Article

Neuron

The Persistence of Hippocampal-Based Memory Requires Protein Synthesis Mediated by the Prion-like Protein CPEB3

Highlights

- CPEB3 forms aggregates after synaptic activity in the hippocampus
- After aggregation, CPEB3 promotes the translation of AMPA receptors
- CPEB3 conditional knockout mice have impaired long-term memory and LTP
- CPEB3 mediates the persistence of memory through a prion-like mechanism

Authors

Luana Fioriti, Cory Myers, Yan-You Huang, ..., Bettina Drisaldi, Elias Pavlopoulos, Eric R. Kandel

Correspondence

erk5@columbia.edu

In Brief

Fioriti et al. found that the translational regulator CPEB3 is a prion-like molecule that aggregates to form an amyloid-like structure in the brain of mice. CPEB3 aggregates mediate the maintenance of hippocampal-based long-term memories.

Fioriti et al., 2015, Neuron *86*, 1433–1448
June 17, 2015
http://dx.doi.org/10.1016/j.neuron.2015.05.021

CellPress

Neuron
Article

The Persistence of Hippocampal-Based Memory Requires Protein Synthesis Mediated by the Prion-like Protein CPEB3

Luana Fioriti,[1] Cory Myers,[1] Yan-You Huang,[1] Xiang Li,[1] Joseph S. Stephan,[1,2] Pierre Trifilieff,[1] Luca Colnaghi,[1,2] Stylianos Kosmidis,[1,2] Bettina Drisaldi,[1] Elias Pavlopoulos,[1] and Eric R. Kandel[1,2,3,*]
[1]Department of Neuroscience, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA
[2]Howard Hughes Medical Institute, New York, NY 10032, USA
[3]Kavli Institute for Brain Science, New York, NY 10032, USA
*Correspondence: erk5@columbia.edu
http://dx.doi.org/10.1016/j.neuron.2015.05.021

SUMMARY

Consolidation of long-term memories depends on de novo protein synthesis. Several translational regulators have been identified, and their contribution to the formation of memory has been assessed in the mouse hippocampus. None of them, however, has been implicated in the persistence of memory. Although persistence is a key feature of long-term memory, how this occurs, despite the rapid turnover of its molecular substrates, is poorly understood. Here we find that both memory storage and its underlying synaptic plasticity are mediated by the increase in level and in the aggregation of the prion-like translational regulator CPEB3 (cytoplasmic polyadenylation element-binding protein). Genetic ablation of CPEB3 impairs the maintenance of both hippocampal long-term potentiation and hippocampus-dependent spatial memory. We propose a model whereby persistence of long-term memory results from the assembly of CPEB3 into aggregates. These aggregates serve as functional prions and regulate local protein synthesis necessary for the maintenance of long-term memory.

INTRODUCTION

Based on its duration, memory can be divided into at least two overlapping phases, short-term and long-term. Short-term memory is temporary and involves covalent modification of preexisting proteins and is mediated by alteration in strength of preexisting synaptic connections. Long-term memory requires gene transcription, new protein synthesis, and the growth of new synaptic connections (Dudai, 2002).

Memory consolidation is a process that stabilizes a long-term memory trace after its initial acquisition. Cellular consolidation is achieved by means of intracellular transduction cascades, which culminates in the activation of transcription factors that lead to changes in gene expression. The resulting gene products are transported to the activated synapse leading to synapse-specific remodeling and growth (Kandel, 2001). Local protein synthesis at the activated synapses accounts for the specificity of the functional and morphological changes that occur after learning (Martin et al., 2000; Sutton and Schuman, 2006). However, the half-life of newly synthesized proteins is shorter than that of the memory. To understand how long-term memory is maintained at the synaptic level, we need to identify how the memory trace persists despite the protein turnover (Bailey et al., 2004; Dudai, 2002; McGaugh, 2000).

One possible answer to this problem comes from recent studies in the invertebrates *Aplysia* and *Drosophila*. Here it was found that the persistence of synaptic plasticity and memory is mediated by the prion-like translational regulator *Aplysia* CPEB and its *Drosophila* homolog Orb2 (Si et al., 2003, 2010; Majumdar et al., 2012). CPEB binds to a six nucleotide-specific sequence in the 3′ UTR of mRNAs called cytoplasmic polyadenylation element (CPE) (Hake and Richter, 1994). In the basal state, this binding represses the translation of mRNAs, but, upon activation, CPEB promotes the polyadenylation of dormant target mRNAs and thereby activates their translation into proteins that maintain synaptic plasticity, synaptic growth, and memory storage (Si et al., 2003, 2010; Du and Richter, 2005; Wu et al., 1998; Alarcon et al., 2004).

We earlier identified four mammalian CPEBs, CPEB1, 2, 3, and 4. In the present paper, we focused on CPEB3 because it is the only one that contains a prion-like domain similar to that found in its homolog, the memory-related *Aplysia* Q/N-rich CPEB (Theis et al., 2003). In addition, the ubiquitin-ligase Neuralized that exerts enhancing effects on learning and memory (Pavlopoulos et al., 2011) requires the presence of CPEB3. Finally, in parallel studies (Stephan et al., 2015), we find that CPEB3 manifests all the three defining properties of a prion in yeasts: amyloid fiber formation, SDS-resistant oligomers, and heritability.

In the present study, we have tested the hypothesis that a CPEB-mediated prion-like mechanism is conserved in mammals. To assess the contribution of CPEB3 to the different phases of long-term memory—acquisition, consolidation, and persistence—we generated a conditional knockout strain of CPEB3. Using this strain, as well as viral-mediated knockdown

of CPEB3, we here provide behavioral, electrophysiological, and molecular evidence that CPEB3 is a key mediator of the consolidation and persistence of hippocampal-based explicit memory in the mouse. These findings provide the first evidence that a CPEB-mediated prion-like mechanism is conserved in mammals for the maintenance of memory.

RESULTS

CPEB3 Protein Is Increased and Becomes Aggregated following Synaptic Activity

We have previously found that CPEB3 transcript is upregulated 2 hr after injection of kainic acid in the mouse hippocampus (Theis et al., 2003). In *Aplysia*, stimulation of sensory neurons with serotonin induces an increase in the protein level of ApCPEB, required for the maintenance of long-term facilitation (Si et al., 2003). We wanted to know whether a similar phenomenon might operate for CPEB3 in the hippocampus. We found an increase in CPEB3 protein 30 min after LTP induction (Figure 1B) and in hippocampal neurons in culture stimulated with glutamate or glycine (Figures 1A and S1) (Jaafari et al., 2013). We also detected an increase in the levels of CPEB3 when we applied dopamine, essential for L-LTP, to hippocampal slices (Figure S1) (Smith et al., 2005).

We detected a similar increase in CPEB3 levels in vivo following behavioral learning. We measured CPEB3 protein in hippocampal extracts of naive mice and mice trained in the Morris water maze and found that training induced an increase in CPEB3 protein ($p < 0.005$, Figure 1C). In addition, we observed an increase of CPEB3 after contextual fear conditioning ($p < 0.01$, Figure 1D). Interestingly, we did not find a significant increase in any of the other CPEBs in all the conditions we tested (Figure S1).

Since aggregation-prone molecules tend to aggregate when they reach a critical concentration (Michelitsch and Weissman, 2000), we reasoned that it might be possible to detect aggregation of CPEB3 in response to stimulation. We examined the aggregated state of CPEB3 following contextual fear conditioning by using a detergent insolubility assay. Typically, aggregates from amyloid and prion-like proteins are found in the detergent-insoluble fraction. We found CPEB3 in the detergent-insoluble fraction after fear conditioning (Figure 1D). We then studied the nature of CPEB3-insoluble aggregates with two additional techniques. First, we examined CPEB3 following sucrose gradient ultracentrifugation of hippocampal extracts from naive and fear-conditioned mice (Figure 1D). We found CPEB3 to sediment in the high molecular weight fractions in response to fear conditioning, while PSD95 and CPEB4 did not comigrate with CPEB3 (Figure S1), suggesting that fear-induced formation of the CPEB3 complex does not lead to nonspecific aggregation of synaptic proteins.

Another particular difficulty of the study of amyloid proteins is to resolve the heterogeneity of the aggregates, since these usually exhibit a variable degree of polymorphism. Semi-denaturating detergent agarose gel electrophoresis (SDD-AGE) takes advantage of both (1) the property of prions and prion-like polymers to be resistant to solubilization by SDS detergent, and (2) the large pore sizes of agarose, which allow the resolution of high molecular weight complexes. We found that CPEB3 forms aggregates that are resistant to SDS after fear conditioning (Figure 1D).

This learning-induced upregulation and aggregation of CPEB3 in the hippocampus suggested that CPEB3 might contribute to synaptic plasticity and memory storage through a prion-like mechanism. Consistent with this view, the prion-like Orb2A was found to limit memory storage in *Drosophila* (Majumdar et al., 2012; Keleman et al., 2007). We therefore asked, is CPEB3 also critical for the persistence of memory and memory-related synaptic plasticity?

To address these questions, we deleted CPEB3 from the mouse brain by generating conditional knockout mice (CKO), which allowed us to regulate the excision of the CPEB3 gene in the adult forebrain.

Regional- and Temporal-Specific Deletion of CPEB3 in Conditional Mutant Mice

To generate mice with forebrain loss of CPEB3, we used the Cre/loxP system. We flanked exon II of the CPEB3 gene and the neomycin selection marker with two loxP sites by homologous recombination in embryonic stem (ES) cells (Figure 2A). To generate conditional lines that have both spatial and temporal patterns of CPEB3 recombination, we crossed the CPEB3 floxed mice to CaMKII-Cre transgenic mice (Nolan et al., 2004). In situ hybridization revealed an almost complete ablation of CPEB3 mRNA expression in the hippocampus and cortex in the CPEB3 CKO mice, compared with wild-type (WT) control mice (Figure 2B). The cerebellum was unaffected.

We also quantified the degree of CPEB3 deletion using RT-PCR (Figure S2) and western blot in the hippocampus (Figure 2C). We found a greater than 90% reduction of CPEB3 mRNA in whole hippocampus of CPEB3 CKO mice compared with WT animals ($n = 3$, $p < 0.001$ by t test). By contrast, we found no significant differences in the cerebellum (data not shown). We also measured the protein levels and confirmed the results obtained with the mRNA analysis (Figure 2C).

We next performed histological examination of brain tissues (via Nissl staining), which did not reveal any gross morphological or developmental defects in CPEB3 CKO mice (Figure 2B). Immunohistochemistry on brain slices also confirmed the almost complete absence of CPEB3 in the hippocampus and cortex. Only a few sparse cells, positive to calbindin, showed expression of CPEB3. This is consistent with the fact that CamKII CRE line drives the deletion of CPEB3 in pyramidal neurons, whereas CPEB3 expression in GABAergic interneurons is spared (Figures 2D and S2B).

CPEB3 CKO Mice Have Normal Locomotor, Exploratory, and Anxiety Behavior

We assessed potential neurological defects in the CPEB3 CKO mice at 4, 12, and 54 weeks of age by exploring eight key characteristics: body weight, pelvic elevation, tremors, kyphosis, tail rigidity, cage walking, foot clasp, and whisker response. The CPEB3 CKO mice showed no neurological phenotype for each age. Based on this analysis, we concluded that this knockout condition resulted in viable mice with no gross neurological deficits.

