Phosphorylation of Eukaryotic Translation Initiation Factor 4E and Eukaryotic Translation Initiation Factor 4E-binding Protein (4EBP) and Their Upstream Signaling Components Undergo Diurnal Oscillation in the Mouse Hippocampus

IMPLICATIONS FOR MEMORY PERSISTENCE*

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Background: mRNA translation is crucial for formation of long-term memory.

Results: Phosphorylation of eIF4E, 4EBPs, rpS6, Akt, and mTOR, and ERK1/2 undergoes diurnal oscillation in mouse hippocampus.

Conclusion: Diurnal oscillation in markers of mRNA translation initiation is critical for memory consolidation and persistence.

Significance: Circadian oscillation in mRNA translation initiation provides a potential mechanism for persistence of long-term memory.

Translation of mRNA plays a critical role in consolidation of long-term memory. Here, we report that markers of initiation of mRNA translation are activated during training for contextual memory and that they undergo diurnal oscillation in the mouse hippocampus with maximal activity observed during the daytime (zeitgeber time 4–8 h). Phosphorylation and activation of eukaryotic translation initiation factor 4E (eIF4E), eIF4E-binding protein 1 (4EBP1), ribosomal protein S6, and eIF4F cap-complex formation, all of which are markers for translation initiation, were higher in the hippocampus during the daytime compared with night. The circadian oscillation in markers of mRNA translation was lost in memory-deficient transgenic mice lacking calmodulin-stimulated adenylyl cyclases. Moreover, disruption of the circadian rhythm blocked diurnal oscillations in eIF4E, 4EBP1, rpS6, Akt, and ERK1/2 phosphorylation and impaired memory consolidation. Furthermore, repeated inhibition of translation in the hippocampus 48 h after contextual training with the protein synthesis inhibitor anisomycin impaired memory persistence. We conclude that repeated activation of markers of translation initiation in hippocampus during the circadian cycle might be critical for memory persistence.

Long-term memory (LTM)3 formation is dependent on the de novo translation of mRNA (1, 2). De novo mRNA translation plays a crucial role by increasing the synthesis of proteins required for enhanced synaptic activity, which then can contribute to memory engrams. A critical step in mRNA translation is the initiation phase, which requires several eukaryotic initiation factors, including eIF4E, an mRNA cap-binding protein. The rate-limiting step for cap-dependent mRNA translation initiation is the formation of the eIF4F complex containing eIF4E (cap-binding protein), eIF4G (scaffold protein), and eIF4A (ATP-dependent mRNA helicase) (3). As eIF4E is the limiting factor in eIF4F complex formation, its binding to the mRNA cap is tightly regulated by members of a family of small molecular weight proteins, the eIF4E-binding proteins (4E-BPs). 4E-BPs binding to eIF4E is modulated by phosphorylation involving multiple serine and threonine residues (4). Hypophosphorylated 4E-BPs bind firmly to eIF4E, and conversely increased phosphorylation of 4E-BP1 or 4EBP2 results in the dissociation of 4E-BPs from eIF4E. Hyper-phosphorylation of 4EBP1/2 thus stimulates cap-dependent translation by making increased amounts of eIF4E available for eIF4F complex formation (3–5). Furthermore, eIF4E itself can undergo phosphorylation at Ser209 causing a concomitant increase in mRNA translation initiation (6–8). In addition, phosphorylation of ribosomal protein S6 (rpS6), a component of the 40 S ribosomal subunit, correlates well with increased translation of 5’-terminal oligopyrimidine tract (TOP) mRNAs (9, 10). These mRNAs encode for ribosomal proteins and other molecules involved in translation machinery. Consequently, phosphorylation of 4EBP1/2 at Thr37/Thr46, eIF4E at Ser209, and rpS6 at Ser235/Ser236 can be used to monitor translation initiation.

A striking feature of memory is the persistence of LTM, which can last for periods exceeding the lifetimes of proteins produced during the initial memory consolidation. The goal of this study was to examine the hypothesis that initiation of mRNA translation in the hippocampus may undergo periodic reactivation, perhaps during the circadian cycle. Here, we report that phosphorylation of eIF4E, 4EBP1/2, rpS6, and mRNA cap-complex formation, all of which are crucial for
mRNA translation initiation, undergoes circadian oscillations in the mouse hippocampus, reaching a peak during the daytime. This diurnal oscillation in translation initiation was associated with increased activity of ERK1/2 MAPK and mammalian target of rapamycin (mTOR), and it was lost in memory-deficient mice lacking calmodulin-stimulated adenyl cyclases 1 and 8. Disruption of this diurnal oscillation physiologically by constant light exposure resulted in impairment of LTM. Moreover, inhibition of protein synthesis during the daytime by anisomycin 2 days after training impaired memory persistence measured 14 and 28 days after contextual training. This suggests the interesting possibility that reactivation of protein synthesis in the hippocampus during the circadian cycle contributes to the persistence of hippocampus-dependent memory.

