments ($n = 28–29$ per point). The test was carried out on the second [per^{01}(OR)] or third (OR) day after 5-h conditioning. (*E*) LTM formation of clock mutants (tim^{01}, $dClk^{Jrk}$, and cyc^{0}). The test was carried out on the fifth day after 7-h conditioning ($n = 26–30$ for each column). (*F*) CS males that had been kept under constant light were used ($n = 30$ for each column).

whereas, without a heat shock, it was detected (Fig. 2 *B* and *C*, HS−). Timing of heat shock was critical for this suppression of LTM formation because S30 males acquired LTM (Fig. 2*B*) when they were heat-shocked after conditioning [Fig. 2 *A Lower* and *B*, HS+(2)]. A heat shock by itself did not affect LTM formation because CS males acquired LTM regardless of heat-shock treatment (Fig. 2*D*). Heat-shocked S30 or S64 naive males yielded significantly lower CIs than nonheat-shocked males (Fig. 2 *B* and *C*, open columns), suggesting that overexpression of *dCREB2-b* directly reduces male courtship activities toward immobilized virgins. Because the CI of heat-shocked *hs-dCREB2-b* naive males was lower than that of nonheat-shocked males (Fig. 2 *B* and *C*), it is possible that low courtship activities of naive males contribute to the lack of difference in the CI between naive and conditioned males that had been heat-shocked before conditioning [Fig. 2 *B* and *C*, HS+(1)]. However, LTM was detected in wild-type OGS-4 which showed a low CI (26%) (Fig. 1*D*). Therefore, the low CI *per se* is not the only cause for our failure in detecting LTM after overexpression of *dCREB2-b*. It is also possible that the lack of LTM in flies overexpressing *dCREB2-b* prior to conditioning is caused by their reduced courtship activity during the conditioning period. We found, however, that this might not be the case because overexpression of *dCREB2-b* did not significantly affect courtship activity toward free-moving mated females used for conditioning. [We observed courtship behaviors for 10 min at 3 h after heat-shock ($CI_{HS-} = 19.65 \pm 4.08\%$, $CI_{HS+} = 11.43 \pm 2.86\%$; $n = 20$ in each female; Mann–Whitney $U = 140.5$, $P = 0.108$).]

We next examined whether overexpression of *dCREB2-b* affects 1-h memory. S30 males were first heat-shocked then conditioned for 30 min and tested 1 h after conditioning (Fig. 2*E*). In this paradigm, 1-h memory was detected in CS males but not in amn^{28A} males (Fig. 2*F*). Despite the fact that the CI in naive S30 males was extremely low 4.5 h after heat shock, we found that heat-shocked (Fig. 2*H*) as well as nonheat-shocked (Fig. 2*G*) S30 males showed statistical differences between naive and conditioned CIs measured 1 h after conditioning. The statistical difference was also detected when freeze-killed females that gave a higher initial CI value were used as target females. Thus, we deduce that overexpression of *dCREB2-b* does not significantly affect 1-h memory but blocks LTM formation.

LTM Is Defective in *per* Mutants. Because *dCREB2* seems to be involved in LTM formation in the courtship conditioning paradigm and *per* is under the control of CREB in the circadian rhythm regulation, it is possible that *per* also plays a role in LTM formation. To test this possibility, we used three types of *per*-null mutants with different genetic backgrounds [per^{01}(+), per^{01}(OR) and per^{01}(CS)], and a per^{S} mutant that has a shortened circadian rhythm. As shown in Fig. 3*A*, LTM after 7 h of conditioning was not detected in these four *per* mutant males. Because this is the

crucial experiment in this study, we carried out single-blind experiments (the experimenters were not informed of the strain of flies tested) to eliminate the bias of experimenters using OR (control) and per^{01}(OR). In these single-blind experiments, LTM was detected in OR males, but not in per^{01}(OR) males (data not shown). The LTM defect was also observed in *per* mutants when 7-h conditioning was performed during nighttime (Zeitgeber time, 12–19), suggesting that defective LTM of *per* doesn't depend on the assay timing (Fig. 6 *A* and *B*, which is published as supporting information on the PNAS web site). LTM was not detected in per^{01}(CS) males even after 9-h conditioning, but was detected after 11-h conditioning (Fig. 3*B*), indicating that LTM is defective but not completely abolished by *per* mutations.