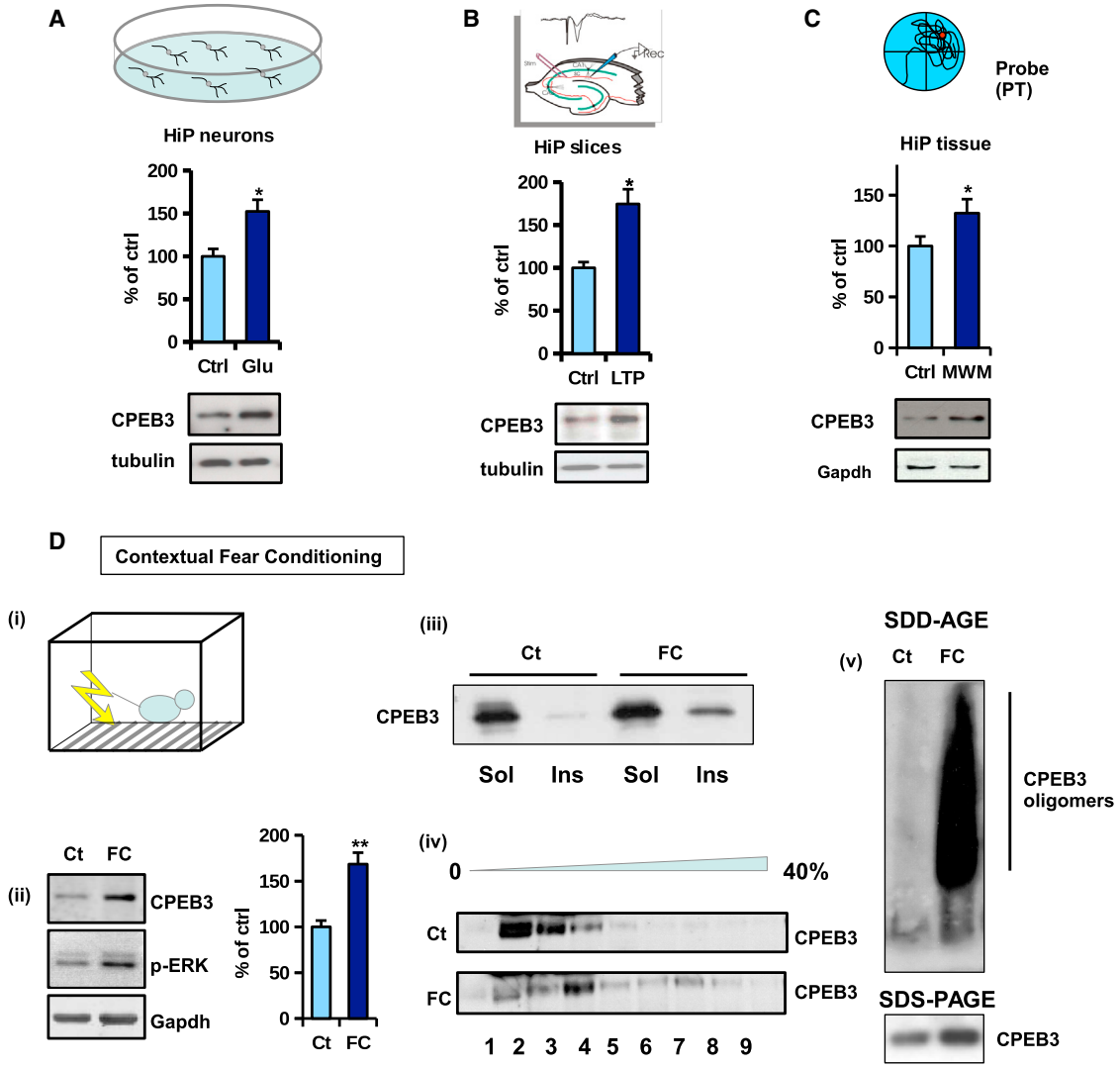

Figure 1. CPEB3 Increases and Aggregates following Synaptic Activity

(A) Synaptic stimulation by glutamate (Glu) induces the increase of CPEB3 in hippocampal neurons. Top: mean ± SEM from four independent experiments (n = 12 replicates) of endogenous CPEB3 30 min after glutamate application in 16 days in vitro hippocampal cultures (Ctrl versus Glu: $p < 0.01$). Bottom: representative image of western blots showing CPEB3 protein and tubulin, used as a loading control.

(B) CPEB3 protein level in the CA1 area is increased 30 min after LTP induction at the Schaffer collateral pathway using 4 × 100 Hz protocol. Ctrl, unstimulated controls. Top: mean ± SEM from two independent experiments ($p < 0.05$). Bottom: representative image of western blots showing CPEB3 protein and tubulin, used as a loading control.

(C) CPEB3 protein level in the hippocampus after Morris water maze experiment. Top: mean ± SEM from two independent experiments (n = 5 mice *$p < 0.05$). Bottom: representative image of western blots showing CPEB3 protein and Gapdh, used as a loading control (see also Figure S1).

(D) CPEB3 protein level in the hippocampus after (i) contextual fear conditioning. (ii) Mean ± SEM from three independent experiments (n = 12 mice, $p < 0.01$). Left: representative image of western blots showing CPEB3 protein and Gapdh, used as a loading control. (iii) Insolubility assay. Hippocampal extracts from naive (Ct) and trained (FC) mice were centrifuged at high speed in the presence of detergents. Soluble proteins are in the Sol fraction, aggregated are in the Insoluble (Ins) fraction. Training induces a significant shift of CPEB3 in the insoluble aggregated fraction (n = 3 replicates, $p < 0.05$). (iv) Sucrose gradient (0%–40% sucrose in Tris buffer in the presence of detergents). After fear conditioning training, CPEB3 redistributes from the light fractions to heavy fractions. Representative image of western blot showing CPEB3 (n = 3 replicates; see also Figure S1 for negative control). (v) SDD-AGE analysis of hippocampal extracts from naive and trained mice. Fear conditioning training induces the formation of SDS-resistant oligomeric species. Representative image of western blot showing total CPEB3 levels (bottom) and oligomers (top).

To investigate anxiety-related behaviors, we performed elevated plus-maze and open-field. In both cases CPEB3 CKO were indistinguishable from their control littermates (Figure S3), showing that CPEB3 CKO mice have normal exploratory behavior and do not display alterations in anxiety levels.

To investigate the role of CPEB3 in hippocampal-based associative and reference memory, we carried out, respectively,

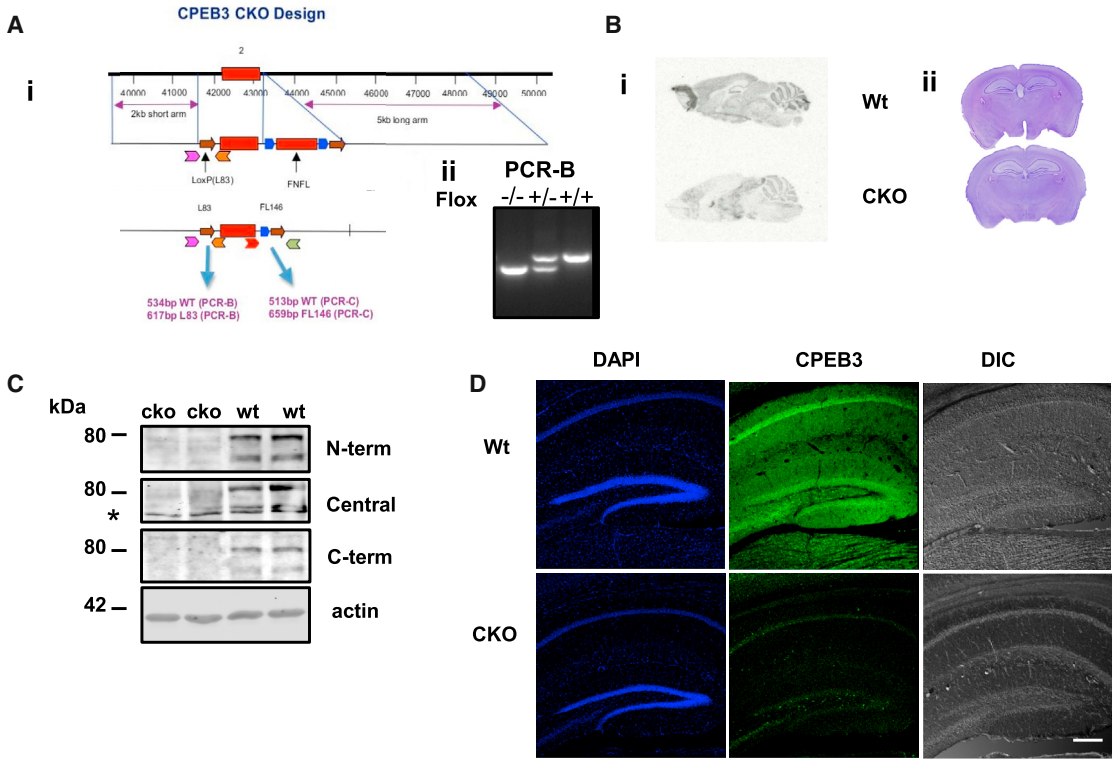

Figure 2. Conditional Targeted Disruption of the CPEB3 Locus

(A) (i) Schematic showing the portion of CPEB3 that is conditionally deleted. Schematic showing the strategy used to genotype CPEB3 CKO mice. Arrows above the schematic of the mutated CPEB3 gene show the approximate positions of oligonucleotide primers used for polymerase chain reaction (Pcr). (ii) Representative image of a PCR reaction from mice homozygous for wild-type (Wt) CPEB3 allele (lane 1), heterozygous mice (lane2), and floxed allele (lane 3).

(B) Radioactive oligonucleotide in situ hybridization analysis of CPEB3 mRNA expression on sagittal brain slices from 3.5-month-old mice. (i) CPEB3 mRNA is absent in the forebrain including cerebral cortex, hippocampus, striatum. (ii) Nissl staining shows no gross anatomical differences between WT and CKO mice.

(C) Immunoblots of hippocampal homogenates, 20 μg of hippocampal lysate were loaded onto an 8% SDS-PAGE, transferred on PVDF, and probed for CPEB3. Three different antibodies, one against the deleted region (top panel), one against the central portion (second panel), and one against the C-terminal region (third panel) of CPEB3 were used to confirm the complete absence of the protein or shorter fragments derived from a non-complete deletion. (*) indicates a non-specific band recognized by the antibody in all samples. Actin was used as loading control.

(D) Confocal images of hippocampal sections derived from Wt or CPEB3 CKO animals. An antibody against the deleted region was used to stain the sections (bar inset corresponds to 150 μm) (see also Figure S2).

contextual fear conditioning or object placement recognition and the Morris water maze tasks.

CPEB3 CKO Mice Have Impaired Memory Consolidation in Contextual Fear Conditioning

We first explored the role of CPEB3 in a form of associative memory that requires the integrity of the hippocampus, contextual fear conditioning (Fanselow and Poulos, 2005; LeDoux, 2000). Following habituation on day 1, mice were trained using a one-trial protocol on day 2 and tested for memory retention on day 3. During the habituation phase, both groups of mice explored the chambers equally (Figure 3B). Similarly, during the training phase, CPEB3 CKO and controls (CT) had similar freezing behavior following the shock. However there was a significant effect of genotype for freezing during context recall (pc < c0.05; Figure 3B). Interestingly, while the CT still retained a high level of freezing in a second test performed a week later, the KO did not, showing a failure to retain long-term memory for this form of associative learning.

Memory for Object Placement Recognition Is Impaired in CPEB3 CKO Mice

Object placement recognition is a hippocampal-dependent task that is based on the ability to discriminate between different spatial configurations of the same familiar objects (Vnek and Rothblat, 1996; Bevins and Besheer, 2006). During the training phase, CPEB3 CKO and CT had similar total exploration times and explored the two objects equally (Figure 3A).

Twenty-four hours after training, the animals were placed back in the arena where one of the objects was moved to a different position. Control and CKO mice displayed similar total exploration time. However, CPEB3 CKO animals spent significantly less time exploring the object placed in a different position compared to CT mice, suggesting that the CKO animals had impaired memory for the spatial configuration of the objects

(Figure 3B). Conversely, when we tested another group of CPEB3 CKO mice using a shorter interval (15 min after training), they showed a similar preference for the newly positioned object as did the controls, indicating that short-term memory was intact.