EXPERIMENTAL PROCEDURES

Mice—Adult (10–18 weeks old) male C57/BL6 and double transgenic AC1/AC8 (DKO) mice were used for all experiments. DKO (AC1−/− × AC8−/−) mice were generated as described previously (11) and bred into the C57/BL6 background for at least 10 generations. Animals were housed in a 12-h light/12-h dark schedule and allowed to eat and drink ad libitum. Experiments were carried out in accordance with the Institutional Animal Care and Use Committee’s recommendations at the University of Washington.

Circadian Time Course—Mice were housed for at least 10 days in a 12-h light/12-h dark cycle prior to experiments. For diurnal oscillation experiments, animals were sacrificed every 4 h during the 24-h period. In dark lighting conditions, mice were sacrificed under 1–2 lux, provided by a Kodak GBX-2 red light with a safelight filter. Lux was measured using a light meter (VWR Scientific).

Contextual Fear Conditioning—One week before the conditioning experiment, mice were housed individually in a cage and handled daily. On the day of training, a mouse was placed into a conditioning chamber (context) made of transparent walls (10 inches wide × 10 inches long × 16 inches high) with a metal grid floor. The floor of the cage consisted of a circuit board that delivered shocks to metal grids (Coulbourn Instruments). The mice were allowed 2 min to explore the chamber prior to receiving a 2-s, 0.7 mA foot shock delivered through the floor. After an additional 1 min in the chamber, the mouse was returned to its home cage. Mice used as unpaired controls were immediately shocked after they were placed in chamber (context) and then quickly returned to their home cages. Unpaired controls did not develop memory for the training context. Mice were sacrificed 30 min after fear conditioning training. Mice used for behavioral experiments were trained and then returned 24 h later to the chamber and videotaped for freezing behavior (no movements except for breathing). Freezing behavior of each mouse was scored every 5 s for 2 min.

Passive Avoidance—The training chamber for measuring step-through latency was a shuttle box that was equally divided into light and dark compartments by a metal partition with a trap door (Coulbourn Instruments). During the training, mice were allowed to explore the lit side freely for 1 min before the trap door was opened. Immediately after mice entered the dark side, the door was closed and a mild foot shock (0.7 mA, 2 s) was delivered. Mice were kept in the dark side for 30 s and then returned to their home cages. Then 24 h after training, mice were put back into the lit side, and the time mice spent before crossing over into the dark side (step-through latency) was recorded. The cutoff value for crossover latency was set to 8 min.

Actogram Acquisition—Mice were individually housed in cages for circadian voluntary activity data acquisition. This was monitored with the use of QA-4 activity input modules coupled to infrared motion detectors. Data were generated with VitalView Data Acquisition System (Mini Mitter, version 4.1) and were later transferred to the ActiView Biological Rhythm Analysis program (Mini Mitter, version 1.2) for actogram generation. The actograms shown are the representative locomotion plots for a group of mice in the corresponding light/dark (L/D), dark/dark (D/D), or light/light (L/L) conditions.

Cannulation and Intra-hippocampal Infusion—Mice were bilaterally implanted with cannulae (Plastics One, VA) using the following coordinates from bregma: anterior/posterior −1.65 mm, medial/lateral 1.5 mm, dorsal/ventral −1.5 mm, and were fixed to the skull with dental acrylic. Anisomycin (125 μg/μl, Sigma) was dissolved in 1x HCl, diluted with sterile PBS, and adjusted to pH 7–7.5 with NaOH. Vehicle solution consisted of same amount of HCl and NaOH as in the anisomycin solution. Mice received bilateral intra-hippocampal injections (0.5 μl/side) of either anisomycin (62.5 μg/0.5 μl per side) or vehicle through an infusion cannula (injectors) connected to a Hamilton syringe through polyethylene tubing. Animals were only briefly restrained to remove the stylets and to insert/remove the injectors. Each side was injected individually, one occurring immediately after the other. Injections were administered over 1 min (each side), and injectors were left in place for an additional 30 s to ensure diffusion of the solution into the hippocampus.

