T1 and T2 contingent on correct identification of T1 were considered separately and submitted to two independent mixed-design ANOVAs. In addition, control, LTL and RTL difference scores (negative minus neutral) were submitted to an additional ANOVA. The valence and arousal evaluation ratings for the control, RTL and LTL groups were submitted to separate between-group *t*-tests.

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α-CaMKII-dependent plasticity in the cortex is required for permanent memory

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Cortical plasticity seems to be critical for the establishment of permanent memory traces[1–3]. Little is known, however, about the molecular and cellular processes that support consolidation of memories in cortical networks[4,5]. Here we show that mice heterozygous for a null mutation of α-calcium-calmodulin kinase II (α-CaMKII$^{+/-}$) show normal learning and memory 1–3 days after training in two hippocampus-dependent tasks. However, their memory is severely impaired at longer retention delays (10–50 days). Consistent with this, we found that α-CaMKII$^{+/-}$ mice have impaired cortical, but not hippocampal, long-term potentiation. Our results represent a first step in unveiling the molecular and cellular mechanisms underlying the establishment of permanent memories, and they indicate that α-CaMKII may modulate the synaptic events required for the consolidation of memory traces in cortical networks.

A homozygous null mutation of α-CaMKII blocks hippocampal long-term potentiation (LTP) and learning[6,7], and disrupts experience-dependent plasticity in the sensory cortex without affecting cortical topography[8,9]. In contrast, α-CaMKII heterozygous mice learn normally in hippocampus-dependent tasks under moderate-to-intensive training conditions[7]. We were, therefore, able to study the impact of reduced levels of α-CaMKII on the establishment of permanent memories. We trained α-CaMKII$^{+/-}$ mutants and their wild-type littermate controls in contextual fear conditioning. In contextual fear conditioning, an association is formed between a distinctive context and an aversive event that takes place in that context. When placed back in the context, mice exhibit a range of conditioned fear responses, including freezing (the absence of all but respiratory movement). This is a particularly robust and long-lasting form of learning that is dependent on the hippocampus[10,11]. Importantly, post-training lesions of the hippocampus disrupt recent, but not remote, contextual fear memories[10].

After training with three shocks, α-CaMKII$^{+/-}$ mutants and wild-type mice were placed back in the context at different times (1, 3, 10, 17 or 50 days), and freezing behaviour was recorded using automated procedures[12]. To avoid the confounding effects of extinction, separate groups of mice were tested at each retention delay. Whereas wild-type mice showed stable contextual freezing at all test delays, contextual freezing declined sharply at longer test delays in α-CaMKII$^{+/-}$ mutants (genotype × delay interaction, $F(4, 102) = 17.6$, $P < 0.01$) (Fig. 1a).

During training, α-CaMKII$^{+/-}$ mutants were around 20% more active than the wild-type mice before shock delivery ($F(1, 102) = 14.8$, $P < 0.05$) (data not shown). We therefore assessed contextual memory with a different measure that is not affected by differences in basal activity. In the same mice, we calculated activity suppression by comparing activity levels before a shock delivery during training with activity levels during the test, thus normalizing for initial differences in activity[12]. Although wild-type and α-CaMKII$^{+/-}$ mice showed indistinguishable levels of activity suppression one day after training, at longer delays, activity

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suppression was impaired in α-CaMKII$^{+/-}$ mutants but not in wild-type controls (genotype × delay interaction, $F(4, 102) = 28.5$, $P < 0.05$) (Fig. 1b). Post-hoc analyses confirmed that levels of activity suppression were greater in wild-type controls than in α-CaMKII$^{+/-}$ mutants at all retention delays except at day one ($P < 0.05$).