CPEB3 CKO Mice Have Impaired Memory Consolidation in the Morris Water Maze Task

We next explored the role of CPEB3 in long-term spatial memory in the Morris water maze. We first compared the performance of the CPEB3 CKO and control animals in a visible-platform version of the Morris water maze, which is not dependent on the hippocampus (Morris, 1981). Both groups displayed similar swimming speed, time spent floating, and thigmotaxis.

We then moved to the hippocampal-dependent hidden version of the Morris water maze to test spatial learning and memory. During acquisition of the task, there were no significant differences between genotypes in any parameters analyzed (Figure S3). With training, the latency for finding the hidden platform decreased significantly for both CT and CKO animals. A probe trial was performed 24 hr after the last training session by removing the platform. We found that CPEB3 CKO mice spent significantly less time than CT mice exploring the target quadrant and displayed a lower number of crossings of the platform position, showing a deficit in memory retention for spatial information (Figure 3C).

To explore memory flexibility, we examined the ability of the mice to learn a new location of the platform in a transfer phase. Again, the control and CPEB3 CKO animals displayed similar performance (Figure 3D). In the probe trial, however, CKO mice showed a significantly impaired memory for the new platform location (Figure 3C).

During a second probe trial, 8 days later, the performance of CPEB3 CKO mice was further decreased compared to CT and was also significantly different from the performance reached by the CKO in the first probe trial, suggesting that the absence of CPEB3 negatively affects the stability of long-term memory (Figure 3E).

As with the object placement recognition test, we also wanted to test in the Morris water maze whether short-term memory was spared in CPEB3 CKO mice. We therefore trained a different cohort of animals and tested them 1 hr after the last training session. At this time point, we did not observe any difference either in the time spent in the target quadrant or in the number of crossings in the target annulus (Figure S3E).

The Maintenance of Long-Term Memory Is Specifically Impaired by Knocking Down CPEB3 after Consolidation

To investigate the role of CPEB3 during maintenance of long-term memory, we used an inducible CRE-lox system to suppress the expression of CPEB3 only after memory has been consolidated. We trained both control and floxed animals in the presence of CPEB3. As expected, both groups displayed identical learning curves and showed a similar memory for the platform position during a probe trial (Figure 4A). After that first probe trial, we injected a CRE virus into the hippocampus of CPEB3-floxed homozygous mice to induce the removal of CPEB3 gene and loss of CPEB3 expression. As a control, we injected the same CRE virus in the control group animals. Two weeks later, when CPEB3 had been eliminated (Figure S4), the conditional induced KO mice showed a significant impairment in the retrieval of the long-term memory (Figure 4A), whereas the control animals remembered the position of the target quadrant zone. These data corroborate the findings obtained with the forebrain-specific CPEB3 KO and further indicate that CPEB3 is not only critical for consolidation but also for the stability of memory.

By performing the first probe trial and then injecting the CRE virus, however, we could interfere with the process of memory reconsolidation (Morris et al., 2006). To avoid that, we repeated the experiment but omitted the first probe trial and waited 2 weeks before injecting the CRE virus. We used a homogeneous group of 16 floxed mice, which were implanted with bilateral cannula and injected either with CRE or GFP, as a control virus. When we tested the mice during a probe trial, we observed a significant loss of memory for the target quadrant in the knocked-down group. By contrast the control group had a good memory (Figure 4B).

Finally we explored whether we could rescue the deficit of the CPEB3 knockdown animals by reintroducing CPEB3. A positive result would suggest that is the presence of CPEB3 during recall, rather than its constant presence during the maintenance phase, that allows the mice to perform successfully during a probe trial. We found that reintroducing CPEB3 had no effect on the performance of the mice, suggesting that CPEB3 is indeed essential for the maintenance of memory and not for memory retrieval (Figure 4B).

Together, these data indicate that CPEB3 is critically involved in both consolidation and maintenance of memory. Moreover, we find that even when the initial consolidation is normal, the maintenance of long-term spatial memory is severely compromised by the absence of CPEB3.

Ablation of CPEB3 in the Adult Hippocampus Impairs Long-Term Synaptic Plasticity

To address whether the deficits in memory storage observed in CPEB3 CKO mice are paralleled by impairment in long-term synaptic plasticity in the hippocampus, we examined long-term potentiation (LTP) at the Shaffer Collateral pathway of the CA1 region of hippocampus. We first examined the basal synaptic transmission and found that there is no significant difference in the stimulus-response curves (Figure 5A) and the paired-pulse facilitation (Figure 5B) between the CT and CKO mice, indicating that the CKO of CPEB3 did not affect the basal properties of synaptic transmission in this pathway. We next examined LTP induced by a single tetanus (1 × 100 Hz). In CT mice, a single train of tetanus induced a short-lasting synaptic potentiation (E-LTP) and LTP decayed to 122% ± 8% (90 min after high-frequency stimulation (HFS), n = 6). The single train of tetanus induced a similar form of LTP in CPEB3 CKO mice (114% ± 4% 90 min after HFS, n = 6) and there was no significant difference between CT and CKO mice in this form of LTP (Figure 5C, $p > 0.05$). By contrast, we found that LTP induced by four-repeated tetanus is different between CT and CKO mice (Figure 5D). Four repeated trains of 100 Hz stimuli give rise to a form of LTP lasting several hours and that requires RNA and protein synthesis. In the CKO mice, LTP induced by 4 × 100 Hz was

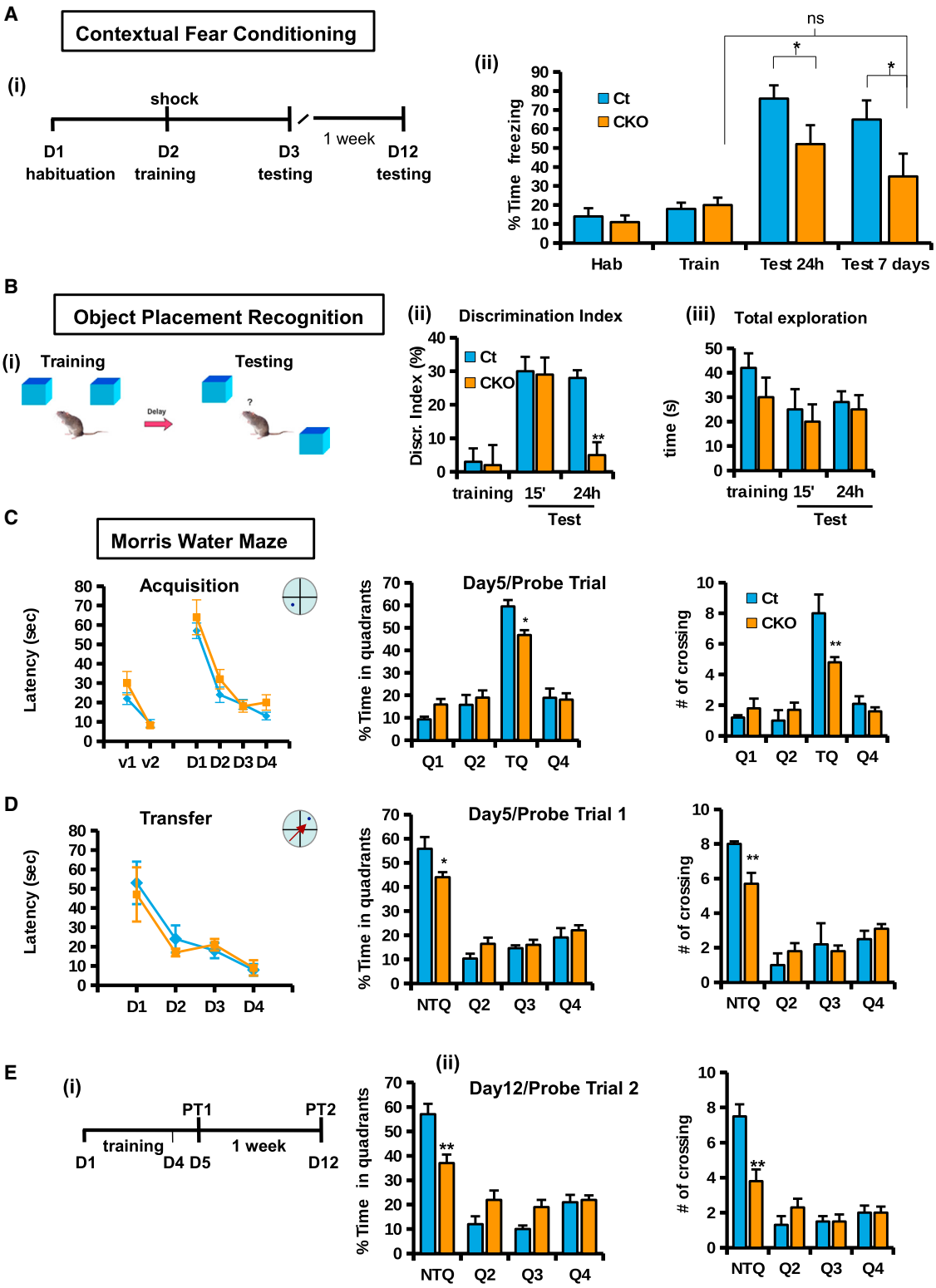

Figure 3. Impaired Associative and Spatial Memory in the CPEB3 Conditional Knockout Mice

(A) Contextual fear conditioning. (i) Scheme of the experiment. (ii) Freezing behavior of Ct and CKO mice during training and testing. Memory is affected in the CKO mice. ANOVA reveal a significant genotype effect; $F(1,18) = 5.28$, $p = 0.038$; and time effect, $F = 25.56$, $p < 0.001$). t test at 24 hr and 7 days, $p < 0.05$ between Ct (flox/flox) and CKO mice (flox/flox CRE).

(legend continued on next page)

only 122% ± 7% (n = 6) 3 hr after HFS, which was significantly smaller compared to the LTP in the CT mice (186% ± 22%, 3 hr after HFS, n = 6, $p < 0.05$). These results indicate that the knocking out of CPEB3 selectively impaired the RNA and protein synthesis-dependent late phase of LTP in the hippocampus. Interestingly, we did observe a faster decline of LTP in the CKO mice, which does not quite reach significance until 1 hr after the stimulation ($p > 0.05$).

The Absence of CPEB3 Impairs the Activity-Dependent Translation of AMPA Receptors

Consolidation of long-term memory (Izquierdo et al., 2008) relies on alteration in levels of AMPA receptors (AMPARs), critical components of synaptic transmission and plasticity (Nayak et al., 1998). In its basal state, CPEB3 represses the translation of both GluA1 (Pavlopoulos et al., 2011) and GluA2 (Huang et al., 2006) in hippocampal neurons in vitro. We confirmed CPEB3 function in vivo by checking the expression level of GluA1 and GluA2 proteins in hippocampal extracts of CPEB3 CKO mice. We found a significant increase of both GluA1 and GluA2 in the hippocampus of CPEB3 CKO mice compared to littermate controls (t test, $p = 0.02$, Figure S6). As a further control, we examined the mRNA levels of these gene products by RT-PCR and found no differences between CPEB3 CKO and controls (Figure S6).

We previously found that mono-ubiquitination of CPEB3 by the ubiquitin ligase Neuralized1 reverses the inhibitory effect of CPEB3 on the translation of GluA1 and GluA2 (Pavlopoulos et al., 2011). This increased translation of GluA1 and GluA2 could be explained either by a suppression of the inhibitory activity of CPEB3 in its basal state or by a change in CPEB3 function, with CPEB3 becoming an activator of protein synthesis. We reasoned that if CPEB3 is indeed changing function and becomes an activator after neuronal activity, then removing CPEB3 would cause a lack of activity-dependent synthesis of its target mRNAs, and this might explain the synaptic and behavioral defects of the CPEB3 CKO mice.

