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Translational Control by MAPK Signaling in Long-Term Synaptic Plasticity and Memory

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Summary

Enduring forms of synaptic plasticity and memory require new protein synthesis, but little is known about the underlying regulatory mechanisms. Here, we investigate the role of MAPK signaling in these processes. Conditional expression of a dominant-negative form of MEK1 in the postnatal murine forebrain inhibited ERK activation and caused selective deficits in hippocampal memory retention and the translation-dependent, transcription-independent phase of hippocampal L-LTP. In hippocampal neurons, ERK inhibition blocked neuronal activity-induced translation as well as phosphorylation of the translation factors eIF4E, 4EBP1, and ribosomal protein S6. Correspondingly, protein synthesis and translation factor phosphorylation induced in control hippocampal slices by L-LTP-generating tetanization were significantly reduced in mutant slices. Translation factor phosphorylation induced in the control hippocampus by memory formation was similarly diminished in the mutant hippocampus. These results suggest a crucial role for translational control by MAPK signaling in long-lasting forms of synaptic plasticity and memory.

Introduction

Storage of long-term memory, or memory consolidation, requires new mRNA and protein synthesis (reviewed in Davis and Squire, 1984; McGaugh, 2000). In contrast, short-term memory is insensitive to inhibitors of transcription and translation. Long-lasting forms of synaptic plasticity, such as "late LTP" (L-LTP), exhibit a similar dependence on macromolecular synthesis, whereas more transient modifications of synaptic strength, such as "early LTP" (E-LTP), can be established in the absence of new mRNA and protein synthesis (Kandel, 2001). Investigation of the molecular mechanisms underlying long-term memory and synaptic plasticity has largely focused on the roles of signal transduction to the nucleus and regulation of gene expression at the transcriptional level. Conversely, the possibility that regulation at the translational level may play a role in these processes has not been explored.

Translation of eukaryotic mRNAs is primarily regulated at the level of initiation (Mathews et al., 2000). Studies in mitotic cells have defined 5′ cap recognition and ribosomal recruitment by translation initiation factors as key events in this multistep process (Raught et al., 2000). Cap recognition is accomplished by eukaryotic initiation factor 4E (eIF4E), and the eIF4E-associated factor eIF4G then recruits the 40S ribosomal subunit. Cap-dependent translation accounts for the synthesis of the vast majority of cellular proteins, since all mRNAs transcribed in the nucleus bear a 5′ cap. The synthesis of the translation machinery itself is additionally regulated by an inhibitory *cis*-acting element, termed a 5′ terminal oligopyrimidine tract (5′ TOP), which occurs adjacent to the cap in mRNAs encoding ribosomal proteins and translation factors (Meyuhas, 2000).

The efficiency of translation initiation is tightly coupled to the growth state of mitotic cells, with translational induction occurring in response to growth factors and mitogens. Phosphorylation events that regulate the activity and/or availability of eIF4E and the ribosomal protein S6 play major roles in this coordinate control of protein synthesis and cellular growth (Raught et al., 2000). Hypophosphorylated eIF4E binding proteins (4E-BPs) sequester eIF4E and block its association with eIF4G, but sequential phosphorylation of multiple 4E-BP residues in response to mitogens results in eIF4E release. Mitogen-induced phosphorylation of eIF4E itself promotes cap-dependent translation, and hyperphosphorylation of S6 is associated with enhanced 5′ TOP-dependent translation.

The inducible translation of mRNAs localized in dendrites, "local protein synthesis," has been proposed as a control point in neuronal plasticity (Martin et al., 2000; Steward and Schuman, 2001). Studies of the "capture" of L-LTP by "tagged" synapses have highlighted the importance of more global, neuron-wide translation in the establishment of hippocampal L-LTP (Barco et al., 2002; Frey and Morris, 1997). Cytoplasmic polyadenylation has been proposed as an alternative mechanism through which neuronal activity may influence translational efficiency. Recent studies have implicated a pair of cytoplasmic polyadenylation elements (CPEs) in the distal α*CaMKII* 3′ UTR in neuronal activity-dependent polyadenylation (Richter and Lorenz, 2002; Wu et al., 1998). Nevertheless, little is known about the contribution of translational regulatory mechanisms to long-term synaptic plasticity and memory.

The ERK MAPK signaling pathway is a highly conserved kinase cascade linking transmembrane receptors to downstream effector mechanisms (Chang and Karin, 2001; Pearson et al., 2001). In neurons, the ERK pathway is activated by stimuli associated with synaptic activity and plasticity, most notably calcium influx and

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neurotrophins (McAllister et al., 1999; Tyler et al., 2002; West et al., 2001). An important role for the ERK pathway in transcriptional regulation, but not in translational regulation, is well established across many cell types, including neurons (Treisman, 1996).