We next tested whether intense training could compensate for the memory deficits of the α-CaMKII$^{+/-}$ mutants. We trained mice with eight shocks and tested separate groups 1, 3 or 17 days after training. Consistent with our previous experiment, freezing declined in mutants, but not in wild-type controls, at longer test delays (genotype × delay interaction, $F(2, 41) = 9.1$, $P < 0.05$) (Fig. 1c). Post-hoc analyses confirmed that freezing was lower in α-CaMKII$^{+/-}$ mutants only in the 3- and 17-day tests ($P < 0.05$). A similar pattern was observed when activity suppression was used as an index of contextual fear (Fig. 1d). Activity suppression was impaired in α-CaMKII$^{+/-}$ mutants, but not wild-type mice, at longer test delays (genotype × delay interaction, $F(2, 41) = 11.0$, $P < 0.05$). Therefore, after either moderate (three shocks) or intense (eight shocks) training, memory loss in α-CaMKII$^{+/-}$ mutants is specific to longer retention delays. Protein-synthesis blockers impair contextual memory 3–5 hours after training[13]. The similar levels of contextual fear in the mutant and wild-type mice 24 h after training indicate that protein synthesis-dependent processes underlying the initial formation of hippocampus-dependent memories are unaffected in these mutant mice. Notably, the time course of memory decay in α-CaMKII$^{+/-}$ mutants corresponds to the shift from hippocampus-dependent to hippocampus-independent retrieval inferred from imaging and lesion studies in mice and rats[10,14,15]. For example, the genetic disruption of NMDA (*N*-methyl-D-aspartate) receptors in the CA1 region of the hippocampus during the 0–14-day period after training blocks contextual memories in mice. In contrast, similar disruption three weeks after training leaves these memories intact[15].

Under less intensive training conditions, α-CaMKII$^{+/-}$ mutants exhibit learning impairments that are affected by genetic background (data not shown)[7]. Thus, despite equivalent levels of freezing in the retention test at day one, the initial underlying memory trace might be weaker in α-CaMKII$^{+/-}$ mutants, and hence less durable. To address this, we compared contextual memory in wild-type mice after weak training (one shock) with contextual memory in α-CaMKII$^{+/-}$ mutants that had been trained intensively (eight shocks). Wild-type mice, trained at the same time as the α-CaMKII$^{+/-}$ mutants, were tested 1, 3 or 17 days after training. WT mice exhibited much lower freezing levels than α-CaMKII$^{+/-}$ mutants at the one-day retention delay. However, unlike the mutants, their levels of freezing were similar at all retention intervals (genotype × delay interaction, $F(2, 54) = 13.7$, $P < 0.05$) (Fig. 1e), indicating that lower initial levels of freezing do not necessarily result in unstable memory. Post-hoc analyses revealed that α-CaMKII$^{+/-}$ mutants froze more than wild-type controls one day after training, but significantly less than wild-type controls 17 days after training ($P < 0.05$).

To assess the severity of memory loss in mutants at long delays, we compared the acquisition of contextual fear conditioning in naive (non-trained) α-CaMKII$^{+/-}$ mutants and α-CaMKII$^{+/-}$ mutants that had been trained 30 days before with three shocks. Both naive and previously trained mutants showed similar rates of acquisition of contextual freezing when trained with one shock per day over four days (Fig. 1f); this was supported by an absence of a condition (trained versus naive) × day (1–4) interaction ($F(3, 30) = 0.20$, $P = 0.89$). The absence of accelerated acquisition (or behavioural savings) in the previously trained mutants is consistent with the hypothesis that memory loss at long retention delays is substantial.

To test whether our initial findings applied to another form of hippocampal learning, we trained α-CaMKII$^{+/-}$ mutants in the Morris water maze[16]. In this spatial learning task, mice learn to navigate to a submerged platform by using extra-maze cues. Mice were trained with 12 trials per day over 5 days, as under these conditions α-CaMKII$^{+/-}$ mutants performed this task as well as wild-type controls[7]. At the completion of training, spatial learning was assessed in a 60-s probe test where the platform was removed from the pool (Fig. 2a–c). To assess spatial memory at different retention delays, separate groups of mice were tested 3, 10 or 17 days after completing the training (Fig. 2d–f). No other training was given between the probe test at the end of training and the memory probe test. In probe tests at the completion of training, wild-type and α-CaMKII$^{+/-}$ mice spent equivalent amounts of time in the target quadrant of the pool ($F(1, 49) = 0.93$, $P = 0.34$). Similarly, α-CaMKII$^{+/-}$ mutants exhibited normal spatial memory when tested three days after completing the training ($F(1, 16) = 0.37$, $P = 0.55$). Therefore, consistent with the fear conditioning data, α-CaMKII$^{+/-}$ mutants showed normal acquisition and normal