To explore this hypothesis, we performed metabolic labeling in synaptosome to measure the extent of stimulus-induced translation of AMPAR in control and CPEB3 CKO mice. We found that stimulation of control synaptosomes induces a robust increase in the amount of newly synthesized GluA1, while only a small increase is observed in the KO synaptosomes (Figure 6A). These findings suggest that a misregulation of activity-dependent increase in the number of synaptic AMPAR might contribute to the phenotype of the CPEB3 CKO mice. Indeed, we found that after performing the Morris water maze CPEB3 CKO mice have impaired translation of AMPAR in vivo. We found that in the controls, both GluA1 and GluA2 levels were significantly increased after the probe trial (Figure 6B). By contrast, CPEB3 CKO mice showed a significantly smaller increase (Figure 6B).

To confirm that this increase was translationally dependent, we performed two additional experiments. First, we injected control mice with either anisomycin or saline and performed a probe trial. The anisomycin-injected animals did not show the same increase in AMPAR proteins as the saline group (Figure 6C), suggesting that the increased amount of AMPAR proteins is due to translation. Second, we performed a polyA tail assay and compared the size of GluA1 and GluA2 polyA tail. We found that water maze training induced elongation of AMPAR polyA tail in CT mice, but not in CPEB3 CKO mice (Figure S6E), thus confirming that the increase in AMPAR protein is indeed due to increased translation through polyadenylation.

The N-Terminal Domain of CPEB3 Mediates the Stimulation-Induced Changes in CPEB3 Activity

We have previously found that the N-terminal domain of CPEB3 is critical for the interaction with Neuralized (Pavlopoulos et al., 2011). We now ask, is this domain important for the synthesis of AMPAR? To address this question, we transduced hippocampal neurons with a viral vector encoding a truncated form of CPEB3 that lacks the first 220 amino acids and found that whereas the truncated protein is still able to repress the translation of the AMPAR at basal state, it fails to promote their synthesis after glutamate application (Figure 7A). Interestingly, this domain contains features, such as a polyQ stretch and a series of hydrophobic residues, which have been implicated in

(B) Object placement recognition task. Scheme of the experiment (i); the total exploration time (iii) and the mean discrimination index (ii) + SEM during the training, 15 min and the 24 hr memory test are shown. CPEB3 CKO mice (KO; n = 12) had total exploration time similar to the controls (n = 12) during both phases of the task (ANOVA did not reveal significant genotype effect; training: $F(1,22) = 2.9$, $p = 0.152$; test 15 min: $F(1,22) = 1.96$, $p = 0.183$, test 24 hr: $F(1,22) = 0.9$, $p = 0.8915$). The discrimination index displayed by the KO mice was similar to controls during the training phase (ANOVA for genotype effect: $F(1,22) = 0.152$, $p = 0.7133$) or the 15 min test (ANOVA for genotype effect: $F(1,22) = 0.375$, $p = 0.55$) but it was significantly lower during the 24 hr test (ANOVA for genotype effect: $F(1,22) = 9.69$, $p = 0.006$).

(C) Data from Morris water maze task. The mean escape latency (+SEM) for mice to reach the platform in the visible and the hidden version of the water maze is plotted against the day of the experiment (CPEB3 CKO = 10; controls = 10). The escape latency was similar among controls and CKO mice both in the visible version of the task (repeated-measures ANOVA for escape latency; $F(1,18) = 0.987$, $p = 0.453$) and in the hidden version of the task, (genotype effect for escape latency: $F(1,18) = 0.721$, $p = 0.675$). Probe trial on day 5 showed significantly reduced performance of the CKO in the training quadrant (TQ) compared to controls (time in quadrant; repeated-measures ANOVA; genotype effect: $F(1,18) = 5.33$, $p = 0.034$; quadrant*genotype interaction effect: $F(3,54) = 3.21$, $p = 0.0301$; Bonferroni test in the training quadrant, $p < 0.01$; crossings; genotype effect: $F(1,18) = 4.22$, $p = 0.0297$; quadrant*genotype interaction effect: $F(3,54) = 4.961$, $p = 0.0297$; Bonferroni test in the training quadrant, $p < 0.01$).

(D) Data from the same group of mice tested in the transfer phase. CKO mice displayed similar performance as the controls (repeated-measures ANOVA; genotype effect: $F(1,18) = 0.562$, $p = 0.372$ for escape latency). CKO mice performed significantly worse in the new training quadrant (NTQ) compared to their control siblings during a probe trial at day 5; (genotype effect: $F(1,18) = 4.13$, $p = 0.036$; quadrant*genotype interaction effect: $F(3,54) = 8.36$, $p = 0.001$; Bonferroni test in the training quadrant, $p < 0.01$). Similarly, for the number of platform crossing, genotype*quadrant interaction effect: $F(3,54) = 4.86$, $p = 0.0049$; t test for crossings in NTQ: $p = 0.01$.

(E) (i) schematic of the experiment; (ii) during a probe trial 8 days later, CKO mice performed even worse than in the first probe trial (repeated-measures ANOVA; genotype*quadrant interaction effect: $F(3,54) = 4.2$, $p = 0.0029$; t test for NTQ: $p = 0.001$). Similarly for the number of platform crossing, genotype*crossing interaction effect of repeated-measures ANOVA: $F(3,54) = 6.27$, $p = 0.0013$; t test for time spent in the training quadrant: $p = 0.01$. See also Figure S3.

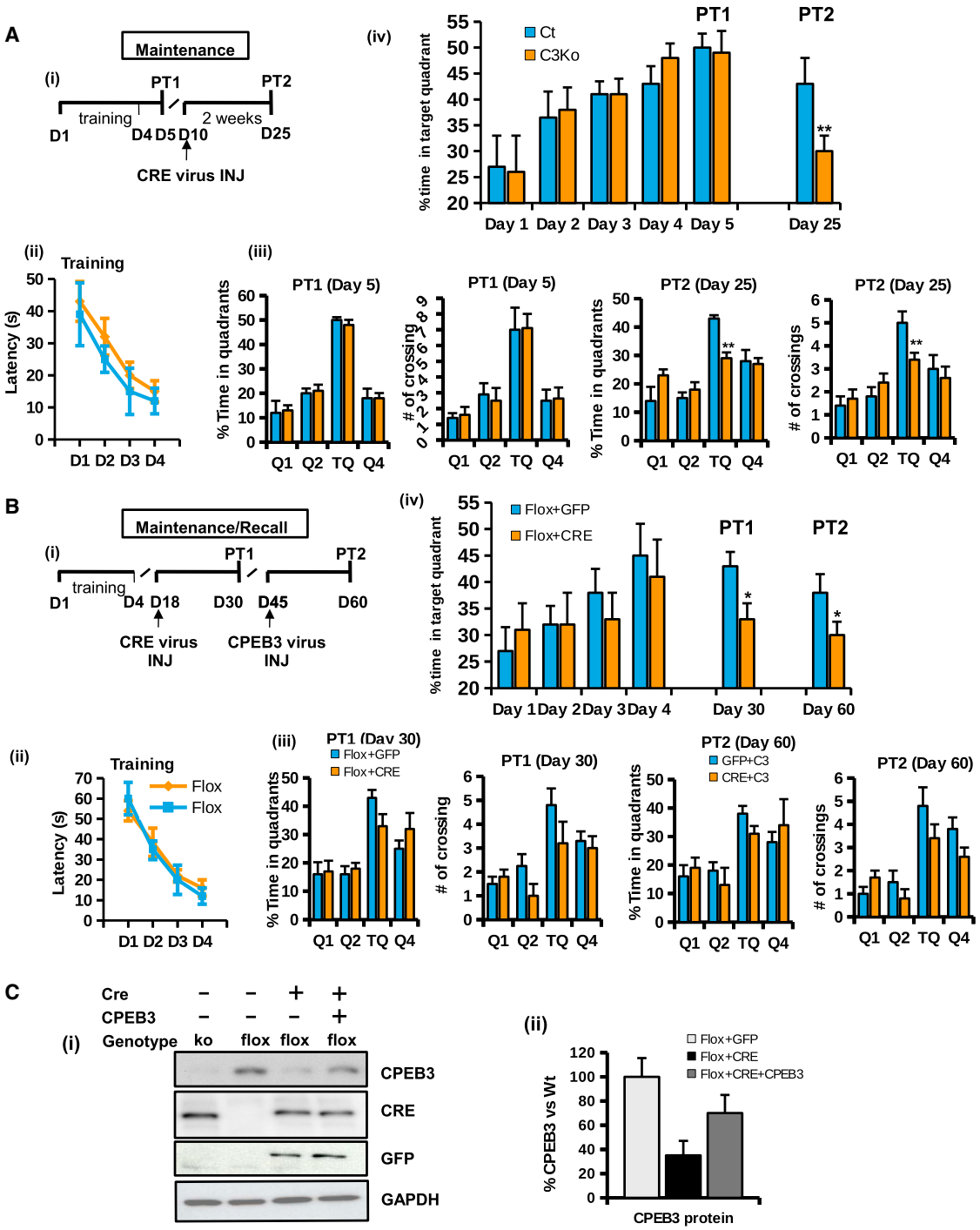

Figure 4. Impaired Memory Maintenance in the CPEB3 CKO Mice

(A) Data from viral injected mice. (i) Scheme of the experiment. (ii) The latency to reach the platform during the training was similar (ANOVA for escape latency, genotype effect: $F(1,16)<1$, $p = 0.72$). (iii) The percentage of time spent in the quadrants (+SEM) (CPEB3 CKO, C3flox/flox = 9; controls, WT = 9). (iv) The percentage of time spent in the first trial of each day over the entire experiments is also shown. There are no differences in the first probe trial. 5 days after the first probe, both groups were injected with NLS-CRE and expressed the virus for 2 weeks. ANOVA shows statistically significant difference between controls and CKO mice at the second probe trial (interaction effect genotype*quadrant: $F(1,16) = 6.24$, $p = 0.00246$; ANOVA for crossings in GFP: $F(3,28) = 4.764$, $p = 0.0219$; ANOVA for CRE group: $F(3,28) = 0.2853$, $p = 0.7547$).

(B) Data from a separate cohort of viral injected mice. (i) Scheme of the experiment. (ii) Latency to reach the platform. (iii) The percentage of time spent in the pool quadrants and the number of crossing are shown (+SEM) (Flox, C3flox/flox = 8; controls, C3flox/flox = 7). (iv) The percentage of time spent in the first trial of each day over the entire experiments is also shown. Flox mice were injected either with CRE (n = 8) or GFP (n = 7) and expressed the virus for 2 weeks. Mice were

(legend continued on next page)