Previous studies have demonstrated the general involvement of the ERK signaling pathway in synaptic plasticity, learning and memory (Impey et al., 1999; Mazzucchelli and Brambilla, 2000; Sweatt, 2001). The relative contributions of ERK signaling to distinct temporal phases of LTP and memory, however, have not been systematically addressed. Furthermore, the molecular mechanisms by which the ERK pathway may regulate the temporal phases of LTP and memory have not been established. Such mechanisms may involve ERK-dependent regulation not only at the transcriptional level, but also at the translational level.

To investigate these issues, we adopted a conditional, region-restricted genetic approach to target ERK activation in the postnatal forebrain. Our analysis of the resulting mutant mice disclosed selective defects in the protein-synthesis dependent components of hippocampal LTP and memory. We further found that ERK activation was required for translational induction in response to neuronal activity, L-LTP, and hippocampal memory formation through regulation of the activity of multiple translation initiation factors. The results of these multidisciplinary investigations suggest an important function for ERK signaling in the control of the translational events underlying L-LTP and memory consolidation.

Results

Generation and Characterization of Conditional Transgenic Mice Expressing Dominant-Negative MEK1 in the Postnatal Forebrain

To investigate the possible involvement of ERK signaling in the protein synthesis-dependent phases of memory and LTP, we generated mutant mice in which a dominant-negative form of the ERK kinase MEK1 (dnMEK1) is expressed selectively in the postnatal forebrain (Figure 1A). This dominant-negative form of MEK1 bears a K→M substitution in the ATP binding site, abolishing its kinase activity but preserving its ability to interact with ERK1 and 2, thereby inhibiting their MEK-dependent activation (Mansour et al., 1994). The conditional approach to restrict the spatiotemporal pattern of transgene expression required the generation of transgenic mice in which a floxed (flanked by loxP sites) transcriptional/translational stop cassette prevents expression of the dnMEK1 cDNA from the chicken β-actin promoter. In the absence of Cre recombinase, the transgene was not detectably expressed in the brains of these "floxed" single transgenic mice (Figure 1B, left). Floxed single transgenic mice were then crossed to *αCaMKII-Cre* transgenic mice previously shown to mediate preferential excision of floxed sequences in a subset of excitatory neurons in the postnatal forebrain (Tsien et al., 1996; Zeng et al., 2001). Expression of the dnMEK1 transgene in the brains of the resulting double transgenic mice (designated "dnMEK1 mice") was largely restricted to hippocampal area CA1 and the neocortex (Figure 1B, right). Consistent with the prior reports employing the same *αCaMKII-Cre* mice, expression was undetectable in hippocampal area CA3 (Tsien et al., 1996; Zeng et al., 2001).

Although ERK signaling has been reported to promote the survival of cultured neurons under conditions of nutrient or growth factor deprivation (Xia et al., 1995), we detected no evidence of compromised neuronal survival in dnMEK1 mice (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/116/3/467/DC1).

To examine the effects of transgene expression on ERK activation in the brains of adult transgenic mice, we stimulated acute hippocampal slices from control and mutant mice with membrane depolarization, a procedure shown to result in robust ERK activation (Wu et al., 2001). Stimulation produced strong ERK activation in control slices, as measured by levels of dually phosphorylated ERK1/2 (Figure 1C), but levels of ERK activation were significantly reduced in mutant slices. We subsequently found that ERK activation was also significantly reduced in the dnMEK1 hippocampus in response to L-LTP induction and contextual memory formation (see below).

Impaired Spatial Reference Memory in dnMEK1 Mice

To determine whether inhibition of ERK activation in the hippocampus and neocortex compromises long-term learning and memory, we subjected single transgenic control and double transgenic mutant mice to the hidden-platform version of the Morris water maze, a hippocampus-dependent reference memory task (Morris et al., 1982). The performance of both control and mutant mice improved during the course of training, with a trend toward longer escape latencies in the mutant group (Figure 2A). Since escape latencies are an insensitive measure of reference memory, probe trials were performed upon the completion of training. Both groups displayed a preference for the pool quadrant in which the platform was located during training, but the mutant mice spent significantly less time than control mice searching in the target quadrant (Figure 2B). In addition, mutant mice were significantly less accurate in identification of the precise platform location, as indicated by a reduced number of platform crossings (Figure 2C). No significant differences in swimming speed (controls 16.6 $\pm$ 0.8 cm/s, mutants 16.7 $\pm$ 0.7 cm/s, $p > 0.05$) or thigmotaxis (swimming near the pool perimeter; controls 30.9 $\pm$ 6.0%, mutants 30.8 $\pm$ 3.8%, $p > 0.05$) were observed between the two groups in the hidden-platform task, and both groups performed similarly in the visible platform version of the task (escape latency, controls 9.5 $\pm$ 1.5 s, mutants 10.5 $\pm$ 1.4 s, $p > 0.05$), indicating that the impairments observed in mutant mice reflect a specific spatial memory deficit.