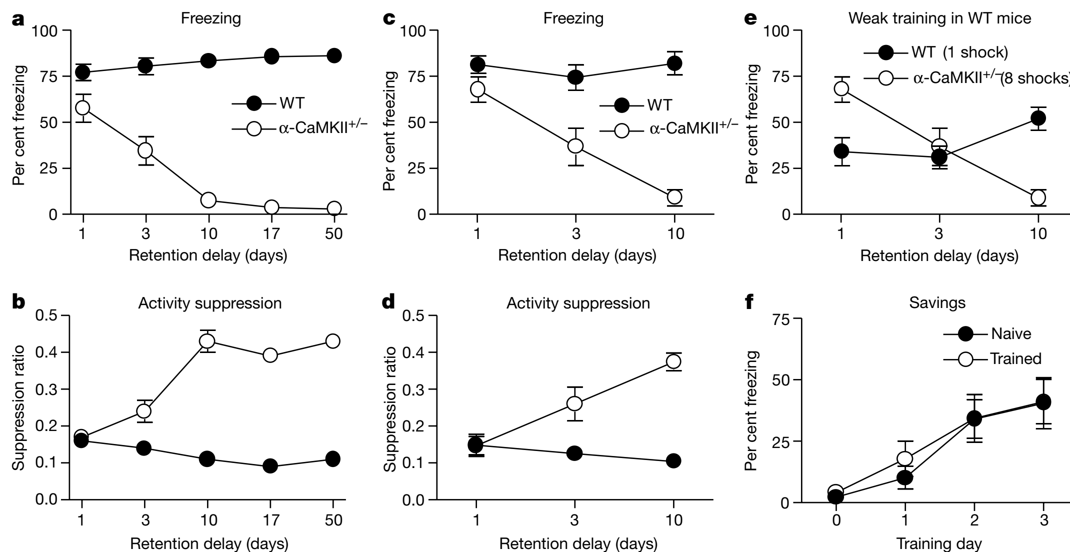

Figure 1 Contextual memory in α-CaMKII$^{+/-}$ mutants. **a**, **b**, Freezing and activity suppression for wild-type (WT) and α-CaMKII$^{+/-}$ mice trained with three shocks and tested at different retention delays. **c**, **d**, Freezing and activity suppression for WT and α-CaMKII$^{+/-}$ mice trained with eight shocks and tested at different retention delays. **e**, Freezing at different retention delays in WT mice after weak training (one shock) and α-CaMKII$^{+/-}$ mutants after intensive training (eight shocks). **f**, Acquisition of contextual freezing in previously trained and naive α-CaMKII$^{+/-}$ mutants.

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memory at short retention delays, indicating that motivation, vision and motor skills required for the task were not affected. However, α-CaMKII$^{+/-}$ mutants spent significantly less time searching the target quadrant when tested 10 days ($F(1, 15) = 15.2$, $P < 0.05$) and 17 days ($F(1, 18) = 10.1$, $P < 0.05$) after the completion of training. Notably, there was a significant genotype × delay interaction ($F(2, 49) = 3.23$, $P < 0.05$), reflecting poorer spatial memory in α-CaMKII$^{+/-}$ mutants only at long retention delays (Fig. 2g). Retrograde amnesia after hippocampal lesions is typically not graded in water maze studies[17], possibly because the hippocampus is required for some aspects of performance during navigation, such as path integration[18–20]. However, under the training conditions used, α-CaMKII$^{+/-}$ mutants learned the water maze normally, indicating that the processing required for performance of the task, such as path integration, is not affected under these conditions in these mice. Rather, the poor performance at long retention delays is likely to reflect loss of spatial memory.