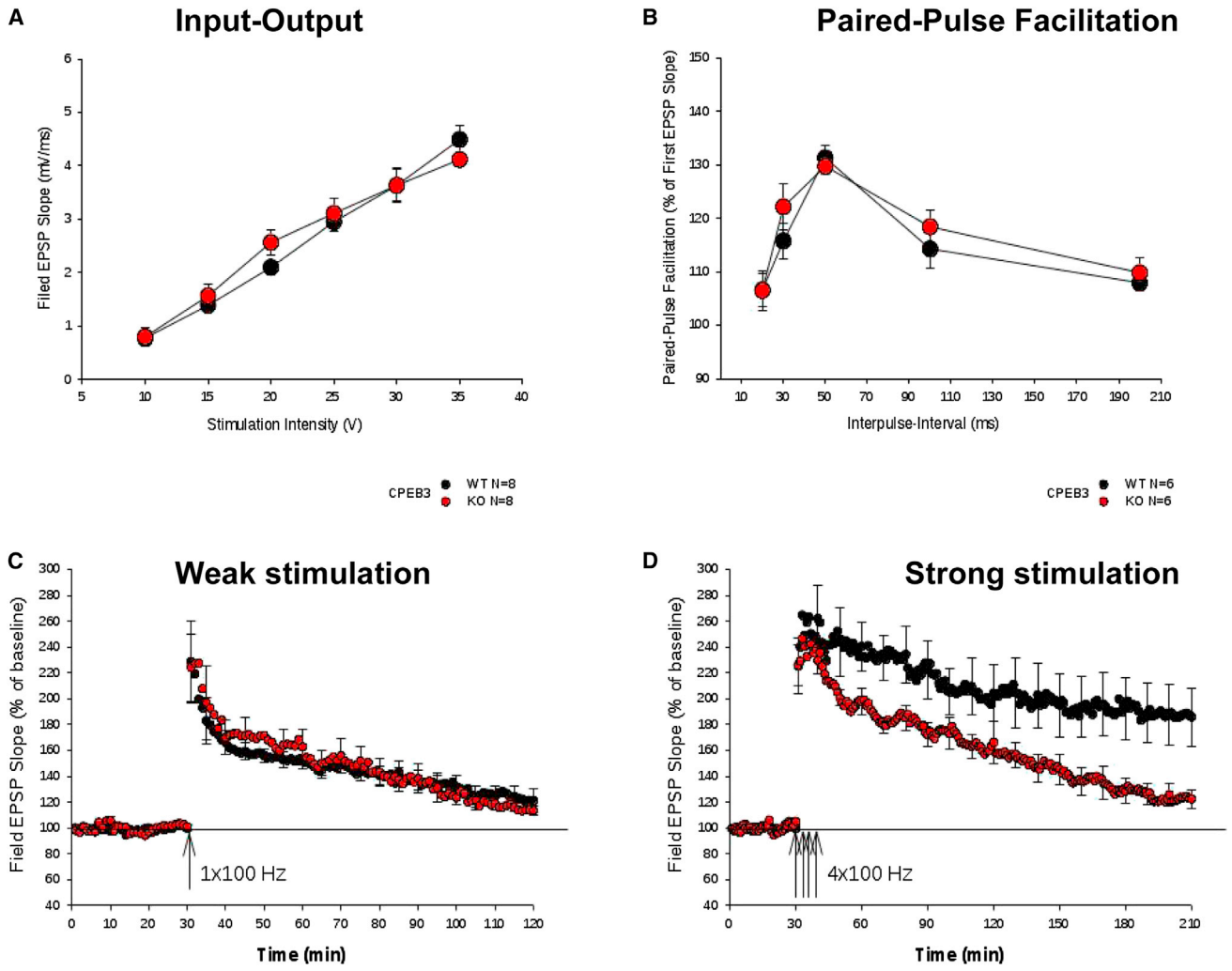

Figure 5. Reduced LTP in the CPEB3 CKO Mice

(A) Basal synaptic transmission measured by the input-output relationship was not affected in slices from KO mice compared to WT controls (KO: n = 6, 6 animals; controls: n = 6, 6 animals; genotype effects: 10 V, p = 0.84, 15 V, p = 0.75; 20 and 30 V, p = 0.6).

(B) Paired-pulse facilitation (PPF) of the field excitatory postsynaptic potential (fEPSP) amplitude in the hippocampal CA1 region of control (n = 6), and CPEB3CKO mice (n = 6) showed that there are no differences in short-term forms of plasticity, two-way ANOVA, p = 0.3.

(C) LTP induced by one train at 100 Hz (KO: n = 8, 6 animals; control: n = 6, 6 animals) was not affected in CPEB3 CKO mice (1 × 100 Hz: 1 hr recording, mean percent of baseline: WT = 122.4% ± 8.0%, KO = 114.3% ± 4%; unpaired t test: p > 0.05).

(D) LTP induced by four trains at 100 Hz (KO: n = 10, 6 animals; control: n = 9, 6 animals) was reduced in CPEB3 CKO mice compared to their control siblings (mean percent of baseline; after 60 min: control = 240 ± 22 KO = 200.6 ± 15, unpaired t test p > 0.05, after 120 min: control = 200 ± 20, KO = 170.6 ± 10; unpaired t test: p > 0.05, after 180 min: KO = 123.6 ± 7, control = 186 ± 22; unpaired t test: p < 0.05).

prion-like conformation changes (Si et al., 2003; Fiumara et al., 2010; Raveendra et al., 2012).

We have observed aggregation of CPEB3 after fear conditioning training (Figure 1D). We then tested whether this aggregation was dependent on the presence of the N terminus prion-like domain. We transfected CPEB3 WT and N terminus truncated protein into cells and determined whether these two proteins would form aggregates. We performed both detergent

challenged with a probe trial and ANOVA shows statistically significant difference between controls (GFP) and knocked-down mice (CRE) at probe trial (ANOVA for time in GFP group: F(3,24) = 11.10, p = 0.0011; ANOVA for CRE group: F(3,28) = 0.77, p = 0.4737). Reintroducing CPEB3 did not rescue the impaired memory observed in the first probe (ANOVA for time in GFP group: F(3,24) = 8.12, p = 0.0021; ANOVA for CRE group: F(3,28) = 0.57, p = 0.3437). There is no improvement between probe 1 (PT1) and probe 2 (PT2) in the number of crossings of CRE-transduced animals after reintroducing CPEB3 protein (ANOVA for crossings in GFP: F(3,28) = 3.647, p = 0.0321; ANOVA for CRE group: F(3,28) = 0.5235, p = 0.8535). See also Figure S4.

(C) Representative western blot (i) and quantification (ii) of CPEB3 knockdown in flox mice injected with GFP, CRE, and rescued with CPEB3. Cre injection induces a significant reduction in the level of CPEB3 in the dorsal hippocampus.

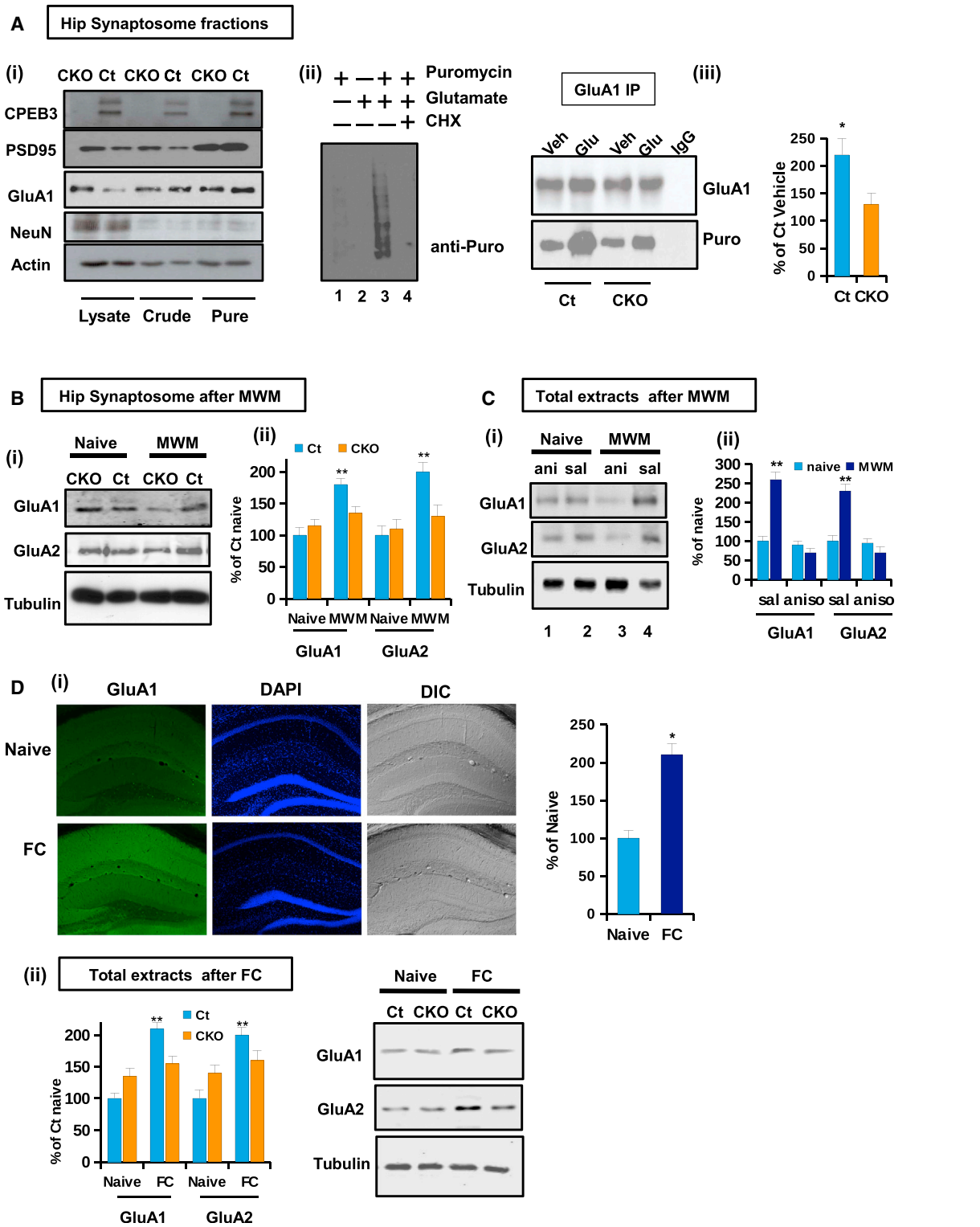

Figure 6. Knockdown of CPEB3 Prevents Activity-Dependent Increase of AMPAR

(A) (i) Representative image of western blot analysis of synaptosome preparation from hippocampal tissue; (ii) pure synaptosome fractions were stimulated with Glutamate and Glycine in the presence of Puromycin; (iii) immunoprecipitation of GluA1 from stimulated synaptosome shows higher amount of activity-induced translation of AMPAR in Ct mice versus CKO.

(legend continued on next page)

insolubility (DI) and SDD-AGE assays. Using the DI assay, we found that overexpressing the wild-type protein induced its aggregation (Figure 7B). By contrast, the truncated mutant was more soluble (Figure 7B). Using the SDD-AGE assay, we found that overexpression of CPEB3 induced the formation of amyloid-like oligomers, while the truncated CPEB3 failed to do so (Figure 7B).

We also observed oligomerization of the protein in vivo after animals performed the Morris water maze. We found that CPEB3 is partially aggregated after training and after a probe trial. We also noted that during a second probe trial CPEB3 is significantly more aggregated than during the first one, suggesting that the protein is either more prone to aggregate after it has been previously activated or that the aggregates have a long half-life and persist over a long period of time (Figure 7D). To test these hypotheses, we performed time-course analyses of the stability of CPEB3 aggregates and measured the amount of CPEB3 in the insoluble fraction 1 hr or 24 hr after the probe trial. The aggregates persist over a period of few hours, but they almost completely disappear 24 hr later (Figure 7E). This suggests that the increased amount of aggregated CPEB3 during the second probe trial is probably due to recruitment of newly synthesized CPEB3 into a small seed that although still present cannot be measured by conventional biochemical technique. We confirmed our results in extracts of fear-conditioned animals and glutamate-stimulated hippocampal neurons (Figures 7C and 7D).

To better understand the nature of CPEB3 aggregates, we performed additional experiments in hippocampal neurons in vitro. Indeed, one possibility is that CPEB3 might form oligomers induced by the binding of RNA molecules. To test this idea, we digested the RNA in the samples and found that treatment with RNase enzyme did not alter the molecular state of CPEB3, indicating that CPEB3 forms oligomeric, aggregated structures through protein-protein interactions.

To further understand whether CPEB3 forms macromolecular complexes with other proteins or is forming homo-oligomers, we performed co-transfection of wild-type protein labeled with red fluorescent protein (RFP) with the mutant protein lacking the N-terminal domain fused to green fluorescent protein (GFP) and performed co-immunoprecipitation experiments. We found that the N-terminal domain is fundamental for the establishment of CPEB3 oligomers. Deletion of the N-terminal region almost completely abolishes the interaction between CPEB3 molecules (Figure S6).

We conclude from these findings that in hippocampal neurons CPEB3 regulates the translation of GluA1 and GluA2 bi-directionally and that this change in function results from a change in the molecular state of CPEB3, from a soluble to an aggregated form.