Selective Impairment in Long-Term Contextual Memory in dnMEK1 Mice

To examine the process of memory consolidation more closely, we turned to contextual fear conditioning, a hippocampus-dependent behavioral paradigm in which robust long-term memory for an experimental context is established following a single training session (Kim and Fanselow, 1992; Phillips and LeDoux, 1992). Admin-

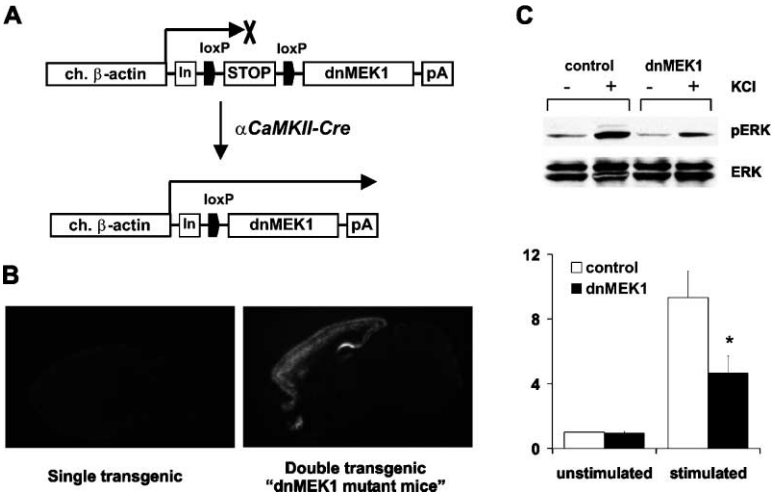

Figure 1. Generation of Forebrain-Specific dnMEK1 Mice

(A) The construct used to produce single transgenic floxed mice is depicted at top. When these single transgenic mice are crossed to α*CaMKII-Cre* transgenic mice, the stop cassette is excised and the dnMEK1 cDNA is expressed only in the postnatal forebrain of the resulting double transgenic mice (depicted at bottom).

(B) Representative results of in situ hybridization with a transgene-specific probe are shown for the single transgenic "floxed" mice (left) and double transgenic "dnMEK1" mice (right) diagrammed above.

(C) Neuronal activity-dependent ERK activation is impaired in the hippocampus of dnMEK1 mice. Top, representative Western analysis using antisera directed against dually phosphorylated ERK and total ERK. Hippocampal slices from control and mutant mice were treated with aCSF alone or aCSF containing 90 mM KCl. Bottom, quantification of mean normalized levels of pERK (n = 4 each).

istration of protein synthesis inhibitors prior to fear conditioning in rodents has been shown to disrupt long-term memory within 24 hr following training, while short-term memory remained intact (Abel et al., 1997; Schafe et al., 1999). Groups of control and mutant mice were therefore subjected to contextual fear conditioning and tested for memory of the experimental context after retention delays of 1 hr and 24 hr. Both control and

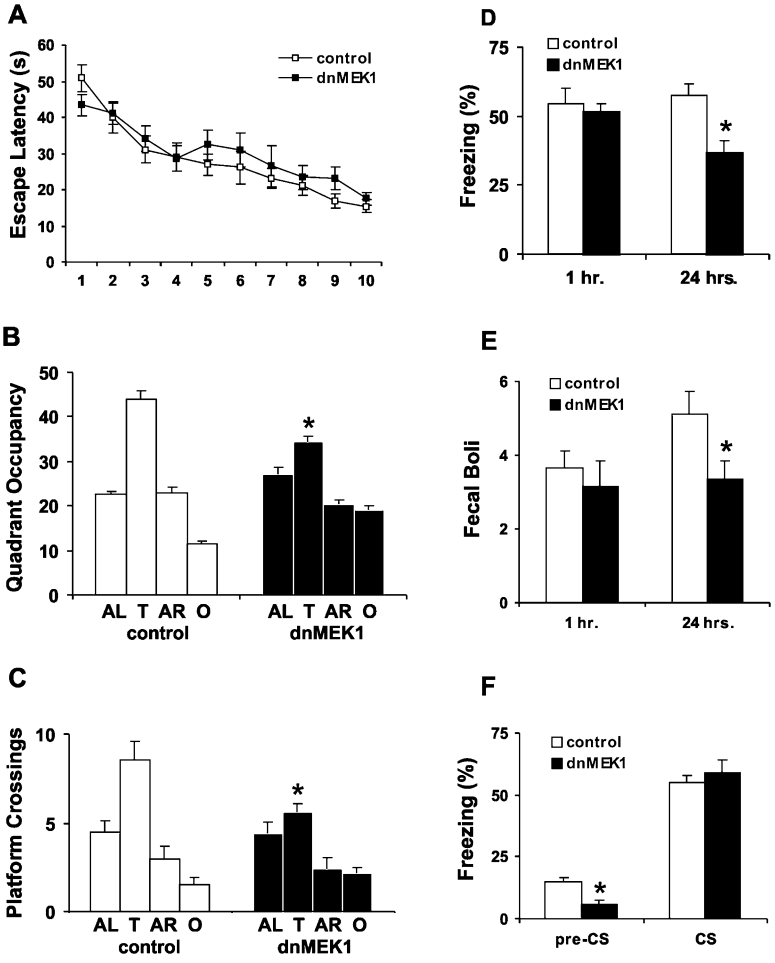

Figure 2. Impaired Hippocampal Memory Consolidation in dnMEK1 Transgenic Mice

(A) Escape latencies in the hidden platform version of the Morris water maze are plotted as a function of training block number for control (n = 20) and dnMEK1 (n = 12) mice.