Tissue Extract Preparation and Western Analysis—Adult mice were euthanized by cervical dislocation; their hippocampi were quickly dissected on ice, flash-frozen in liquid nitrogen, and stored at −70 °C until use. For total extract preparation, frozen hippocampal tissue was pulverized and homogenized in ~10 volumes of homogenization buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 100 mM NaF, 1 mM Na3VO4, 1 mM PMSF, a protease inhibitor tablet (Roche Applied Science), and 1:100 dilutions of Sigma phosphatase 2 and phosphatase 3 inhibitors). Homogenates were sonicated and cleared by centrifugation (20 min at 20,000 × g). An equal volume of 2× SDS-PAGE sample buffer (Bio-Rad) was added to lysates, and samples were heated at 95 °C for 5–10 min. Protein concentrations were determined by a dot blot assay, and an equal amount of proteins was resolved on either 12.5 or 4–15% Tris-HCl (v/v) precast polyacrylamide gels (Bio-Rad). Proteins were transferred to a PVDF membrane (Millipore), and the membrane was blocked with 2% BSA (w/v) in Tris-buffered saline with 0.1% Triton X–100 (v/v) (TBST). Western blots were visualized using ChemiDoc XR5 (Bio-Rad), and bands were quantified by using ImageJ software (National Institutes of Health). Quantifications were made from exposures on which band intensities were within the linear range of optical density calibration curves. Primary antibodies used...
were at the following dilutions: rabbit anti-p-ERK1/2 (Thr202/Tyr204, 1:1000, Cell Signaling); mouse anti-ERK1/2 (1:2000, BD Transduction Laboratories); rabbit anti-p-elF4E (Ser209, 1:1000, Cell Signaling); rabbit anti-elF4E (1:1000, Cell Signaling); rabbit anti-p-4EBP1 (Thr37/46, 1:1000, Cell Signaling); rabbit anti-4EBP1 (1:1000, Cell Signaling); rabbit anti-4EBP2 (1:1000, Cell Signaling); rabbit anti-rpS6 (Ser235/236, 1:1000, Cell Signaling); rabbit anti-rpS6 (1:1000, Cell Signaling); rabbit anti-p-Akt (Ser473, 1:1000, Cell Signaling); rabbit anti-Akt (1:1000, Cell Signaling); rabbit anti-p-mTOR (Ser2448, 1:1000, Cell Signaling); and rabbit anti-elF4G1 (1:500, Bethyl Laboratories). HRP-conjugated corresponding secondary antibodies (1:1000, Cell Signaling) were used at room temperature for 1 h, and Western blots were developed using Supersignal West Pico reagent (Thermo Scientific Pierce). Phosphorylated protein blots were stripped in stripping buffer (25 mM glycine, pH 2.0, and 7% SDS) for 30 min, washed three times with TBST for 10 min each, blocked with BSA, and re-probed for total protein signals.

**Cap Analog Pulldown Assay**—Hippocampal lysates were prepared as described above from mice except sonication was omitted. Tissue lysates were incubated with 25 μl of 7-methylGTP-Sepharose 4B beads (GE Healthcare) at 4 °C for 12 h with moderate shaking to precipitate elF4E and the associated proteins. The beads were pelleted, washed four times with homogenization buffer, suspended in 2X SDS-PAGE loading buffer, and denatured at 95 °C for 10 min. Samples were separated on a 4–15% precast gradient gel and analyzed by Western analysis.

**Administration of SL327**—Mice were injected intraperitoneally with either SL327 (2 ml/kg, dissolved in 100% DMSO, Tocris Bioscience) or vehicle (100% DMSO) 30 min before the training in a contextual fear-conditioning paradigm. Then mice were trained for context and sacrificed 30 min later. Hippocampi were isolated from mice on ice, flash-frozen in liquid nitrogen, and stored at −70 °C for further use.

**Statistical Analysis**—Unless otherwise specified, values are expressed as means ± S.E. Significance between the two groups was analyzed by Student’s t tests and was set at p ≤ 0.05. Circadian time course data were analyzed by one-way ANOVA analysis, followed by Dunnett’s multiple comparison test for post hoc analysis.