These data show that α-CaMKII$^{+/-}$ mutants have deficits in memory at a time when memories become more dependent on cortical function, and less on the hippocampus[10,14]. Whereas hippocampal plasticity is required for initial learning and memory formation, cortical plasticity is thought to be critical for the establishment of permanent memory traces[1–3,14]. Therefore, hippocampal and cortical synaptic plasticity may be differentially affected in α-CaMKII$^{+/-}$ mutants[7,21]. To investigate this possibility, we examined LTP induced by five θ-burst stimulations (TBS) in the hippocampus and cortex in the same slices from α-CaMKII$^{+/-}$ mutants and wild-type mice (Fig. 3). This strong induction protocol saturates LTP in the cortex (A.K., unpublished observations). Memory is likely to be widely distributed within cortical networks[3], so we chose to study the visual cortex as a representative of primary sensory cortex and the temporal cortex as a representative of associational cortex. Stimulation of Schaffer collaterals produced stable LTP in the CA1 region of the hippocampus of wild-type ($t(10) = 7.17$, $P < 0.05$) and of α-CaMKII$^{+/-}$ mice ($t(8) = 4.54$, $P < 0.05$), and the magnitude of LTP did not differ between the two ($F(1, 18) = 1.83$, $P = 0.19$). In addition, protein-synthesis-

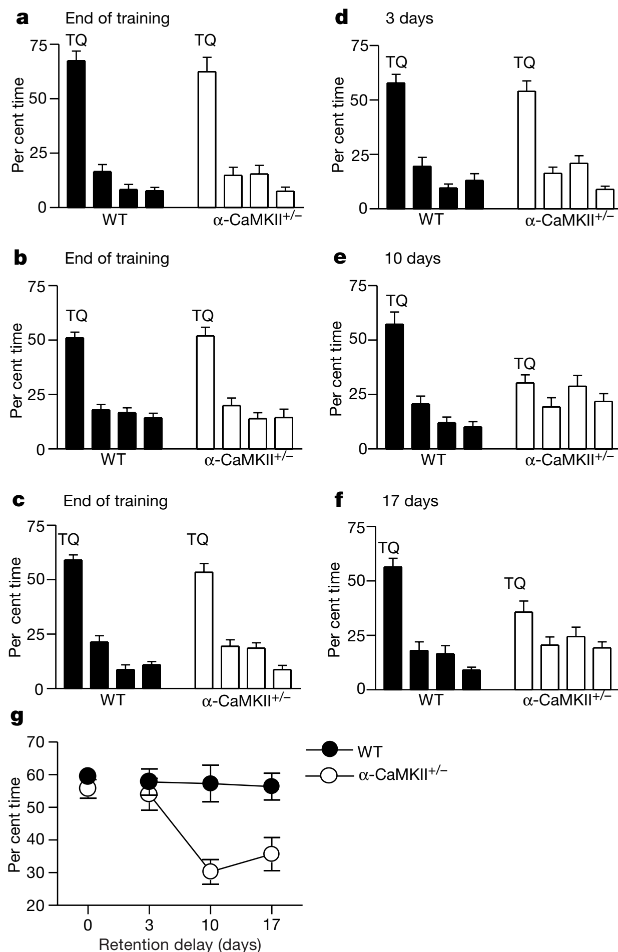

Figure 2 Spatial memory in α-CaMKII$^{+/-}$ mutants. Mice were given a probe test at the end of training (**a**–**c**) and then tested 3, 10 or 17 days after training (**d**–**f**). The per cent time searching the target quadrant (TQ) of the pool in which the platform was located during training versus each of the other three quadrants is plotted for WT and α-CaMKII$^{+/-}$ mutants. After training, WT mice and α-CaMKII$^{+/-}$ mutants searched selectively in the TQ. Spatial memory in α-CaMKII$^{+/-}$ mutants was normal three days after training, but impaired in the 10- or 17-day retention tests. **g**, Summary of results of probe tests for WT and α-CaMKII$^{+/-}$ mice. The per cent time searching the TQ is plotted for each of the probe tests at different times after the completion of training.