The N-Terminal Domain of CPEB3 Mediates the Stability of LTP and Spatial Memory

Is the effect of CPEB3 on the maintenance of memory and synaptic plasticity mediated by its prion-like N-terminal domain? CPEB3 CKO mice fail to sustain L-LTP. We therefore thought to re-introduce into the hippocampus of CPEB3 CKO mice either WT or mutant CPEB3 lacking the N terminus. As a control we also injected CPEB3 CKO mice with viruses expressing only GFP. We found that injections of WT CPEB3 viral particles completely rescued the synaptic plasticity defect of CPEB3 CKO mice, while slices expressing CPEB3 lacking its prion domain still showed a profound deficit in L-LTP (Figure 8A). As expected, transduction with control GFP virus did not alter the synaptic properties of CPEB3 CKO neurons. In addition, we injected CT mice with the same viruses and found that none of them induced a significant change compared to slices that were not transduced. Interestingly, we did find a significant difference between CT slices transduced with WT and truncated CPEB3, suggesting that the two different constructs work in opposite directions, the WT protein inducing an increase in the stability of LTP, while the truncated one causes a reduction.

We then used the same strategy to perform Morris water maze experiments. We injected GFP, WT CPEB3, and mutant CPEB3 viruses in the dorsal hippocampus of CKO mice. We found that both GFP- and mutant-transduced CKO mice had lower performances than CT animals, while CPEB3 WT-transduced mice were indistinguishable from CT animals (Figure 8B). However, we observed a partial rescue of CPEB3 CKO defects when we injected the CKO mice with the mutant construct. We then tested whether reintroducing CPEB3 would also rescue the maintenance of memory. Consistent with our previous data, we found that 1 week later the CKO animals injected with GFP had a worse performance than in their first probe (Figure 8B). By contrast, mice transduced with WT CPEB3 continued to show a strong preference for the target quadrant. Interestingly, during the second probe trial, mice expressing the mutant form of CPEB3 lacking the N-terminal aggregating domain did not maintain their memory for the target quadrant.

Together, these findings indicate that CPEB3 N terminus, which mediates CPEB3 aggregation, is required for the stability of LTP and long-term spatial memory.

DISCUSSION

The main finding of our study is that CPEB3-mediated protein synthesis is required for long-term hippocampal-based memory storage—i.e., both consolidation and maintenance—but not for memory formation per se. We observe a deficit in memory storage in three different behavioral paradigms, contextual fear

(B) (i) Representative image of western blot analysis of hippocampal synaptosomes from mice after MWM (see also Figures S5A–S5C). (ii) Quantification of two independent experiments, n = 4 replicates. $p < 0.05$, t test between naive and MWM mice.

(C) Local infusion of Anisomycin in the dorsal hippocampus prevents the increase of AMPAR protein levels. (i) Representative western blot and (ii) quantification of GluA1 and GluA2 in Ct animals infused with anisomycin (n = 5) or saline (n = 4). (ANOVA for treatment effect; $F(1,14) = 3.65$, $p = 0.012$ and $F(1,14) = 5.32$, $p = 0.017$, for GluA1 and GluA2, respectively).

(D) Representative images and quantification of IHC staining (i) and western blot (ii) of dorsal hippocampus after contextual fear training. Both IHC and WB analyses show a significant increase in the level of GluA1 ($p < 0.01$) and GluA2 ($p < 0.05$) following training. In the CKO mice there is not a significant increase ($p > 0.05$).

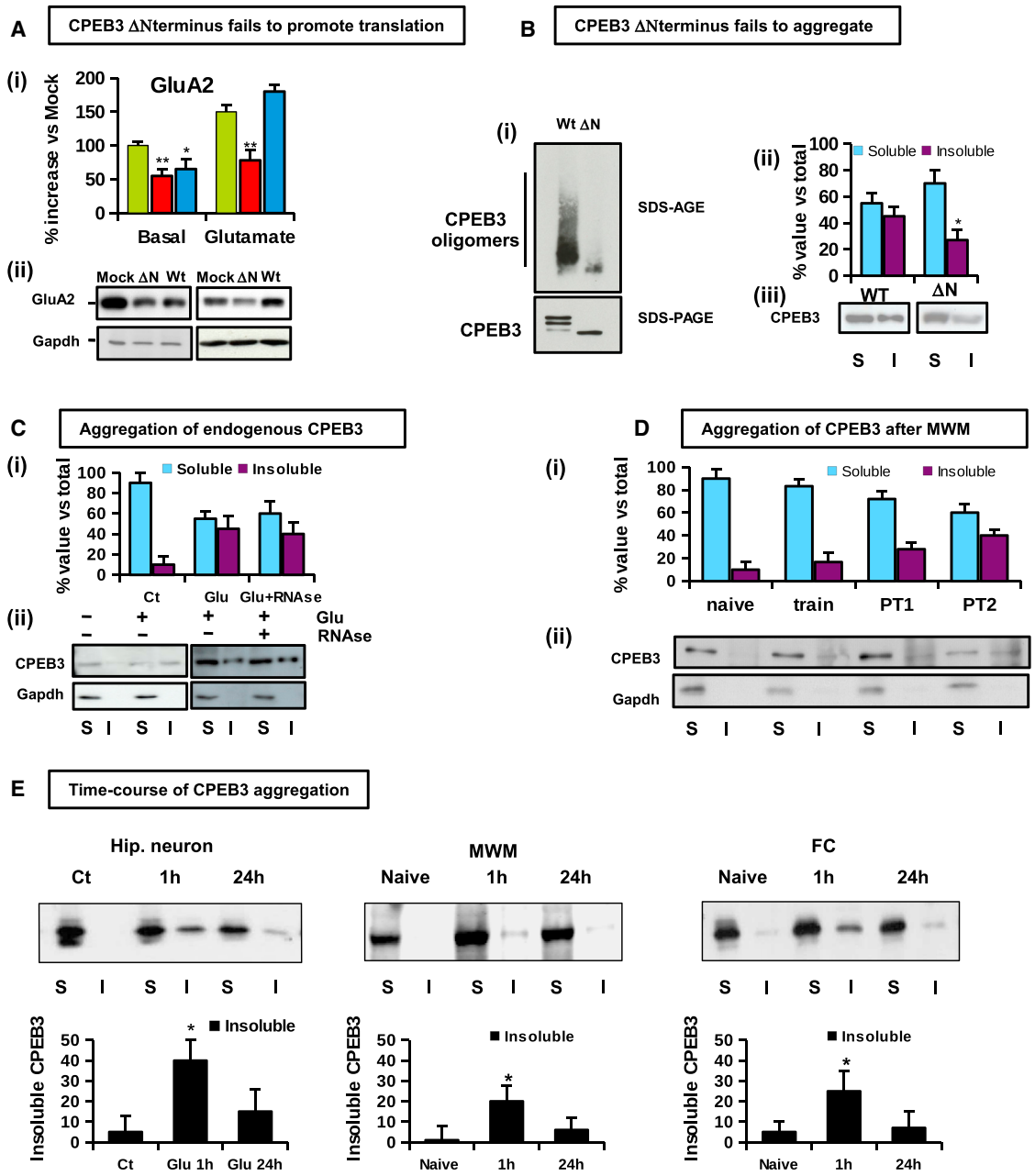

Figure 7. CPEB3 N-Terminal Domain Mediates the Aggregation and Activity of CPEB3

(A) (i) Quantification of three independent experiments, with n = 12 replicates (t test, $p < 0.01$). (ii) Representative western blot of lysates from neurons transduced with WT or truncated CPEB3. Top panel shows the amount of GluA2 after glutamate and bottom panel shows Gapdh, used as loading control.

(B) (i) Semi-denaturing Agarose gel shows lack of aggregates in the truncated CPEB3 (top panel); bottom panel shows equal loading of the CPEB3 proteins on a conventional SDS-PAGE; (ii) quantification of three independent experiments. $p < 0.05$, t test between pellet fractions; (iii) representative western blot of HEK cell extracts transfected with Wt or truncated CPEB3. Homogenates were ultracentrifuged at 62,000 × rpm and soluble (S) and insoluble (I) fractions were loaded on SDS-PAGE.

(C) (i) Quantification of 4 independent experiments, n = 8 replicates. $p < 0.01$, t test between pellet fractions. (ii) Representative image of western blot analysis of hippocampal neurons extracts from mock (Ct) or glutamate-treated cells (glu). Top panel shows CPEB3, bottom panel shows gapdh.

(D) Representative image of western blot analysis of dorsal hippocampal homogenates from mice after MWM. Quantification of two independent experiment, n = 4 replicates. $p < 0.05$, t test between pellet fractions.

(E) Representative images of time course analysis of CPEB3 aggregates in hippocampal cultures and in hippocampal tissue. Aggregates show a significant reduction after 24 from the stimuli. Quantification from two independent experiments, n = 4, $p < 0.05$, t test between pellet fractions.

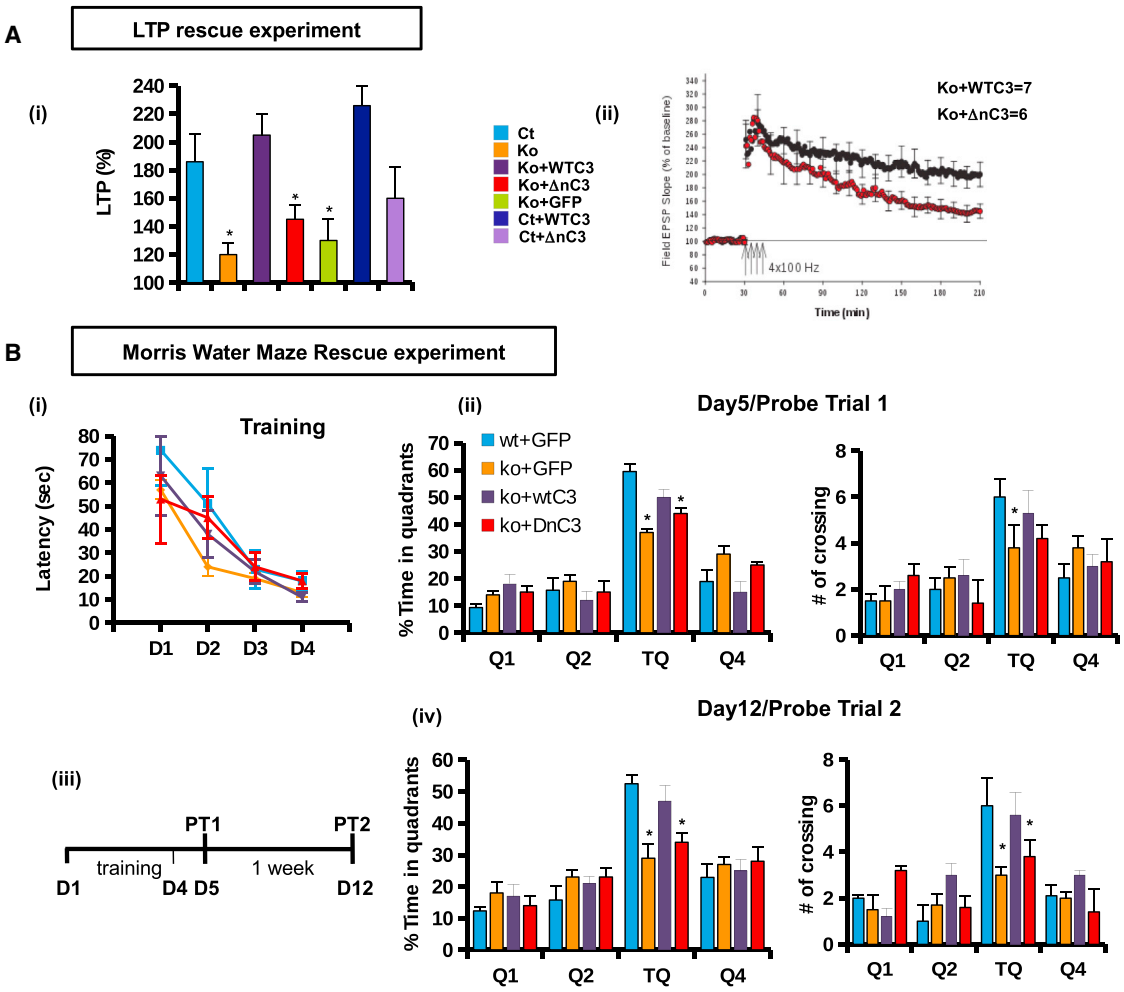

Figure 8. CPEB3 N-Terminal Domain Mediates the Maintenance of L-LTP and the Persistence of Memory

(A) LTP induced by four trains at 100 Hz (KO transduced with WT CPEB3: n = 7, 7 animals; KO transduced with mutant CPEB3: n = 6, 6 animals) is rescued in CKO mice transduced with WT CPEB3 protein but is not with a truncated CPEB3 (mean percent of baseline; after 180 min: CKO+WT = 200 ± 18, CKO+mutant = 145 ± 11; unpaired t test: $p = 0.016$). (i) Summary of LTP experiments performed on Ct and KO animals transduced with GFP, WT CPEB3, and truncated CPEB3. Significant differences versus Ct mice are indicated with an asterisk ($p < 0.05$). See also Figure S7.