**RESULTS**

**Training for Contextual Fear Memory Increases elF4E, 4EBPs, and Ribosomal Protein S6 Phosphorylation in the Hippocampus**—Contextual fear conditioning was used to determine whether biochemical markers for initiation of translation were increased when mice were trained for hippocampus-dependent memory. Contextual fear conditioning is a form of associative learning that has been widely used for studying hippocampus-dependent memory (12, 13). Context-trained mice and unpaired controls were sacrificed 30 min after exposure to context and probed for initiation of translation. Context-trained mice exhibited a significant increase in hippocampal Ser(D)^209-ElF4E levels (132.1 ± 3.9) as compared with control mice 30 min after training (n = 5 mice/group, **, p < 0.01, Fig. 1, A and B). No change in total elF4E protein levels was observed between control and context-trained groups. Phosphorylation of 4EBP at Thr^37/Thr^46 sites was also significantly increased in context-trained mice (147.9 ± 15.1) as compared with control mice (**, p ≤ 0.05, Fig. 1, A and B). In this experiment, the antibody used recognizes both p-4EBP1 and p-4EBP2, the latter being the predominant isoform present in the brain. Mice trained for context also exhibited a significant increase in hippocampal rpS6 phosphorylation at Ser^235/236 sites (160.5 ± 21.6) as compared with control mice (**, p ≤ 0.05, Fig. 1, A and C). Because increased phosphorylation of elF4E (6–8), 4EBPs (4, 5, 14), and rpS6 (9, 10) correlates with increased mRNA translation initiation, these data collectively suggest that training for contextual memory is associated with increased mRNA translation in the hippocampus.

**ERK1/2 MAPK Mediates Stimulation of elF4E and 4EBPs Phosphorylation during Training for Context**—Phosphorylation of elF4E is stimulated by the extracellular signal-regulated kinase (ERK1/2)/Mnk1 pathway in several different systems (6–8), 4EBPs (4, 5, 14), and rpS6 (9, 10) correlates with increased mRNA translation initiation, these data collectively suggest that training for contextual memory is associated with increased mRNA translation in the hippocampus.

**elF4E, 4EBP Phosphorylation Undergo Diurnal Oscillation**

![Image of Western blots](image-url)

**FIGURE 1.** elF4E, 4EBPs, and ribosomal protein S6 phosphorylations are stimulated by training for contextual fear memory in hippocampus. **A**, Western analysis of hippocampal lysates from wild type mice 30 min after training for contextual fear conditioning. Mice exhibited an increase in phosphorylation of Thr^{187}/Tyr^{189} of ERK2 (p-ERK2), Ser^{209} phosphorylation of elF4E (p-elF4E), Ser^{235/236} phosphorylation of S6 (p-rpS6), and Thr^{2448} phosphorylation of 4EBP1 (p-4EBP1) compared with controls (shock). **B** and **C**, quantification of changes observed in phosphorylated proteins normalized to either their respective nonphosphorylated protein or actin levels. Data are mean ± S.E. and are expressed as a percentage of control (shock). N (number of mice) is 5 each for control and context-shock. *, p < 0.05; **, p < 0.01 context-shock versus control (shock) using Student’s t test.

To test directly whether ERK1/2 is required for increased phosphorylation of elF4E during contextual fear conditioning, we inhibited ERK1/2 activation by SL327. SL327 acts by blocking the activation of MEK, an upstream kinase activator of...
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ERK1/2 (22, 26). SL327 was injected intraperitoneally at 50 mg/kg dose 30 min prior to contextual training of mice (22). Control mice injected with vehicle (DMSO) exhibited a significant increase in ERK1/2 phosphorylation (161.9 ± 5.2), 30 min after contextual training (vehicle groups, ***, p < 0.001 context-shock versus control (shock), Fig. 2A). However, mice treated with SL327 did not demonstrate an increase in ERK1/2 phosphorylation after training for contextual fear memory (Fig. 2A).

Pretreatment with SL327 blocked training-induced increases in eIF4E phosphorylation at the Ser209 site (Fig. 2B). SL327 also inhibited 4EBP1 phosphorylation at Thr37/Thr46 sites in context-trained mice as compared with control mice (Fig. 2C). These data therefore indicate that an increase in markers for initiation of mRNA translation in the hippocampus triggered by training for contextual fear memory is regulated by the ERK1/2 MAPK pathway.