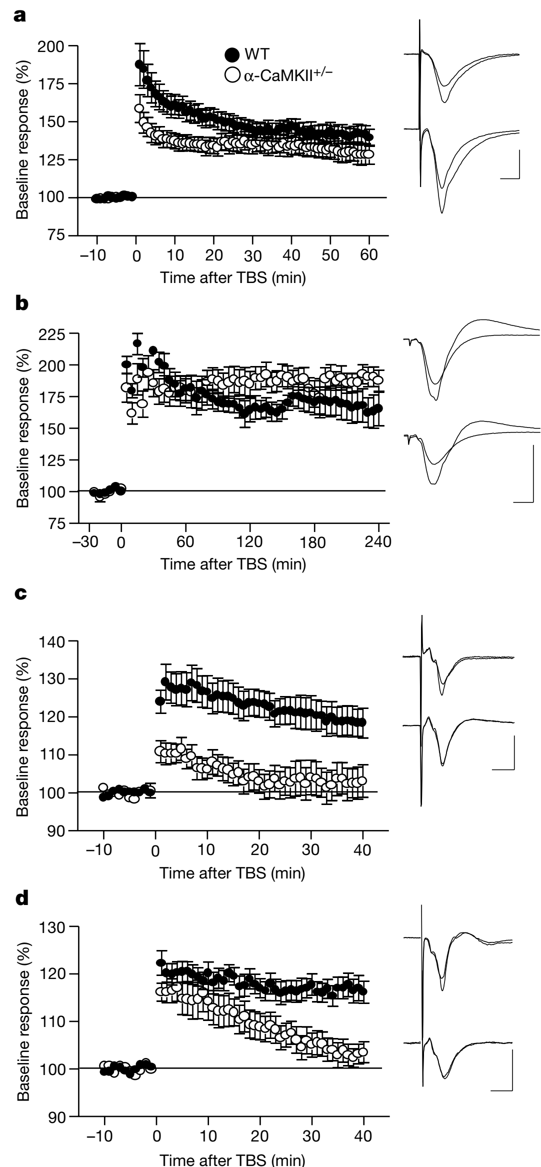

Figure 3 Reduced cortical LTP in α-CaMKII$^{+/-}$ mutants. The graphs depict the time course of the changes in magnitude of synaptic responses following TBS for recordings in the CA1 region of the hippocampus (**a**, LTP; **b**, late LTP), visual cortex (**c**), and temporal cortex (**d**) for slices from WT and α-CaMKII$^{+/-}$ mice. Examples of LTP are shown at the right of each graph (WT, upper; α-CaMKII$^{+/-}$, lower). These superimposed traces are the average of four consecutive responses recorded 1 min before TBS and at the end of the experiment (60 min for CA1 LTP, 240 min for CA1 late LTP, 40 min for visual and temporal cortex LTP). Calibration bars: 5 ms, 1 mV.

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dependent late LTP, induced by three trains of ten TBS, was normal in α-CaMKII$^{+/-}$ mutants ($F(1, 11) = 2.87$, $P > 0.05$). Post-tetanic potentiation was reduced in α-CaMKII$^{+/-}$ mutants[7]. This is consistent with our behavioural data showing that memory is normal in α-CaMKII$^{+/-}$ mutants up to three days after training. In contrast, stimulation of layer IV failed to produce LTP in visual cortex ($t(10) = 1.30$, $P > 0.05$) and temporal cortex ($t(22) = 1.82$, $P > 0.05$) in α-CaMKII$^{+/-}$ mutants. Similar stimulation produced stable LTP in visual cortex ($t(18) = 4.23$, $P < 0.05$) and temporal cortex ($t(21) = 7.00$, $P < 0.05$) in wild-type mice, and these levels of potentiation were significantly higher than those in α-CaMKII$^{+/-}$ mutants (visual cortex: $F(1, 28) = 5.87$, $P < 0.05$; temporal cortex: $F(1, 43) = 15.3$, $P < 0.05$). Consistent with previous data, these results indicate that LTP impairments in α-CaMKII$^{+/-}$ mutants are restricted to the cortex[7,21]. Both hippocampal and cortical LTP are impaired in α-CaMKII homozygous mice[7,21]. The α-CaMKII protein is expressed more abundantly in the hippocampus than in the cortex, so it is likely that the loss of half the normal levels of this kinase affects cortical plasticity more severely.