It has been reported that 5-h conditioning in a large chamber (15 × 75 mm) is sufficient to induce LTM in a triple mutant, *yellow* per^{01} *white* (23). Under the experimental condition copied from the above experiments, we found that per^{01}(OR) did not show LTM on the second day after 5-h conditioning and that OR males acquired LTM lasting at least 3 d after 5-h conditioning (Fig. 3*D* and *Supporting Materials and Methods*). Presente *et al.* (29) have also reported that CS males acquires LTM lasting at least 2 days after 5-h conditioning in a similar chamber (12 × 75 mm). Taken together, our result indicates that *per* mutants are defective in the formation of LTM regardless of the size of conditioning chambers. Thus, the formation of LTM reported in *yellow* per^{01} *white* males cannot be explained simply by the difference in the size of apparatus. It is possible that *yellow* or *white* mutation somehow compensates for the *per*-null mutation in LTM formation.

It is known that *per*-null mutations do not affect short-lasting memory (24, 25). We confirmed 1-h memory formation in per^{01}(CS) and per^{01}(+) (Fig. 2*I*). Thus, mutations in *per* are selectively affecting LTM.

Induction of a Wild-Type *per* Transgene Rescues LTM Formation in the *per*-Null Background. Having found that *per* plays a role in LTM formation, we next asked whether expression of a wild-type *per* transgene driven by heat-shock promoter (hsp) rescues LTM formation in the *per*-null background. Transgenic flies, s13, (genotype, per^{01}; +; *hsp-per*) show rhythmic locomotor and mating activities at high temperatures under free-running conditions (30, 31). We confirmed that after a heat shock these flies show rhythmic mating activities (data not shown). Furthermore, per^{01};+;*hsp-per* males acquired LTM when they were heat-shocked before conditioning [Fig. 3*C*, HS+(1)], whereas they did not without a heat shock (Fig. 3*C*, HS−). Nor did they acquire LTM when they were heat-shocked after conditioning [Fig. 3*C*, HS+(2)], indicating that *per* plays a critical role in formation of LTM.

It is possible that courtship activities of heat-shocked per^{01};+;*hsp-per* males toward mated females are more vigorous than those in nonheat-shocked per^{01};+;*hsp-per* males and that these vigorous courtship activities lead to acquisition of LTM. To test whether induction of the *per* transgene affects male courtship activities during 7-h conditioning, courtship behaviors of heat-shocked and nonheat-shocked per^{01};+;*hsp-per* males toward mated females were observed for 10 min at 0, 0.3, 1, 3, and 5 h during 7-h conditioning. No significant difference of the CI was detected during 7-h conditioning (Fig. 7*A*, which is published as supporting information on the PNAS web site). Thus, the rescue of LTM formation in heat-shocked per^{01};+;*hsp-per* males can't be simply attributed to an enhancement of courtship activities.

Overexpression of *per* Enhances LTM Formation in the Wild-Type Background. To determine whether overexpression of *per* enhances LTM formation, we conditioned for 5 h the transgenic flies, +;+;*hsp-per* (Fig. 4*A*). The 5-h conditioning was not sufficient in forming LTM in wild-type males (Figs. 1*B* and 4*E*), nor was it in nonheat-shocked +;+;*hsp-per* males (Fig. 4*B*, HS−). However, +;+;*hsp-per* males that were heat-shocked before conditioning did acquire LTM (Fig. 4*B*, HS+). Because there was no significant difference in the CI toward mated females during conditioning between nonheat-shocked and heat-shocked males (Fig. 7*B*), we conclude that overexpression of *per* enhances LTM formation in the wild-type background.