Phosphorylation of eIF4E and 4EBP Undergoes Diurnal Oscillation in the Hippocampus—Although the training for memory leads to an increase in markers of translation initiation at 30 min (Figs. 1 and 2), it is not known how fear memory is maintained and consolidated over multiple days or weeks. The proteins synthesized after contextual training that play role in memory consolidation generally have half-lives ranging from a few hours to at the most a few days. We hypothesized that mRNA translation initiation is activated repeatedly in a circadian manner for memory consolidation and persistence. To address this hypothesis, we used mice maintained under 12-h light/12-h dark (L/D) conditions for 10 days and analyzed their hippocampal lysates obtained from wild type mice during the 24 h (ZT0 to ZT20) were evaluated by Western analysis for phosphorylated Ser209-eIF4E and total eIF4E levels. p-eIF4E/eIF4E levels were elevated at ZT4 h (*, p < 0.05 ZT4 h versus ZT0 h) and ZT8 h (**, p < 0.001 ZT8 h versus ZT0 h), one-way ANOVA followed by Dunnett’s post hoc test. c, hippocampal lysates from wild type mice were analyzed by Western blot analysis for phosphorylated Thr37/Thr46 4EBP1, total 4EBP1, and total 4EBP2 levels. p-4EBP1/4EBP1 levels were increased at ZT4 h (*, p < 0.05 ZT4 h versus ZT16 h) and ZT8 h (**, p < 0.01 ZT8 h versus ZT16 h). Data are shown as mean ± S.E., one-way ANOVA followed by Dunnett’s post hoc test. c. Western analysis of hippocampal lysates from ZT4 h and ZT16 h for phosphorylated eIF4E, total eIF4E, phosphorylated 4EBP1, and actin (as a loading control), D, quantification of changes observed in phosphorylated eIF4E and 4EBP1 proteins normalized to total eIF4E and actin levels, respectively, and expressed as a percentage of ZT4 h. Data are shown as mean ± S.E., ***, p < 0.001 ZT4 h versus ZT16 h using Student’s t test. E, hippocampal lysates isolated from wild type mice at ZT4 h and ZT16 h were incubated with 7-methyl-GTP-Sepharose 4B beads for a mRNA-cap pulldown assay. Precipitated proteins were run and subjected to Western analysis. Shown here is a representative Western blot of hippocampal lysates from ZT4 h and ZT16 h for phosphorylated eIF4E, total eIF4E, phosphorylated 4EBP1 and actin (as a loading control). F, bar graph represents ratio of eIF4E/eIF4G1 at ZT4h and ZT16 h. Data are represented as mean ± S.E. and are expressed as a percentage of ZT4 h. n = 11 mice for each time point, and *, p < 0.05, ZT4 h versus ZT16 h using Student’s t test.
nounced diurnal oscillation (Fig. 3B). 4EBP1 phosphorylation was significantly higher at ZT4 h (111.10 ± 7.07) and ZT8 h (121.7 ± 5.5) as compared with ZT16 h (F(5,24) = 5.58, one-way ANOVA, *, p < 0.05; **, p < 0.01, ZT4 h versus ZT16 h; Fig. 3B). Total 4EBP1 and 4EBP2 levels did not change over the 24-h timeline. Phosphorylation of both eIF4E and 4EBPs demonstrated a significant decrease at ZT16 h compared with ZT4 h (Fig. 3C and D).

Because hyper-phosphorylation of 4EBPs is associated with reduced binding to eIF4E, we directly monitored the amount of eIF4E associated with the mRNA cap complex. An mRNA cap pulldown assay for eIF4E was carried out using methyl-GTP-Sepharose beads. GTP-Sepharose beads bind with high affinity to mRNA cap and pull down the proteins associated with it. Western blot analysis revealed an increased amount of eIF4E bound to GTP-Sepharose at ZT4 h compared with ZT16 h (*, p < 0.05, Fig. 3E and F). In this experiment, eIF4E levels were normalized to eIF4G1, a scaffold protein that binds to eIF4E and is important for eIF4F complex formation at the mRNA cap. As eIF4E is the rate-limiting factor in eIF4F complex formation, which is critical for translation initiation, the increased amount of eIF4E in the mRNA cap complex at ZT4 h compared with ZT16 h provides additional evidence that mRNA translation initiation undergoes a diurnal oscillation in the hippocampus.

Ribosomal S6 Protein, ERK1/2, Akt, and mTOR Phosphorylation in the Hippocampus Exhibit Diurnal Oscillations—Phosphorylation of rpS6 leads to increased synthesis of translation factors with 5’-TOP sequences, which can then function to enhance translational capacity (9, 10). Therefore, we next examined rpS6 phosphorylation in the hippocampus during the circadian cycle. Western analysis of hippocampal lysates revealed a significant difference in Ser235/Ser236 phosphorylation of rpS6 between ZT4 h and ZT16 h (***, p < 0.001, Fig. 4A) thus suggesting diurnal oscillation in translation of mRNAs containing 5’-TOP sequences.