(B) Data from Morris water maze task of CKO mice transduced with GFP (n = 5), WT CPEB3 (8), mutant CPEB3 (n = 6), and Ct mice transduced with GFP. (i) The mean escape latency (+SEM) to reach the platform is plotted against the day of the experiment. The escape latency was similar among all groups; (ii) probe trial on day 5 showed significantly reduced performance of the CKO transduced with GFP and mutant CPEB3 in the training quadrant (TQ) compared to CKO transduced with WT CPEB3 (genotype*quadrant interaction effect of ANOVA: $F(6,48) = 7.28$, $p = 0.0028$; Tukey post hoc test for time spent in training quadrant: $p < 0.05$). For the number of platform crossing, genotype*quadrant interaction effect of repeated-measures ANOVA: $F(6,48) = 6.32$, $p = 0.0034$; Tukey post hoc test for crossings, $p = 0.01$; (iii) schematic of the experiment; (iv) during a probe trial 8 days later, the CKO mice transduced with GFP, and mutant CPEB3 performed even worse than in the first probe trial, training quadrant (repeated-measures ANOVA; genotype*quadrant interaction effect: $F(6,48) = 4.2$, $p = 0.0029$; Tukey post hoc test for training quadrant: $p = 0.001$). For the number of platform crossing, genotype*crossing interaction effect of repeated-measures ANOVA: $F(6,48) = 6.27$, $p = 0.0013$; Tukey post hoc test for crossings in the training quadrant: $p < 0.05$.

conditioning, object placement recognition, and Morris water maze task, suggesting that this process is conserved across different types of hippocampal-based learning tasks. We also find that CPEB3 loses its ability to maintain both long-term synaptic plasticity and long-term memory when its prion-like N terminus domain is deleted. We therefore propose that, like *Aplysia* CPEB and *Drosophila* Orb2A, CPEB3 can sustain the persistence of memory through a stimulus-induced conformation change, which causes protein aggregation and a change in function that allows enhanced translation of the targets mRNA of CPEB3, such as the AMPAR subunits GluA1 and GluA2.

Misregulation of AMPA Receptor Translation in CPEB3 CKO Mice

In the basal state, CPEB3 binds to and represses the translation of its target mRNAs, the AMPA receptor subunits GluA1 (Pavlopoulos et al., 2011) and GluA2 (Huang et al., 2006). In addition, Hosoda et al. (2011) found that CPEB3 represses the translation

of its target mRNA by interacting with the protein Tob (transducer of Erb2). In turn, our laboratory has found that CPEB3 promotes the translation of the AMPAR following mono-ubiquitination by the ubiquitin ligase Neuralized (Pavlopoulos et al., 2011). Together, these data suggest that CPEB3 is a translational regulator with a dual role: it acts as a repressor in the basal state and as an activator in response to learning-related activity.

We have recently found that CPEB3 is SUMOylated in the basal state. Following stimulation of hippocampal neurons either in cultures or in vivo, CPEB3 is rapidly de-SUMOylated. This allows the oligomerization and aggregation of CPEB3 necessary for its activation as a regulator of translation (Drisaldi et al., 2015). These findings raised the possibility that a loss of CPEB3-mediated regulation of the translation of AMPA receptors contributes critically to the phenotype of the CPEB3 CKO mice.

Slipczuk et al. (2009) had earlier found that learning of spatial tasks induces the synthesis of AMPA receptors through an mTor and BDNF-mediated mechanism. We here provide evidence that CPEB3 is also implicated in the translation of AMPAR following learning. Interestingly it has been recently suggested that CPEB proteins might control the translation of BDNF itself (Oe and Yoneda, 2010), suggesting that CPEB3 might act not only directly but also indirectly, through BDNF signaling, to promote the increase of AMPAR protein levels.

The number, synaptic distribution, and subunit composition of the AMPA receptors regulate synaptic plasticity and synaptic strength (Groc and Choquet, 2006; Kessels and Malinow, 2009). The most abundant subunit of AMPA receptors in the adult brain is GluA2, which plays a critical role in synaptic plasticity and memory storage (Mead and Stephens, 2003). GluA1 also mediates certain forms of synaptic plasticity (Lee et al., 2010). But whereas GluA1 KO mice display impaired working memory, they show normal spatial learning (Zamanillo et al., 1999; Reisel et al., 2002). By contrast, GluA2 KO mice show impaired spatial and non-spatial learning in the water maze (Gerlai et al., 1998; Kessels and Malinow, 2009). Moreover, pharmacological inhibition of AMPAR affects learning, consolidation, and retrieval of spatial memory (Liang et al., 1994; Bast et al., 2005; Bannerman et al., 2006). These data support the idea that the regulated synthesis and trafficking of AMPAR is critical for several stages of memory storage. We find that the CPEB3 CKO mice show a reduction in learning-induced synthesis of AMPAR compared to control mice. This failure to increase the expression of AMPAR in response to learning might contribute to the defects in synaptic plasticity and behavior of these mice. It is quite possible however that in addition to the AMPAR, other, as yet un-identified, targets of CPEB3 might also contribute to the impairments of the CPEB3 CKO mice. Indeed it has been recently suggested that CPEB3 also regulates the expression of post-synaptic density 95 protein (PSD95) (Chao et al., 2013), a protein that is crucial both for synaptic function and behavioral learning and memory (Skibinska et al., 2001; Muller et al., 2000). Our findings therefore indicate that a subset of newly synthesized CPEB3 targets, including GluA1 and GluA2, play a role in the remodeling process involved in the persistence of long-term memory.

A recent study by Chao et al. (2013) has found that CPEB3 null mice have aberrant memory consolidation. These total knockout animals have decreased locomotor activity and slightly increased anxiety compared to control animals. We have not found any of these features affected in the conditional knockout mice. The total knockout animals also show better spatial memory consolidation but have impaired flexibility and do not have any defect in synaptic plasticity. Our data on the conditional forebrain-restricted CPEB3 CKO instead show defects in synaptic plasticity and impaired spatial memory. The differences between these results could be explained by the fact that a total knockout mouse may have undergone a series of developmental changes and compensatory events that might contribute to the observed phenotype.

A Role for CPEB3 Aggregates in Memory Storage and Synaptic Plasticity

We here provide evidence that CPEB3 is a translation regulator with a dual role; it acts as a repressor in the basal state and as an activator following learning-induced activity. This raises the question: how does CPEB3 switch from one state to the other? We find that this change in activity is correlated with a change in the molecular state of CPEB3 from a soluble form, in the basal state, to an aggregated form, upon synaptic activity. The propensity of CPEB3 to form oligomers derives from its N terminus domain, which comprises a region rich in glutamine, which is predicted to be poorly structured and to form aggregates (Fiumara et al., 2010).

Indeed, in 2000 Michelitsch and Weissman (2000) found that the eukaryotic proteome is enriched in glutamine and asparagine (Q/N)-rich low-complexity sequences, particularly within DNA and RNA binding proteins. Interestingly, Lindquist and colleagues have recently found many candidate prions within the list of Q/N-rich proteins proposed by Weissman (Alberti et al., 2009), thus indicating that there are likely more prions among DNA and RNA binding proteins to be identified.

We found that CPEB3 forms aggregates after synaptic activity in culture and in vivo after the performance of a behavioral task. We propose that this change in CPEB3 molecular state might contribute, at least in part, to the sustained synthesis of learning-related proteins that underlie the maintenance of memory. We have found, however, that differently from *Aplysia* and *Drosophila*, CPEB3 aggregates seem to be shorter lived; they last up to few hours and then are significantly reduced. Later on, though, when we stimulate the animals again, we observe another wave of aggregation, this time even larger than the previous one. One possible explanation for this re-aggregation is that a seed remains from the previous stimulation and it acts as a marker of activity of the functional state of that synapse. This seed probably recruits newly synthesized CPEB3 molecules that will be incorporated into the aggregate to maintain it in a functional state. Intriguingly, since CPEB3 mRNA contains CPE elements (Theis et al., 2003), CPEB3 could promote its own synthesis and sustain this positive feedback loop. In fact, when we knockdown CPEB3, as we did in our virus experiments, we interrupt this supply of CPEB3 molecules required to maintain the functional state of the aggregate. As a result of interrupting the synthesis of learning-related proteins memory could not be maintained.

Our data also suggest a model in which the synapses that contain the CPEB3 seed, once reactivated, become strengthened to sustain long-term synaptic connections and memories. This model predicts a possible role of CPEB3 in memory reconsolidation, and it is, in fact, one possible explanation for why recurrent retrievals can induce memory strengthening, that is because once CPEB3 has been reactivated it can promote the synthesis of synaptic proteins that reinforce the persistence of that memory trace.

EXPERIMENTAL PROCEDURES

Conditional Gene Targeting

Design: a Loxp (L83) site and an FNFL (Frt-Neo-Frt-Loxp) cassette were engineered to flank exon 2 (about 1 kb) of the *CPEB3* gene (Genebank: NC_000085.6) to generate the "floxed/neo" CPEB3 allele. A gene-targeting vector was constructed by retrieving the 2 kb short homology arm (5′ to L83), the 1 kb floxed sequence containing exon 2, the FNFL cassette, and the 5-kb-long homology arm (end of FNFL to 3′). The FNFL cassette conferred G418 resistance during gene targeting in KV1 (129B6 hybrid) ES cells. Several targeted ES cells were identified and injected into C57BL/6 blastocysts to generate chimeric mice (chimeras). Male chimeras were bred to homozygous bACTFlpe females to transmit the floxed CPEB3 allele (the L83/FL146 allele with neo cassette removed by Fple recombinase) through germline. Mice carrying floxed CPEB3 allele were crossed to tissue-specific cre to study CPEB3 CKO. Mice were treated in compliance with the rules of IACUC.

Surgery, Viral Injections, and Immunohistochemistry

Standard techniques were used for surgery, viral injections, perfusion, and immunohistochemistry (eee Supplemental Experimental Procedures).

Poly(A) Assays

Poly(A) tail-length assay kit was used (USB-Affymetrix).

Electrophysiology and Behavior

Adult littermate males (3–5 months) were used. Statistical analyses used ANOVAs with genotype as the between-subject factor. The experimenter was "blind" to the genotype. Standard protocols were followed for behavior and electrophysiology (Supplemental Experimental Procedures).

Hippocampal Culture

Hippocampal neurons were prepared according to Chiesa et al. (2004). For transfection with plasmid DNA and transduction with lentivirus, see Supplemental Experimental Procedures.

Protein Isolation and Western Blotting

Standard protocols were used for extraction and analysis of proteins from hippocampal tissue, cultured neurons, and cell lines (see Supplemental Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.05.021.

ACKNOWLEDGMENTS

We are grateful to Steven Siegelbaum, Craig Bailey, Robert Hawkins, Ferdinando Fiumara, and Amir Levine for their critical comments. We thank Kimberly Kempadoo for her guidance with the cannula implants and local infusions. We also thank Jenna Tarasoff and Deqi Yin for technical help and Joel Richter for the gift of the CPEB3 antibody. This work was supported by HHMI and the Mathers Foundation (to E.R.K) and by the "Fondation pour la Recherche Médicale" (to P.T.).