(B) Mice were subjected to probe trials after the completion of training, and the mean proportion of time spent in each of the training quadrants is presented for both groups. AL, adjacent left quadrant; T, target quadrant; AR, adjacent right quadrant; OP, opposite quadrant.

(C) The mean number of crossings of the platform location during the probe trial is shown for the target quadrant and the corresponding locations in other quadrants.

(D) Mice were subjected to contextual fear conditioning, and context tests were administered after retention delays of one hour and 24 hr. The mean percentage of time spent freezing during the context tests is presented for control (n = 24) and dnMEK1 (n = 12) groups.

(E) Fecal boli were quantified as an independent measure of conditioned fear during the same context tests.

(F) Independent groups of control and mutant mice were subjected to cued fear conditioning. Noncontextual memory for the experimental tone was assessed in a novel chamber after a retention delay of 48 hr. The mean percentage of time spent freezing prior to presentation of the tone ("pre-CS") and during phasic presentation of the tone ("CS") is shown for control (n = 24) and dnMEK1 (n = 12) groups. Statistically significant differences at 95% confidence levels are denoted by asterisks.

mutant mice exhibited equivalent levels of freezing during the training session and after a 1 hr retention delay. In contrast, mutant mice exhibited significantly reduced levels of freezing after a retention delay of 24 hr (Figure 2D; controls 57.5 ± 4.1%, mutants 36.6 ± 4.7%, $p < 0.05$). Similar results were obtained when defecation was monitored as an independent measure of conditioned fear (Figure 2E) (Antoniadis and McDonald, 2000; Godsil et al., 2000).

We then evaluated the responses of control and mutant mice to cued fear conditioning, a hippocampus-independent version of the task in which a tone constitutes the conditioned stimulus. When tested in a distinct context after a 48 hr retention delay, mutant mice exhibited normal associative memory for the tone (Figure 2F). Consistent with their impairment in contextual memory consolidation, the low level of freezing prior to tone presentation was also reduced in mutant mice. A similar reduction in low-level contextual generalization has been observed as a consequence of pretraining hippocampal lesions and anisomycin infusion (Abel et al., 1997; Frankland et al., 1998). Importantly, the normal short-term contextual memory and normal long-term noncontextual memory exhibited by dnMEK1 mice exclude the possibility of any general defect in fear responses. These findings demonstrate a specific impairment in the protein-synthesis dependent phase of hippocampus-dependent contextual memory in dnMEK1 mice.

Selective Impairment in the Translational Component of Hippocampal L-LTP in dnMEK1 Mice

We next investigated hippocampal synaptic transmission and LTP at Schaeffer collateral (SC)-CA1 synapses, hypothesizing that defects in L-LTP might be associated with impaired hippocampal memory consolidation in dnMEK1 mice. Any observed impairments at these synapses should be referable to the postsynaptic neurons, since the dnMEK1 transgene is expressed in area CA1 neurons but not in area CA3 neurons. Basal synaptic transmission was normal in mutant mice, as evidenced by similar synaptic input-output relationship in control and mutant slices (Figure 3A). Paired-pulse facilitation (PPF), a presynaptic form of short-term synaptic plasticity, was also normal in mutant mice at multiple interpulse intervals (Figure 3B).

LTP was next induced with two trains of tetanic stimulation separated by 20 s, a procedure that induces protein synthesis-independent E-LTP (Winder et al., 1998). Stable potentiation was induced in both the control and mutant groups, with the magnitude of potentiation essentially identical throughout the 60 min recording (Figure 3C; E-LTP magnitude at 30 min posttetanization, controls 131.3 ± 4.6%, mutants 128.1 ± 3.2%, $p > 0.05$). We then applied four trains of tetanic stimulation separated by 5 min intervals, a protocol that induces long-lasting, protein synthesis-dependent L-LTP (Huang and Kandel, 1994). This procedure elicited long-lasting potentiation in control slices that persisted for at least 3 hr after the onset of tetanization. In contrast, mutant slices exhibited an unstable potentiation that progressively decayed throughout the duration of recording, with fEPSP slopes returning near unstimulated levels by 3 hr posttetanization (Figure 3D; L-LTP magnitude at 200 min, controls 138.6 ± 5.1%, mutants 108.8 ± 4.8%; $p < 0.05$).