In humans and animals, damage limited to the hippocampal formation produces a severe amnesia for recently acquired information that is declarative or episodic in nature, but spares memory for similar remotely acquired information[10,22–24]. The opposite pattern is observed in cases of semantic dementia associated with atrophy of the temporal cortex, which leads to impairments of remote, rather than recent, memory[2]. Consistent with this, retrieval of recently acquired spatial information is associated with activation of the hippocampus, whereas retrieval of remotely acquired spatial information is associated with activation of the cortex in mice[14]. This has led to the idea that storage and retrieval of recent memories is dependent on hippocampal networks, whereas storage and retrieval of remote memories depends on cortical[1–3] or cortical–hippocampal[25] networks. Although hippocampal lesions preferentially disrupt recent but not remote (or permanent) memories, we found that a functional disruption of cortical plasticity preferentially disrupts the establishment of permanent memories. Furthermore, the time course of the memory decay in the α-CaMKII$^{+/-}$ mutants corresponds to that predicted by imaging and lesion studies in mice and rats[10,14,15,26]. In addition, we found that mice that are heterozygous for a point mutation that substitutes threonine 286 for alanine in α-CaMKII (α-CaMKII$^{T286+/-}$)[27] exhibit normal contextual and spatial memory for up to 50 days (data not shown). Because cortical plasticity is not impaired in these mice[28], this finding strengthens the conclusion that normal cortical plasticity is required for the establishment of permanent memory traces. The consolidation of memory traces in cortical networks is likely to depend on the recurrent activation of cortical traces during, for example, sleep[3]. Our results indicate that repetitive LTP-like events, which are dependent on α-CaMKII, are required for the consolidation of memory traces in cortical networks, and they represent a first step in unveiling the molecular and cellular mechanisms underlying cortical memory consolidation. They further indicate that the molecular processes underlying the establishment of permanent cortical memory traces may share some of the same mechanisms as those underlying initial encoding of hippocampal memories. □

Methods

Mice

The α-CaMKII$^{+/-}$ mice used in the fear conditioning and electrophysiological experiments were the F_1 progeny from a cross between α-CaMKII$^{+/-}$ mice in the C57B1/6 background (> 99%) and 129/SvEms mice. For the water maze studies, mice were tested from two different genetic backgrounds with similar findings. The two backgrounds tested included mice that were the offspring from a cross between α-CaMKII$^{+/-}$ mice in the C57B1/6 background (> 99%) and 129/SvEms mice, and mice resulting from a cross between chimaeras having the α-CaMKII mutation in the 129/Ola background and C57B1/6 mice. The C57B1/6 background exacerbates the effects of the α-CaMKII heterozygous mutation (data not shown). Mice were housed in groups and maintained on a 12 h light/dark cycle. Mice (at least eight weeks old) were tested during the light phase of the cycle. All behavioural and electrophysiological experiments were conducted blind to the genotype.