Received: August 4, 2014
Revised: December 2, 2014
Accepted: May 5, 2015
Published: June 11, 2015

REFERENCES

Alarcon, J.M., Hodgman, R., Theis, M., Huang, Y.S., Kandel, E.R., and Richter, J.D. (2004). Selective modulation of some forms of schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the CPEB-1 gene. Learn. Mem. *11*, 318–327.

Alberti, S., Halfmann, R., King, O., Kapila, A., and Lindquist, S. (2009). A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. Cell *137*, 146–158.

Bailey, C.H., Kandel, E.R., and Si, K. (2004). The persistence of long-term memory: a molecular approach to self-sustaining changes in learning-induced synaptic growth. Neuron *44*, 49–57.

Bannerman, D.M., Rawlins, J.N., and Good, M.A. (2006). The drugs don't work-or do they? Pharmacological and transgenic studies of the contribution of NMDA and GluR-A-containing AMPA receptors to hippocampal-dependent memory. Psychopharmacology (Berl.) *188*, 552–566.

Bast, T., da Silva, B.M., and Morris, R.G.M. (2005). Distinct contributions of hippocampal NMDA and AMPA receptors to encoding and retrieval of one-trial place memory. J. Neurosci. *25*, 5845–5856.

Bevins, R.A., and Besheer, J. (2006). Object recognition in rats and mice: a one-trial non-matching-to-sample learning task to study 'recognition memory'. Nat. Protoc. *1*, 1306–1311.

Chao, H.-W., Tsai, L.-Y., Lu, Y.-L., Lin, P.-Y., Huang, W.-H., Chou, H.-J., Lu, W.-H., Lin, H.-C., Lee, P.-T., and Huang, Y.-S. (2013). Deletion of CPEB3 enhances hippocampus-dependent memory via increasing expressions of PSD95 and NMDA receptors. J. Neurosci. *33*, 17008–17022.

Chiesa, R., Fioriti, L., Tagliavini, F., Salmona, M., and Forloni, G. (2004). Citotoxicity of PrP peptides. In Techniques in Prion Research, S. Lehmann and J. Grassi, eds. (Birkhäuser Basel), pp. 176–197.

Drisaldi, D., Colnaghi, L., Fioriti, L., Rao, N., Myers, C., Snyder, A.M., Metzger, D.J., Tarasoff, J., Konstantinov, E., Fraser, P., et al. (2015). SUMOylation is an inhibitory constraint that regulates the prion-like aggregation and Activity of CPEB3. Cell Rep. *11,* Published online June 11, 2015. http://dx.doi.org/10.1016/j.celrep.2015.04.061.

Du, L., and Richter, J.D. (2005). Activity-dependent polyadenylation in neurons. RNA *11*, 1340–1347.

Dudai, Y. (2002). Molecular bases of long-term memories: a question of persistence. Curr. Opin. Neurobiol. *12*, 211–216.

Fanselow, M.S., and Poulos, A.M. (2005). The neuroscience of mammalian associative learning. Annu. Rev. Psychol. *56*, 207–234.

Fiumara, F., Fioriti, L., Kandel, E.R., and Hendrickson, W.A. (2010). Essential role of coiled coils for aggregation and activity of Q/N-rich prions and PolyQ proteins. Cell *143*, 1121–1135.

Gerlai, R., Henderson, J.T., Roder, J.C., and Jia, Z. (1998). Multiple behavioral anomalies in GluR2 mutant mice exhibiting enhanced LTP. Behav. Brain Res. *95*, 37–45.

Groc, L., and Choquet, D. (2006). AMPA and NMDA glutamate receptor trafficking: multiple roads for reaching and leaving the synapse. Cell Tissue Res. *326*, 423–438.

Hake, L.E., and Richter, J.D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell *79*, 617–627.

Hosoda, N., Funakoshi, Y., Hirasawa, M., Yamagishi, R., Asano, Y., Miyagawa, R., Ogami, K., Tsujimoto, M., and Hoshino, S. (2011). Anti-proliferative protein Tob negatively regulates CPEB3 target by recruiting Caf1 deadenylase. EMBO J. *30*, 1311–1323.

Huang, Y.-S., Kan, M.-C., Lin, C.-L., and Richter, J.D. (2006). CPEB3 and CPEB4 in neurons: analysis of RNA-binding specificity and translational control of AMPA receptor GluR2 mRNA. EMBO J. *25*, 4865–4876.

Izquierdo, I., Bevilaqua, L.R.M., Rossato, J.I., da Silva, W.C., Bonini, J., Medina, J.H., and Cammarota, M. (2008). The molecular cascades of long-term potentiation underlie memory consolidation of one-trial avoidance in the CA1 region of the dorsal hippocampus, but not in the basolateral amygdala or the neocortex. Neurotox. Res. *14*, 273–294.

Jaafari, N., Konopacki, F.A., Owen, T.F., Kantamneni, S., Rubin, P., Craig, T.J., Wilkinson, K.A., and Henley, J.M. (2013). SUMOylation is required for glycine-induced increases in AMPA receptor surface expression (ChemLTP) in hippocampal neurons. PLoS ONE *8*, e52345.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialog between genes and synapses. Biosci. Rep. *21*, 565–611.

Keleman, K., Krüttner, S., Alenius, M., and Dickson, B.J. (2007). Function of the Drosophila CPEB protein Orb2 in long-term courtship memory. Nat. Neurosci. *10*, 1587–1593.

Kessels, H.W., and Malinow, R. (2009). Synaptic AMPA receptor plasticity and behavior. Neuron *61*, 340–350.

LeDoux, J.E. (2000). Emotion circuits in the brain. Annu. Rev. Neurosci. *23*, 155–184.

Lee, H.-K., Takamiya, K., He, K., Song, L., and Huganir, R.L. (2010). Specific roles of AMPA receptor subunit GluR1 (GluA1) phosphorylation sites in regulating synaptic plasticity in the CA1 region of hippocampus. J. Neurophysiol. *103*, 479–489.

Liang, K.C., Hon, W., Tyan, Y.M., and Liao, W.L. (1994). Involvement of hippocampal NMDA and AMPA receptors in acquisition, formation and retrieval of spatial memory in the Morris water maze. Chin. J. Physiol. *37*, 201–212.

Majumdar, A., Cesario, W.C., White-Grindley, E., Jiang, H., Ren, F., Khan, M.R., Li, L., Choi, E.M.-L., Kannan, K., Guo, F., et al. (2012). Critical role of amyloid-like oligomers of Drosophila Orb2 in the persistence of memory. Cell *148*, 515–529.

Martin, K.C., Barad, M., and Kandel, E.R. (2000). Local protein synthesis and its role in synapse-specific plasticity. Curr. Opin. Neurobiol. *10*, 587–592.

McGaugh, J.L. (2000). Memory—a century of consolidation. Science *287*, 248–251.

Mead, A.N., and Stephens, D.N. (2003). Involvement of AMPA receptor GluR2 subunits in stimulus-reward learning: evidence from glutamate receptor gria2 knock-out mice. J. Neurosci. *23*, 9500–9507.

Michelitsch, M.D., and Weissman, J.S. (2000). A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. Proc. Natl. Acad. Sci. USA *97*, 11910–11915.

Morris, R.G.M. (1981). Spatial localization does not require the presence of local cues. Learn. Motiv. *12*, 239–260.

Morris, R.G.M., Inglis, J., Ainge, J.A., Olverman, H.J., Tulloch, J., Dudai, Y., and Kelly, P.A.T. (2006). Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. Neuron *50*, 479–489.

Muller, D., Toni, N., and Buchs, P.A. (2000). Spine changes associated with long-term potentiation. Hippocampus *10*, 596–604.

Nayak, A., Zastrow, D.J., Lickteig, R., Zahniser, N.R., and Browning, M.D. (1998). Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. Nature *394*, 680–683.

Nolan, M.F., Malleret, G., Dudman, J.T., Buhl, D.L., Santoro, B., Gibbs, E., Vronskaya, S., Buzsáki, G., Siegelbaum, S.A., Kandel, E.R., and Morozov, A. (2004). A behavioral role for dendritic integration: HCN1 channels constrain spatial memory and plasticity at inputs to distal dendrites of CA1 pyramidal neurons. Cell *119*, 719–732.

Oe, S., and Yoneda, Y. (2010). Cytoplasmic polyadenylation element-like sequences are involved in dendritic targeting of BDNF mRNA in hippocampal neurons. FEBS Lett. *584*, 3424–3430.

Pavlopoulos, E., Trifilieff, P., Chevaleyre, V., Fioriti, L., Zairis, S., Pagano, A., Malleret, G., and Kandel, E.R. (2011). Neuralized1 activates CPEB3: a function for nonproteolytic ubiquitin in synaptic plasticity and memory storage. Cell *147*, 1369–1383.

Raveendra, B.L., Siemer, A.B., Puthanveettil, S.V., Hendrickson, W.A., Kandel, E.R., and McDermott, A.E. (2012). Characterization of prion-like conformational changes of the neuronal isoform of Aplysia CPEB. Nat. Struct. Mol. Biol. *20*, 495–501.

Reisel, D., Bannerman, D.M., Schmitt, W.B., Deacon, R.M.J., Flint, J., Borchardt, T., Seeburg, P.H., and Rawlins, J.N.P. (2002). Spatial memory dissociations in mice lacking GluR1. Nat. Neurosci. *5*, 868–873.

Si, K., Giustetto, M., Etkin, A., Hsu, R., Janisiewicz, A.M., Miniaci, M.C., Kim, J.-H., Zhu, H., and Kandel, E.R. (2003). A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell *115*, 893–904.

Si, K., Choi, Y.-B., White-Grindley, E., Majumdar, A., and Kandel, E.R. (2010). Aplysia CPEB can form prion-like multimers in sensory neurons that contribute to long-term facilitation. Cell *140*, 421–435.

Skibinska, A., Lech, M., and Kossut, M. (2001). PSD95 protein level rises in murine somatosensory cortex after sensory training. Neuroreport *12*, 2907–2910.

Slipczuk, L., Bekinschtein, P., Katche, C., Cammarota, M., Izquierdo, I., and Medina, J.H. (2009). BDNF activates mTOR to regulate GluR1 expression required for memory formation. PLoS ONE *4*, e6007.

Smith, W.B., Starck, S.R., Roberts, R.W., and Schuman, E.M. (2005). Dopaminergic stimulation of local protein synthesis enhances surface expression of GluR1 and synaptic transmission in hippocampal neurons. Neuron *45*, 765–779.

Stephan, J.S., Fioriti, L., Lamba, N., Colnaghi, L., Karl, K., Derkatch, I., and Kandel, E.R. (2015). The CPEB3 protein is a functional prion that interacts with the actin cytoskeleton. Cell Rep. *11,* Published online June 11, 2015. http://dx.doi.org/10.1016/j.celrep.2015.04.060.

Sutton, M.A., and Schuman, E.M. (2006). Dendritic protein synthesis, synaptic plasticity, and memory. Cell *127*, 49–58.

Theis, M., Si, K., and Kandel, E.R. (2003). Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus. Proc. Natl. Acad. Sci. USA *100*, 9602–9607.

Vnek, N., and Rothblat, L.A. (1996). The hippocampus and long-term object memory in the rat. J. Neurosci. *16*, 2780–2787.

Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M.-A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J.R., and Richter, J.D. (1998). CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of α-CaMKII mRNA at synapses. Neuron *21*, 1129–1139.

Zamanillo, D., Sprengel, R., Hvalby, O., Jensen, V., Burnashev, N., Rozov, A., Kaiser, K.M., Köster, H.J., Borchardt, T., Worley, P., et al. (1999). Importance of AMPA receptors for hippocampal synaptic plasticity but not for spatial learning. Science *284*, 1805–1811.