Previous publications have suggested that transcriptional and translational inhibitors produce distinct kinetic patterns of L-LTP impairment, with transcriptional inhibition producing a delayed decay of L-LTP (typically beginning more than 1 hr after the onset of tetanization), and translational inhibition producing an early, progressive decay of L-LTP (Frey et al., 1996, 1988; Frey and Morris, 1997; Nguyen et al., 1994). The kinetics of L-LTP decay in mutant mice strongly resembled the reported effects of translational inhibition on L-LTP. To examine this relationship more closely, we performed an additional series of L-LTP experiments with the transcriptional inhibitor actinomycin-D and the translational inhibitor anisomycin. Consistent with prior reports, treatment of control slices with anisomycin prior to tetanization caused a progressive inhibition of L-LTP similar to that observed in mutant slices, whereas treatment of control slices with actinomycin-D produced a distinct, delayed pattern of inhibition (Figure 3E). Specifically, L-LTP magnitude in slices treated with actinomycin-D remained indistinguishable from that in untreated slices for approximately 75 min after the onset of tetanization (LTP magnitude at 60 min posttetanization, untreated controls 164 ± 5%, actinomycin-D-treated controls 157 ± 4%, $p > 0.05$), followed by a progressive decay to anisomycin-treated levels thereafter (LTP magnitude at 200 min, untreated controls 143 ± 6%, actinomycin-D-treated controls 107 ± 4%, anisomycin-treated controls 108 ± 5%, $p < 0.05$ for treated relative to untreated conditions). This difference in the kinetic patterns of inhibition by actinomycin-D and anisomycin defines a transcription-independent, translation-dependent phase of L-LTP during the first 60–90 min following tetanization.

The inhibitory effects of both agents were occluded in mutant slices, as treatment with either actinomycin-D or anisomycin did not produce any additional decrement in L-LTP (Figure 3F; L-LTP magnitude at 200 min, untreated mutants 115 ± 6%, actinomycin-D-treated mutants 122 ± 5%, anisomycin-treated mutants 113 ± 5%, $p > 0.05$). These results indicate the presence of a translational defect in mutant slices, since a transcriptional defect alone would not have occluded the inhibitory effect of anisomycin. Further supporting this interpretation, the kinetics of L-LTP decay were indistinguishable between the anisomycin-treated control group and the untreated mutant group throughout the duration of the recording (Figure 3G; L-LTP magnitude at 200 min, anisomycin-treated controls 108 ± 5%, untreated mutants 115 ± 6%, $p > 0.05$). In contrast, the time course of L-LTP during the transcription-independent, translation-dependent phase differed significantly between the actinomycin-D-treated control and the untreated mutant groups (Figure 3H; L-LTP magnitude at 60 min, actinomycin-D-treated controls 157 ± 3%, untreated mutants 139 ± 5%, $p < 0.05$). Taken together, these results demonstrate maximal blockade of the translational component of L-LTP in dnMEK1 mice.

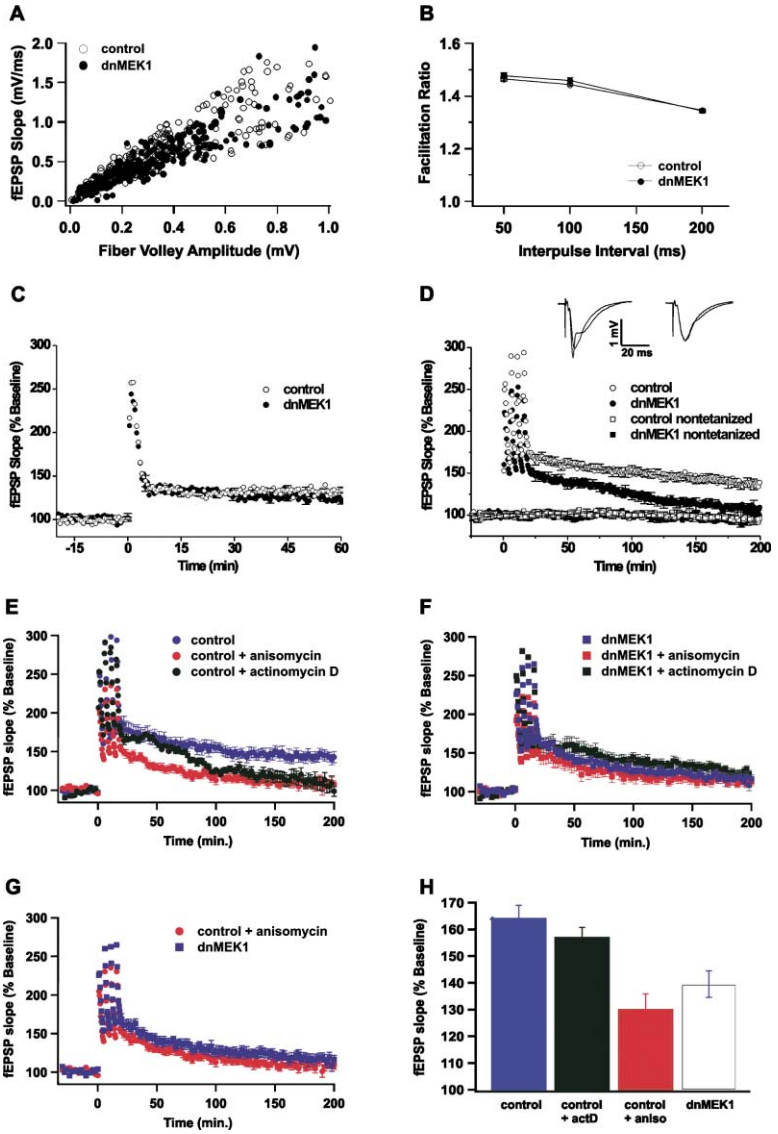

Figure 3. Impairment of the Translation-Dependent, Transcription-Independent Phase of Hippocampal L-LTP in dnMEK1 Transgenic Mice

(A) Normal basal synaptic transmission in dnMEK1 mice. The synaptic input-output curve shows fEPSP slopes as a function of fiber-volley amplitudes for control (n = 16 slices, 12 mice) and mutant (n = 16 slices, 9 mice) slices.