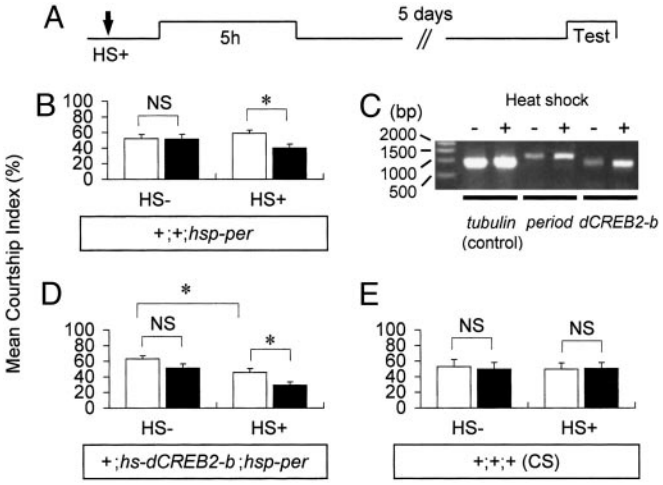

Fig. 4. Enhancement of LTM formation induced by overexpression of *per*. Open columns represent the mean CI of naive males, and filled columns represent the mean CI of conditioned males. *, $P < 0.05$; NS, not significant. (*A*) An experimental paradigm. (*B*) +;+;*hsp-per* males were used ($n = 30$ for each column). (*C*) Results of an RT-PCR analysis of +;*hs-dCREB2-b*;*hsp-per* males. The expected sizes of amplified fragments (908, 1,154, and 849 bp for *tubulin*, *per*, and *dCREB2-b*, respectively) were observed. +, heat-shocked flies; −, nonheat-shocked flies. (*D*) +;*hs-dCREB2-b*;*hsp-per* males were used ($n = 30$ for each column). (*E*) CS males were used ($n = 22$ for each column for HS−; $n = 30$ for each column for HS+).

Simultaneous Overexpression of both *dCREB2-b* and *per* Enhances LTM Formation. *dCREB2* and *per* participate in the same regulatory feedback loop for the normal circadian rhythm (12). To test the epistatic relationship between these two genes in LTM formation, we carried out 5-h conditioning with the transgenic flies, +;*hs-dCREB2-b*;*hsp-per*, which have *hs-dCREB2-b* from S30 on the second chromosome and *hsp-per* from s13 on the third chromosome. First, by using RT-PCR analysis (see *Supporting Materials and Methods*), we confirmed overexpression of both *dCREB2-b* and *per* after heat-shock treatment (Fig. 4*C*). If *dCREB2* acts downstream of *per*, we expect that a heat shock to +;*hs-dCREB2-b*;*hsp-per* males does not enhance LTM formation, regardless of overexpression of *per*. In contrast, if *per* is epistatic to *dCREB2-b*, the LTM enhancement induced by *per* overexpression would not be affected by *dCREB2-b* overexpression. We found that heat-shocked +;*hs-dCREB2-b*;*hsp-per* males acquired LTM after 5-h conditioning (Fig. 4*D*, HS+), whereas +;*hs-dCREB2-b*;*hsp-per* males without a heat shock did not (Fig. 4*D*, HS−), nor did a control [+;+;+(CS)] regardless of heat shock (Fig. 4*E*). These results suggest that *per* is operating downstream of *dCREB2* in LTM formation.

Circadian Clock Activities Are Not Required for LTM Formation. PER forms a heterodimer with TIM, and the PER/TIM heterodimer is considered to be essential for generation of the circadian rhythm (14–16). We found that LTM formation in tim^{01} was normal (Fig. 3*E*). Thus, TIM is not required for LTM formation. dCLK/CYC heterodimers activate transcription of *per* and *tim* by means of E-boxes located in their respective upstream se-

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quences. PER and TIM levels are extremely low in $dClk^{Jrk}$ and cyc^{0} mutants, and their behavioral rhythm is abolished (32, 33), indicating that dCLK and CYC are essential for the circadian timekeeping mechanism. In contrast, LTM formation in $dClk^{Jrk}$ and cyc^{0} mutants was normal (Fig. 3*E*). These results suggest that *per* expression, which is regulated by transcriptional activators other than dCLK/CYC, is involved in LTM formation. Because it is unlikely that *dCREB2* acts downstream of *per* (Fig. 4*D*), *dCREB2*-mediated *per* expression might be involved in LTM formation. Furthermore, these results indicate that *per*-expressing neurons, which are involved in LTM formation, are distinct from those involved in circadian rhythm.

It is known that *per* expression in wild-type flies dampens to arrhythmicity on the fourth day under constant light (34, 35). Hence, under this condition, the circadian clock of *Drosophila* is nonfunctional. We kept CS male flies under constant light after emergence and carried out 7-h conditioning on the fourth day and tested on the ninth day after emergence. LTM was detected in these males (Fig. 3*F*). These results indicate that the molecular mechanism of LTM formation is independent of the circadian clock.