ERK1/2 MAPK (Figs. 1 and 2) and Akt/mTOR are two major signal transduction pathways involved in controlling the activity of the translational machinery in memory (37). To determine whether upstream kinases ERK1/2, Akt, and mTOR also undergo a diurnal oscillation that parallels activation of rpS6, 4EBF, and 4EBPs, we monitored ERK1/2, Akt (Ser473), and mTOR (Ser2448) phosphorylation in parallel in hippocampal lysates. Phosphorylated ERK1/2 was significantly higher at ZT4 h compared with ZT16 h (***, p < 0.001, Fig. 4B) thus suggesting diurnal oscillation in translation of mRNAs containing 5’-TOP sequences.
account for the increased phosphorylation of eIF4E at ZT4 h (Fig. 3, A and C) because the ERK1/2/Mnk1 pathway modulates eIF4E phosphorylation at Ser209 site (Fig. 2). We next found out that Akt phosphorylation (Ser473, **, p < 0.01, Fig. 4C) and mTOR phosphorylation (Ser2448, *, p < 0.05, Fig. 4D) were significantly higher at ZT4 h compared with ZT16 h. Akt has been reported to phosphorylate and activate downstream kinase mTOR, which in turn can stimulate protein synthesis in hippocampal neurons by directly phosphorylating 4EBPs and indirectly activating rpS6 (27, 28). Our results therefore suggest that increase in 4EBPs (Fig. 3, B and C) and rpS6 phosphorylation (Fig. 4A) observed during mid-day could be due to enhanced activation of mTOR pathway. Taken together, our results suggest that diurnal oscillations observed in markers of mRNA translation initiation can be correlated to activation of upstream MAPK and Akt/mTOR pathway.

Diurnal Oscillations in p-eIF4E, p-Akt, p-rpS6, and p-ERK1/2 Are Lost in Mice Lacking Calmodulin-activated Adenylyl Cyclases—ERK1/2 in CNS neurons is activated by cAMP through a B-Raf- and Rap1-dependent pathway (29) and through cAMP-activated EPAC1 (30). The calmodulin-stimulated adenylyl cyclases are expressed in the hippocampus, and consolidation of contextual memory depends on calmodulin-stimulated adenylyl cyclase 1 (AC1) and adenylyl cyclase 8 (AC8) (25). Mice lacking AC1 and AC8 cyclases (referred in here as DKO mice) are deficient in long-term potentiation and hippocampus-dependent LTM (11). Therefore, we examined the phosphorylation of eIF4E, rpS6, Akt, and ERK1/2 at ZT4 and ZT16 h in DKO mice. There was no significant difference between hippocampal eIF4E phosphorylation at ZT4 and ZT16 (Fig. 4, E and F) in DKO mice. Similarly, pERK1/2, which regulates eIF4E phosphorylation (Figs. 1 and 2), was also invariant between ZT4 h and ZT16 h (Fig. 4, E and F). We also evaluated rpS6 and Akt phosphorylation in the same hippocampal samples. Neither rpS6 nor Akt phosphorylation showed a significant change between ZT4 h and ZT16 h in DKO mice (Fig. 4, E and F). These results indicate that the diurnal oscillation observed in markers of mRNA translation initiation in wild type mice (Figs. 3 and 4, A–D) is lost in animals, which lack calmodulin-stimulated adenylyl cyclases AC1 and AC8 and that this loss of oscillation may be associated with deficits in LTM observed in DKO mice.

Oscillations in p-eIF4E, p-Akt, p-rpS6, and p-ERK1/2 Are Circadian in Nature—To confirm that L/D oscillations observed in p-eIF4E, p-4EBP1, p-rpS6, p-ERK1/2, and p-Akt are truly circadian in nature, we tested the mice under free-running conditions. Mice were first exposed to the normal L/D cycle and then placed in constant darkness (D/D) for 7 days. As expected, actograms generated from monitoring voluntary locomotor activity of mice in D/D indicated that mice were undergoing circadian oscillations (Fig. 5A). Hippocampi were then isolated from mice placed in D/D cycle for 7 days, at ZT4 and ZT16 h, and probed for various phosphorylated proteins. Oscillations in p-ERK1/2, p-eIF4E, p-Akt, p-4EBP1, and p-rpS6 were maintained in the hippocampus under D/D conditions with a significant decrease observed at ZT16 h as compared with ZT4 h (Fig. 5, B and C).