(B) Normal paired-pulse facilitation in dnMEK1 mice. The facilitation ratio is shown as a function of interpulse interval for control (n = 12 slices, 8 mice) and mutant (n = 15 slices, 9 mice) slices.

(C) Normal E-LTP in dnMEK1 mice. E-LTP was induced in control (n = 8 slices, 8 mice) and mutant (n = 8 slices, 8 mice) slices with two tetanic trains (100 Hz, 1 s) separated by 20 s.

(D) Impaired L-LTP in dnMEK1 mice. L-LTP was induced in control (n = 10 slices, 10 mice) and mutant (n = 10 slices, 10 mice) slices with four tetanic trains (100 Hz, 1 s) separated by 5 min each. The inset traces at top show the fEPSP responses immediately prior to and 200 min after tetanization for control (left) and mutant (right) slices.

(E) Actinomycin-D and anisomycin produce patterns of L-LTP inhibition with distinct kinetic profiles. L-LTP was induced in slices from control mice in the presence (n = 8 slices, 8 mice) and absence (n = 8 slices, 8 mice) of actinomycin-D and anisomycin.

(F) The inhibitory effects of actinomycin-D and anisomycin treatment are occluded in dnMEK1 slices. L-LTP was induced in slices from dnMEK1 mice in the presence (n = 8 slices, 8 mice) and absence (n = 8 slices, 8 mice) of actinomycin-D and anisomycin.

(G) The effects of anisomycin on L-LTP in control mice are indistinguishable from the effects of dnMEK1 transgene expression in mutant mice. Superimposition of the L-LTP results for anisomycin-treated control slices and untreated mutant slices is shown.

(H) Quantification of the L-LTP magnitude at 60 min posttetanization is shown for each group. L-LTP magnitude in dnMEK1 slices is significantly lower than that in untreated and actinomycin-D-treated control slices, but is indistinguishable from that in anisomycin-treated control slices.

ERK Signaling Regulates Translation in Response to Multiple Forms of Neuronal Activity through a Polyadenylation-Independent Mechanism

To investigate a possible role for ERK activation in the regulation of neuronal protein synthesis, we developed a translation reporter assay in cultured primary hippocampal neurons. This assay relies on transfection of cultured neurons with synthetic mRNAs, which permits isolation of translational regulation from regulation of transcription or mRNA processing and transport. Furthermore, this method allows an assessment of the contribution of *cis*-acting elements and the polyadenylation state of transfected mRNAs to translational regulation. Synthetic reporter mRNAs contained an EGFP reporter coding sequence, preceded by a 5′ m^7GpppG cap and a ~30 nt synthetic 5′ UTR. Given the important role of αCaMKII in synaptic plasticity and the proposed role of its 3′ UTR in translational regulation (Soderling, 2000), the EGFP sequences in our reporter mRNAs were appended with a distal ~160 nt segment of the α*CaMKII* 3′ UTR (Wu et al., 1998), which contained both CPE elements as well as the hexamer sequence (AAAUAA) required for polyadenylation.

Translation of the transfected EGFP-αCaMKII 3′ UTR reporter mRNA was absolutely dependent on the presence of a minimal poly(A) tail (20 nt), and translational efficiency was progressively enhanced by increasing poly(A) tail lengths (Figure 4A). In order to bypass any minimal requirement for polyadenylation, we then analyzed the neuronal activity-dependent translation of reporter mRNA appended with a 20 nt poly(A) tail. Moderate levels of basal translation of EGFP-αCaMKII-A_{20} mRNA were observed in the presence of spontaneous neuronal activity, but translation was significantly inhibited by pretreatment with tetrodotoxin (TTX) or the ionotropic glutamate receptor antagonists AP5 and DNQX (Figure 4B). Reporter mRNA translation was still more strongly inhibited by pretreatment with the specific MEK