Disruption of Dynamin Function Through *per*-Expressing Cells Blocks Retrieval, but Not Storage or Formation, of LTM. Mushroom bodies (MBs) play a central role for establishment of memory induced by olfactory conditioning (36, 37). Our results suggest that *per*-expressing cells are critically involved in LTM formation induced by courtship conditioning although the location of relevant *per*-expressing cells in the brain is not known. We used the GAL4/UAS system to demonstrate whether *per*-expressing cells affects LTM formation. The yeast transcriptional activator, GAL4, activates UAS-linked target genes. The *Drosophila shi* gene encodes Dynamin, which is essential for synaptic vesicle recycling. A temperature-sensitive allele, shi^{ts1}, is defective in Dynamin function at restrictive temperatures (>29°C). In F_1 males between *per-Gal4*, which expresses *Gal4* in *per*-expressing cells (26), and UAS-shi^{ts1} (27), shi^{ts1} is expressed in *per*-expressing cells. In these males, Dynamin function is disrupted at restrictive temperatures in *per*-expressing cells.

First, all procedures in the experiments were carried out at 30°C (restrictive temperature; Fig. 5*D*, 30-30-30) or at 25°C (permissive temperature; Fig. 5*D*, 25-25-25). In these experiments, we used *per-Gal4*/UAS-shi^{ts1} (F_1 males between *per-Gal4* females and UAS-shi^{ts1} males), +/*per-Gal4* (F_1 males between CS females and *per-Gal4* males), and +/UAS-shi^{ts1} (F_1 males between CS females and UAS-shi^{ts1} males) males. We did not use F_1 males between *per-Gal4* females and CS males (*per-Gal4*/+) for the reason described in the Supporting *Materials and Methods*.

LTM was detected in *per-Gal4*/UAS-shi^{ts1} males at 25°C but not at 30°C (Fig. 5*A*). +/*per-Gal4* and +/UAS-shi^{ts1} males acquired LTM either at 25°C or 30°C (Fig. 5 *B* and *C*). The difference of temperature (25°C versus 30°C) did not affect the CI of naive males of any transgenic line (Fig. 5 *A*–*C*, open columns). Furthermore, we confirmed that disruption of Dynamin function in *per*-expressing cells did not affect locomotor activities (data not shown), 1-h memory (Fig. 8, which is published as supporting information on the PNAS web site), or the CI toward mated females during conditioning (Fig. 7*C*). These results indicate that disruption of Dynamin function in *per*-expressing cells specifically interferes with normal LTM, most likely because of the disruption of synaptic transmission.

To determine at what process of LTM formation Dynamin function in *per*-expressing cells is required, we shifted the temperature from permissive to restrictive during three experimental phases, namely, the period for courtship conditioning (acquisition of memory), the test period (retrieval), and the period in-between (storage) (Fig. 5*D*, 30-25-25, 25-25-30, and 25-30-25). When we tested at 30°C (Fig. 5*D*, 25-25-30), there was

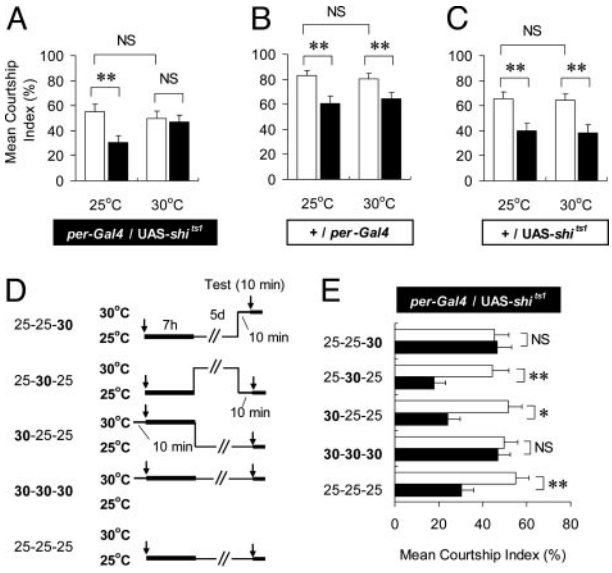

Fig. 5. Disruption of synaptic transmission in the *per*-expressing cells abolishes retrieval, but not formation or storage, of LTM. Open columns represent the mean CI of naive males, and filled columns represent the mean CI of conditioned males. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. (*A*) We used *per-Gal4*/UAS-shi^{ts1} males ($n = 30$ for each column). (*B*) We used +/*per-Gal4* males ($n = 30$ for each column). (*C*) We used +/UAS-shi^{ts1} males ($n = 30$ for each column). (*D*) Experimental paradigms of temperature-shift are indicated. The first and second arrows indicate the beginning of 7-h conditioning and 10-min test, respectively. (*E*) *per-Gal4*/UAS-shi^{ts1} males were used in the experiments: 25-25-30, disruption of synaptic transmission from *per*-expressing cells abolishes retrieval of LTM; 25-30-25, synaptic transmission from *per*-expressing cells is not required for storage of LTM; 30-25-25, synaptic transmission from *per*-expressing cells is not required for formation of LTM ($n = 30$ for each column).