Disruption in Circadian Rhythm Leads to Loss of Diurnal Oscillations in p-eIF4E, p-4EBP1, and p-rpS6—Does disruption of circadian rhythm block the diurnal oscillation of translation in the hippocampus? To perturb the circadian rhythm, mice were exposed to constant bright light (L/L) conditions (31, 32) for at least 10 days (about 300 lux). The advantage of using L/L procedure over lesion of the suprachiasmatic nucleus (SCN) is that neural connections remain intact in L/L-treated animals. Mice exposed to L/L conditions exhibited severe disruptions in circadian rhythm, and by day 10 mice were arrhythmic (Fig. 6A). Exposure to L/L conditions led to loss of oscillations in p-eIF4E, p-4EBP1, p-rpS6, p-ERK1/2, and p-Akt in the hippocampus, thus supporting the notion that diurnal oscillation in markers of translation initiation may be regulated by circadian rhythms (Fig. 6, B and C, ZT4 h versus ZT16 h, no significant difference). We next asked whether disruption of oscillations in markers of translation initiation after constant L/L exposure would lead to loss of contextual fear memory. Animals were exposed to either L/L or L/D conditions for at least 10 days and then trained for contextual fear conditioning. Mice subjected to L/L conditions demonstrated a significant decrease in contextual memory (quantified as a freezing behavior) 24 h after training compared with mice exposed to normal L/D conditions (**, p < 0.001 test, Fig. 6, D and E). These data therefore support the hypothesis that diurnal oscillations in markers of translation initiation in the hippocampus play a critical role in the persistence of contextual associated fear memory.
Inhibition of Protein Synthesis 2 Days after Memory Consolidation Impairs Memory Persistence—To test whether protein synthesis is required for the persistence of contextual memory after consolidation, we used protein synthesis inhibitor anisomycin. Anisomycin has been used to block memory formation in a wide variety of behavioral paradigms (33–35), and it can inhibit protein synthesis up to 6 h after injection in dorsal hippocampus (36, 37). However, in these studies anisomycin was injected either before, immediately after, or a few hours after contextual training, and hence it is not known whether protein synthesis is playing a role in the persistence of contextual memory. Therefore, anisomycin was injected into the dorsal hippocampus 2 days after training at ZT4 h daily for 4 days (Fig. 7A). Anisomycin-infused mice tested 14 and 28 days after training had significant deficits in fear memory as compared with the vehicle group (n = 6 animals). C, anisomycin- and vehicle-injected mice were trained and tested (24 h later) for step-through latency in passive avoidance. Data are expressed as mean ± S.E., **, p < 0.01; ***., p < 0.001, vehicle versus anisomycin group using Student’s t test.

DISCUSSION

Consolidation of hippocampus-dependent memory and various forms of long-lasting synaptic plasticity depend on new protein synthesis (38, 39). However, it seems unlikely that a single round of translation generated during memory acquisition can account for the persistence of LTM, which persists for periods exceeding the lifetime of synaptic proteins generated during memory formation. The objective of this study was to determine whether markers of mRNA translation initiation and their upstream signaling molecules in the hippocampus undergo periodic reactivation during the circadian cycle of mice and whether this translation initiation is critical for memory persistence.

Using several biochemical markers for initiation of translation, including p-eIF4E, p-4EBP1, p-rpS6, we found that training for contextual fear memory increases translation of mRNA in the hippocampus. This increase was blocked by SL327, a MEK inhibitor, which inhibits the ERK1/2 MAPK pathway. These data are consistent with an earlier report that suggested that ERK1/2 MAPK regulates translation during memory formation (21). Interestingly, eIF4E, 4EBP phosphorylation, and mRNA cap-complex showed a diurnal oscillation with a maximum during the daytime, which was maintained in mice exposed to constant dark conditions indicating the circadian nature of these oscillations. The diurnal oscillations in markers of translation initiation were paralleled by changes in the activation of ERK1/2 and Akt/mTOR upstream signaling pathways. The oscillations in ERK phosphorylation observed in the hippocampus has been reported in a number of other tissues, causing nonspecific neuronal damage. These data therefore indicate that protein synthesis 48 h post memory consolidation is required for memory persistence.
including the mouse SCN, the rat pineal gland, the hamster SCN, and the chick pineal gland (40–43). Moreover, the ERK1/2 MAPK pathway is also involved in regulating circadian rhythms in SCN and plays an important role in the clock–resetting mechanisms of mammalian circadian clock (40, 44, 45). Interestingly, mTOR activity also exhibits circadian variations in SCN (46) and has been reported to be crucial for regulating protein synthesis by modulating the phosphorylation of 4E BP and mRNAs with 5′-TOP motifs (47, 48). Our results fit broadly with these studies suggesting that ERK1/2 MAPK and mTOR activity are critical for regulating translation initiation.