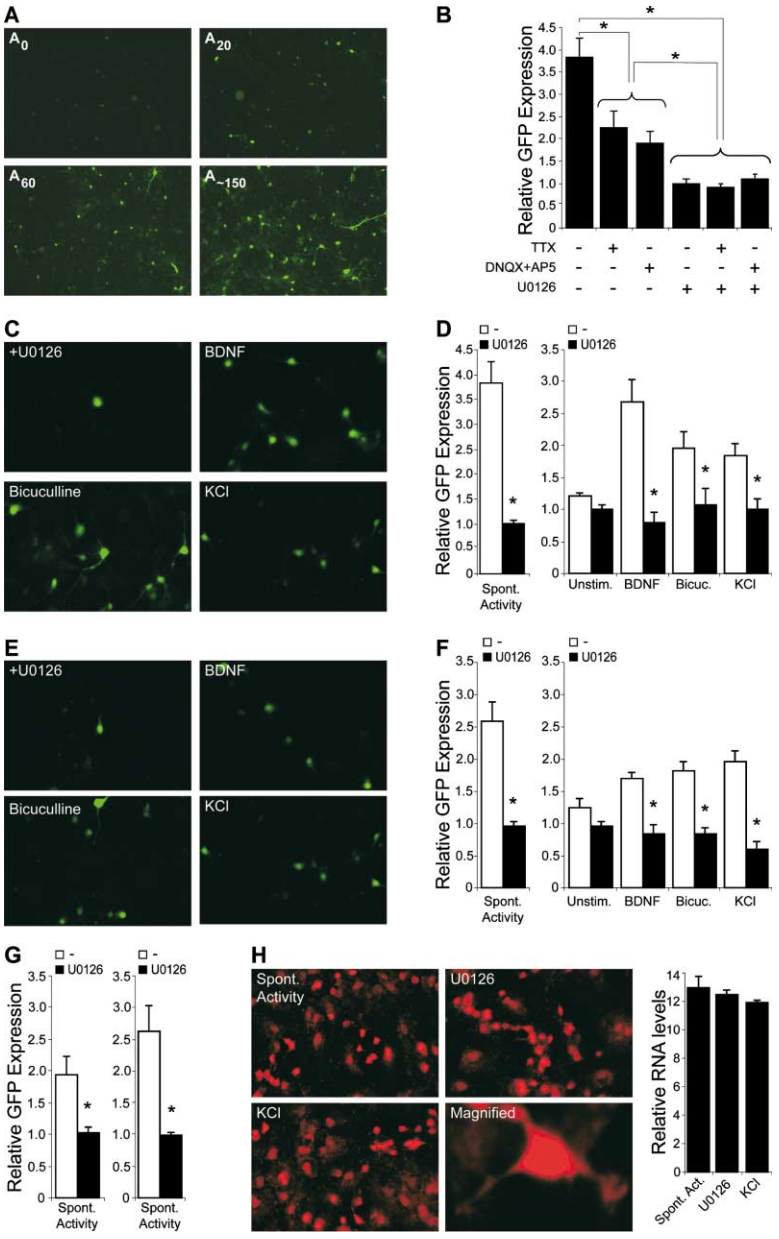

Figure 4. The ERK Signaling Pathway Regulates Neuronal Activity-Dependent Translation of Reporter mRNAs through a Polyadenylation-Independent Mechanism

(A) Reporter mRNA translation is stimulated by increasing poly(A) tail lengths. Representative fluorescent images (10× view) show EGFP expression under conditions of spontaneous neuronal activity as a function of increasing mRNA poly(A) tail length (results with 0, 20, 60, and >150 residues are shown).

(B) Reporter mRNA translation is neuronal activity- and ERK-dependent. The effects of the indicated pharmacologic agents on reporter mRNA translation under conditions of spontaneous activity are shown. Reporter expression levels are normalized to the expression level in the presence of U0126 ($n > 6$ each).

(C–D) Reporter mRNA translation is stimulated in an ERK-dependent manner by multiple forms of neuronal activity. In (C), examples of the stimulation of reporter mRNA translation by neuronal activity are shown (20× view). In (D), reporter expression levels are normalized to the expression level in the presence of U0126 and in the absence of externally added stimulants ($n > 6$ each).

(E–F) ERK-dependent stimulation of reporter mRNA translation does not require the CPEs. In (E), examples of translational stimulation of reporter mRNA bearing mutations in both CPEs are shown. In (F), reporter expression levels are normalized to the expression level in the presence of U0126 and in the absence of externally added stimulants ($n > 6$ each).

(G) ERK-dependent translational stimulation of reporter mRNA does not require the hexamer (AAUAAA) sequence. Left, translational stimulation of reporter mRNA containing mutant hexamer and intact CPE sequences. Right, translational stimulation of reporter mRNA containing mutant hexamer and mutant CPE sequences. Reporter expression levels are normalized to the expression level in the presence of U0126 ($n > 6$ each).

(H) In situ hybridization revealed no differences in neuronal survival or reporter mRNA stability, transfection efficiency, or localization under the indicated conditions. Representative fluorescent images are shown at left. Relative mRNA levels are quantified at right. Statistically significant differences at 95% confidence levels are denoted by asterisks.

inhibitor U0126, which blocks ERK activation (Figure 4B). This effect was specific to the pharmacologic action of U0126 on MEK, since the inactive conformer U0124 had no significant effect (data not shown). Combined pretreatment with U0126 and either TTX or AP5/DNQX produced no additional inhibition (Figure 4B), suggesting that the translational effects of spontaneous neuronal activity may be largely mediated through the ERK signaling pathway. To evaluate the effects of different forms of neuronal activity, we stimulated hippocampal neurons with BDNF, the GABA-A receptor inhibitor bicuculline, and membrane depolarization. In order to reduce background EGFP expression, neurons were incubated with U0126 for 12 hr and then washed just prior to transfection. All three forms of stimulation produced significant increases in EGFP-αCaMKII-A_{20} mRNA translation (Figures 4C–4D). In all cases, concurrent treatment with U0126 significantly attenuated the effects of stimulation.