Contextual conditioning

In each of the experiments, mice were placed in the conditioning chamber and presented with a series of unsignalled foot-shocks (2 s duration, 0.5 mA, 1 min apart). During testing, mice were placed back in the conditioning chamber and freezing and activity were recorded using automated procedures described previously[12]. Activity suppression ratios were calculated by comparing activity levels during the first 2 min of training with the first 2 min of testing according to the formula: $(\text{activity}_{\text{test}}/(\text{activity}_{\text{train}} + \text{activity}_{\text{test}}))$. In the first experiment, mice were trained with 3 foot-shocks and tested 1 (wild-type, $n = 8$; α-CaMKII$^{+/-}$, $n = 9$), 3 (wild-type, $n = 14$; α-CaMKII$^{+/-}$, $n = 12$), 10 (wild-type, $n = 16$; α-CaMKII$^{+/-}$, $n = 10$), 17 (wild-type, $n = 14$; α-CaMKII$^{+/-}$, $n = 11$) or 50 (wild-type, $n = 9$; α-CaMKII$^{+/-}$, $n = 9$) days after training. In the second experiment, mice were trained with 8 foot-shocks and tested 1 (wild-type, $n = 9$; α-CaMKII$^{+/-}$, $n = 6$), 3 (wild-type, $n = 7$; α-CaMKII$^{+/-}$, $n = 9$) or 17 (wild-type, $n = 8$; α-CaMKII$^{+/-}$, $n = 9$) days after training. In the third experiment, wild-type mice were trained with 1 foot-shock (after 3 min of a 5-min session) and tested 1 ($n = 9$), 3 ($n = 14$) or 17 ($n = 13$) days after training. In the fourth experiment, acquisition of contextual fear conditioning was compared in naive mutants and mutants that had been trained with 3 shocks 30 days earlier. During acquisition, naive ($n = 6$) and trained ($n = 6$) mutants were trained with a single foot-shock (delivered after 3 min of a 5-min session) over four consecutive days. Freezing was measured before the shock was delivered on each day.

Water maze

The water maze apparatus and procedures have been described[29]. Mice were trained with 12 trials per day for 5 days (3 blocks of 4 trials; 1 min inter-trial intervals, 1 h inter-block intervals). Spatial learning and memory were assessed in probe tests with the platform removed from the pool. All mice were given a probe test at the end of training, and a memory probe test 3 (wild-type, $n = 10$; α-CaMKII$^{+/-}$, $n = 8$), 10 (wild-type, $n = 9$; α-CaMKII$^{+/-}$, $n = 8$) or 17 (wild-type, $n = 10$; α-CaMKII$^{+/-}$, $n = 10$) days later.

Electrophysiology

Coronal brain slices from 6–8-week-old mice containing visual cortex, temporal cortex and the CA1 region of the hippocampus were prepared as described[21]. Slices were left to recover for at least 1 h before recording in oxygenated (95% O_2 and 5% CO_2) warm (30 °C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose. The experiments were performed in an interface chamber with an atmosphere of humidified 95% O_2 and 5% CO_2, and superfused with 30 °C ACSF at a rate of 1 ml min^{-1}. Field potentials (FP) in layer III evoked by layer IV stimulation, and CA1 FP evoked by Schaffer collateral stimulation, were measured as previously described[30]. Responses were quantified either as the initial slope of the FP, in CA1, or as the amplitude of the FP in cortex. The amplitude, rather than slope, of the FP in layer III was used as a measure of the evoked population excitatory synaptic current, because the initial slope was contaminated by the presynaptic volley. LTP was induced with TBS, which consisted of five brief bursts (four pulses at 100 Hz) of stimuli delivered every 200 ms. Average responses (± s.e.m.) were expressed as percentage of pre-TBS baseline responses (at least 10 min of stable responses). LTP induction was assessed with paired *t*-tests by comparing responses recorded before and 60 min (CA1 LTP) or 40 min (cortical LTP) after TBS. To examine late LTP in CA1, transverse hippocampal slices were maintained in a submerged chamber as previously described[27]. Late LTP was induced by 3 trains of 10 TBS spaced 10 min apart. To determine whether the magnitude of LTP differed between wild-type and α-CaMKII$^{+/-}$ mice, responses from the last 10-min block of recordings (51–60 min for CA1 LTP; 31–40 min for cortical LTP) or the last 30-min block of recordings (210–240 min for CA1 late LTP) were compared using analysis of variance.