no significant difference in the CI between naive and conditioned *per-Gal4*/UAS-shi^{ts1} males (Fig. 5*E*, 25-25-30); i.e., LTM was not observed in these males. In this paradigm (25-25-30), we were testing the contribution of *per*-expressing cells to the retrieval of LTM. When the temperature during courtship conditioning or the interval between conditioning and test was shifted to 30°C, *per-Gal4*/UAS-shi^{ts1} males acquired, stored, and retrieved LTM (Fig. 5*E*, 25-30-25 and 30-25-25). Furthermore, no significant difference was detected in the CI of naive males in five sets of temperature combinations (Fig. 5*E*, open columns; Kruskal–Wallis test, $P > 0.66$), indicating that the temperature shift between 25°C and 30°C does not affect male courtship activities. Taken together, we suggest that Dynamin function, most likely synaptic transmission, in *per*-expressing cells is required during retrieval, but not formation or storage, of LTM.

Concluding Remarks. This study demonstrated in *Drosophila* that the *per* gene is critically involved in LTM formation induced by courtship conditioning. *per* mutations inhibited formation of LTM (Fig. 3*A*), and induction of a wild-type *per*–transgene preceding courtship conditioning rescued formation of LTM in the *per*-null background (Fig. 3*C*). Furthermore, overexpression of *per* in the wild-type background enhanced formation of LTM (Fig. 4*B*). These results indicate that LTM formation depends on the level of *per* expression. Because *per* mutations did not affect 1-h memory (Fig. 2*I*), this gene must be selectively involved in LTM formation.

We found that *per* expression was required for the formation of LTM (Fig. 3*C*), whereas synaptic transmission through *per*-expressing cells might be required for retrieval of LTM but not for formation or storage (Fig. 5*E*). Taken together, these results

(Figs. 3*C* and 5*E*) indicate that *per*-expressing cells are critically involved both in the formation and retrieval of LTM. We still don't know which *per*-expressing neurons are important for LTM, but our results indicate that *per*-expressing neurons, which is involved in LTM formation, are distinct from pacemaker neurons of the circadian clock (Fig. 3*E*). MBs are known to be a critical region of the brain for retrieval of immediate or 3-h memory induced by olfactory conditioning to execute appropriate behaviors (36, 37), and LTM induced by olfactory conditioning is not detected in flies whose MBs are genetically ablated (38). In addition, McBride *et al*. (23) have reported that 1-h memory and LTM induced by courtship conditioning are not detected in flies whose MBs are chemically ablated. Because, apparently, *per* is not expressed in MBs (26), *per*-expressing neurons that directly or indirectly interact with MBs might be involved in formation or retrieval of LTM. Considering that LTM seems to be normal in $dClk^{Jrk}$ and cyc^{0} mutants where overall *per* expression is greatly reduced, it is likely that particular neurons that maintain fairly normal *per* expression in these mutants are essential in LTM formation. One potentially important brain structure for control of LTM through PER function is the central complex (CX). The CX is known to be involved in learning and memory in *Drosophila* (39–41), and *per* is expressed in a part of the CX. Furthermore, we confirmed that overexpression of *per* in the fan-shaped body within the CX enhanced LTM formation (T.S., T.K., and Y.K., unpublished data), suggesting that the CX is involved in formation of LTM induced by courtship conditioning. *per* expression in some CX neurons may not be affected by $dClk^{Jrk}$ and cyc^{0} mutations. For example, *per–Gal4*-directed GFP expression in neurons sending fibers from the large neuronal clusters that are relatively ventral within laterally located neuronal clusters in the posterior optic tract seems unaffected by either $dClk^{Jrk}$ or cyc^{0} (26). Such neurons are not likely to be involved in regulating circadian rhythm but may play a critical role in LTM formation.

In species ranging from invertebrates, such as *Aplysia* or *Drosophila*, to mammals, CREB has been shown to be involved in LTM formation (1, 8–11). Our findings reinforce this connection but also separate the formation of LTM from the circadian clock. Nevertheless, because the molecular mechanism of the mammalian circadian clock is similar to that in *Drosophila* (14–16), it is intriguing to know whether *per* plays a key role in the formation of LTM in mammals.

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