The diurnal oscillations in phosphorylation of eIF4E, 4EBPs, and their upstream signaling components in the hippocampus were lost in mice exposed to constant L/L conditions. This lack of diurnal oscillation in eIF4E, 4EBP, rpS6, ERK, and Akt phosphorylation was associated with reduction in contextual memory. The difference in initiation of mRNA translation between ZT4 and ZT16 was also lost in a transgenic mouse strain lacking the calmodulin-stimulated adenyl cyclases AC1 and AC8. Because cAMP signals generated by the calmodulin-stimulated adenyl cyclases are required for the activation of ERK1/2 MAPK during contextual memory formation (25), these data suggest that the diurnal oscillation of p-ERK1/2 and translation initiation may be driven by oscillation of cAMP in the hippocampus. This idea is supported by the observation that Ca2+ signals generated by NMDA receptors during training for contextual memory activate calmodulin-stimulated adenyl cyclases, which generate a cAMP signal for the activation of ERK1/2 MAPK. Stimulation of ERK1/2 MAPK stimulates translation by several mechanisms, which lead to the phosphorylation and activation of eIF4E and S6.

There is considerable evidence that the circadian rhythms are important for memory consolidation and the persistence of hippocampus-dependent memory. For example, circadian phase-shifting after training impairs hippocampus-dependent long-term spatial memory (50) and passive avoidance memory (51). In addition, the lesion of the SCN, which disrupts circadian rhythm, impairs passive avoidance memory in rats (51) and contextual fear memory in mice (52). For example, the electrolytic lesion of the SCN 48 h after training for context and memory consolidation impairs the persistence of contextual memory. Intriguingly, electrolytic lesion of the SCN also impairs diurnal oscillation of ERK1/2 MAPK activity and cAMP in the hippocampus (52).

What is the physiological importance of diurnal oscillations of mRNA translation in the hippocampus? Because of the importance of mRNA translation for memory consolidation, our findings with anisomycin support the idea that the diurnal oscillation of translation after memory formation may be important for sustaining hippocampus-dependent memories. Reactivation of calmodulin-sensitive adenyl cyclase, ERK1/2 MAPK, and mTOR activities during the circadian cycle may stimulate repeated activation of translation required to sustain hippocampus-dependent memories. These increases in translation may be a mechanism by which the strength of synapses “tagged” during memory formation (53) is maintained over extended periods of time. This general hypothesis is supported by the observation that CAMP-response element-binding protein-mediated transcription also undergoes a circadian oscillation in the hippocampus (49) and exhibits elevated levels in rapid eye movement sleep compared with awake animals (54).

In conclusion, eIF4E, 4EBP, and rpS6 phosphorylations undergo diurnal oscillations in the hippocampus, which are dependent on calcium-stimulated adenyl cyclases, ERK1/2 MAPK, and Akt/mTOR activities. Disruption of circadian rhythms leads to loss of diurnal translation oscillation and causes memory deficits. Moreover, inhibition of protein synthesis during the midday for 4 days post-training impairs memory persistence.

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REFERENCES

1. Davis, H. P., and Squire, L. R. (1984) Protein synthesis and memory: a review. Psychol. Bull. 96, 518–559
2. McGaugh, J. L. (2000) Memory—a century of consolidation. Science 287, 248–251
3. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68, 913–963
4. Pause, A., Belsham, G. I., Gingras, A.-C., Donzé, O., Lin, T.-A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature 371, 762–767
5. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J. 15, 658–664
6. Scheper, G. C., and Proud, C. G. (2002) Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur. J. Biochem. 269, 5350–5359
C. H., Leblanc, L., Lebovic, S. S., Lo, Q., Ralph, M. R., and McDonald, R. J. (2001) Circadian phase-shifted rats show normal acquisition but impaired long-term retention of place information in the water task. *Neurobiol. Learn. Mem.* 75, 51–62

51. Tapp, W. N., and Holloway, F. A. (1981) Phase shifting circadian rhythms produces retrograde amnesia. *Science* 211, 1056–1058

52. Phan, T. X., Phan, T. H., Chan, G. C., Sindreu, C. B., Eckel-Mahan, K. L., and Storm, D. R. (2011) The diurnal oscillation of MAP (mitogen-activated protein) kinase and adenylyl cyclase activities in the hippocampus depends on the suprachiasmatic nucleus. *J. Neurosci.* 31, 10640–10647

53. Frey, U., and Morris, R. G. (1997) Synaptic tagging and long-term potentiation. *Nature* 385, 533–536

54. Luo, J., Phan, T. X., Yang, Y., Garelick, M. G., and Storm, D. R. (2013) Increases in cAMP, MAPK activity, and CREB phosphorylation during REM sleep: implications for REM sleep and memory consolidation. *J. Neurosci.* 33, 6460–6468