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A new role for cryptochrome in a *Drosophila* circadian oscillator

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Cryptochromes are flavin/pterin-containing proteins that are involved in circadian clock function in *Drosophila* and mice. In mice, the cryptochromes Cry1 and Cry2 are integral components of the circadian oscillator within the brain[1–6] and contribute to circadian photoreception in the retina[7]. In *Drosophila*, cryptochrome (CRY) acts as a photoreceptor that mediates light input to circadian oscillators in both brain and peripheral tissue[8–12]. A *Drosophila cry* mutant, *cry^b*, leaves circadian oscillator function intact in central circadian pacemaker neurons but renders peripheral circadian oscillators largely arrhythmic. Although this arrhythmicity could be caused by a loss of light entrainment, it is also consistent with a role for CRY in the oscillator. A peripheral oscillator drives circadian olfactory responses in *Drosophila* antennae[13]. Here we show that CRY contributes to oscillator function and physiological output rhythms in the antenna during and after entrainment to light–dark cycles and after photic input is eliminated by entraining flies to temperature cycles. These results demonstrate a photoreceptor-independent role for CRY in the periphery and imply fundamental differences between central and peripheral oscillator mechanisms in *Drosophila*.

We initially established that the *cry^b* mutant severely reduces or eliminates circadian rhythms in olfactory responses and molecular rhythms in oscillator components during and after entrainment to light–dark cycles. Electroantennogram (EAG) responses to the food odorant ethyl acetate were measured in wild-type and *cry^b* flies under 12 h:12 h light–dark cycles (LD) and on the second day of constant darkness (DD) after LD entrainment. In *cry^b* mutants, we observed EAG responses of similar magnitude throughout the day except for a weak but statistically significant peak at Zeitgeber time 17 (5 h after lights off; Fig. 1a), indicating that the *cry^b* allele is nearly a loss-of-function mutant. This small peak was lost when mutant antennae were recorded in DD (Fig. 1b). Thus, *cry$^+$* is required for a

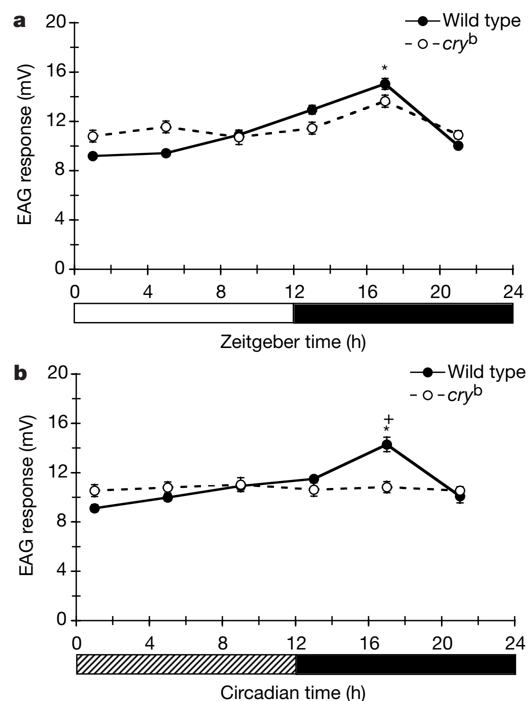

Figure 1 Olfactory responses of wild-type and *cry^b* mutant *Drosophila* under LD and DD conditions. Each point represents mean odorant-evoked EAG responses. Error bars denote s.e.m. **a**, Responses on the fourth day of LD 12 h:12 h. $n = 48$ flies per point. White bar, lights on; black bar, lights off. Overall effects of time of day and interaction were significant ($P < 0.05$) by two-way analysis of variance (ANOVA), but effects of genotype were not. Asterisk, significant ($P < 0.005$) difference in wild-type responses at Zeitgeber time (ZT)17 compared to all other times of day. The *cry^b* response at ZT17 was significantly ($P < 0.0001$) different from that at ZT1, but not other times of day. **b**, Responses on the second day of DD, indicating lack of rhythmicity in the mutant. $n = 24$ flies per point. Hatched bar, subjective day; black bar, subjective night. Cross, significant ($P < 0.005$) difference between wild type at ZT17 and *cry^b* at all times of day. Overall effects of time of day and interaction were significant ($P < 0.000001$), but effects of genotype were not. Responses of *cry^b* in LD and DD were not different ($P > 0.1$) at any time of day by three-way ANOVA.

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