We next examined the role of the *cis*-acting elements in the α*CaMKII* 3′ UTR in translational induction. Neuronal activity-induced stimulation of reporter mRNA translation and its sensitivity to inhibition by U0126 persisted after mutation of the CPEs (Figures 4E–4F) and/or the hexamer (AAUAAA) sequence (Figure 4G). These observations indicate that cytoplasmic polyadenylation is not essential for neuronal activity-induced ERK-dependent translation, since identical mutations in either of these elements have been shown to prevent cytoplasmic polyadenylation (Mendez and Richter, 2001). Consistent with this interpretation, translation of reporter mRNAs with differing poly(A)-tail lengths exhib-

ited comparable sensitivity to U0126 (R.J.K., A.G., and S.T., unpublished data). Parallel analysis by in situ hybridization revealed no detectable differences in reporter mRNA stability or localization, transfection efficiency, or neuronal survival (Figure 4H), arguing that the observed effects are specific to translation.

ERK Signaling Mediates Induction of Neuron-Wide and Local Protein Synthesis through Regulation of Multiple Translation Initiation Factors

In the above analysis, reporter mRNAs remained responsive to ERK-dependent translational stimulation despite mutation of the CPE and hexamer sequences. This mutant reporter contains no other known *cis*-acting elements in the short 5′ and 3′ UTR segments and hence represents a "generic" mRNA. Thus, ERK signaling may provide a general pathway regulating translation of the majority of neuronal mRNAs. To test this prediction, we conducted metabolic pulse labeling in cultured hippocampal neurons. The presence of actinomycin-D and chloramphenicol precluded any confounding effects of transcription or mitochondrial translation. As shown in Figures 5A–5B, the results were remarkably similar to those obtained with transfected reporter mRNAs. Bulk translation of endogenous transcripts in response to spontaneous neuronal activity, BDNF, and bicuculline was strongly inhibited by U0126 in each case. Similar levels of inhibition were obtained with the structurally distinct MEK inhibitor PD98059 (data not shown). Electrophoretic separation of radiolabeled translation products confirmed that ERK-dependent stimulation was not limited to one or few predominant protein species, but rather extended across the entire range of resolved molecular weights (Figure 5A). The inhibitory effect of rapamycin was comparable to that of U0126, supporting a dual requirement for ERK and mTOR signaling in neuronal activity-dependent translation.

These results indicate that ERK-dependent translational modulation is a general rather than gene-specific phenomenon, suggesting that the relevant target(s) of the ERK pathway may reside in the general translation machinery. Inducible phosphorylation of specific residues in the cap binding factor eIF4E (Ser209), its inhibitor 4E-BP1 (Ser65), and ribosomal protein S6 (Ser235/236) are associated with enhanced rates of translation initiation in mitotic cells (Raught et al., 2000). In parallel with metabolic-labeling studies, we therefore performed Western analysis with antisera specific for the phosphorylated forms of these translation factors. Specific phosphorylation of S6, eIF4E, and 4E-BP1 occurred in response to multiple forms of neuronal activity, and phosphorylation was significantly inhibited by U0126 treatment in each case (Figures 5D–5F). Phosphorylation of all three translation factors under conditions of spontaneous neuronal activity was also significantly inhibited by rapamycin, with rapamycin exerting the stronger effect on S6 phosphorylation, and U0126 exerting relatively stronger effects on eIF4E and 4E-BP1 phosphorylation. Analysis of phospho-ERK levels confirmed that ERK phosphorylation was stimulated by neuronal activity and entirely abolished by U0126 treatment (Figure 5C).

The results described above establish an important role for ERK signaling in neuron-wide translational processes. In view of evidence implicating local protein synthesis in long-term synaptic plasticity and memory, we examined the ERK-dependence of protein synthesis and translation factor phosphorylation in synaptoneurosomes prepared from hippocampal neurons under conditions of spontaneous activity. Translation of endogenous synaptodendritic mRNAs and phosphorylation of ERK, S6, and eIF4E in synaptoneurosomes were all significantly reduced by U0126 treatment (Figures 5G–5K), indicating a similarly important role for the ERK pathway in synaptodendritic protein synthesis.

Impaired Translational Induction during L-LTP and Hippocampal Memory Formation in dnMEK1 Mice

To confirm the relevance of ERK-dependent translational regulation to the phenotypes observed in dnMEK1 mice, we analyzed translational activity in the context of hippocampal L-LTP and memory formation. First, we assessed changes in translational activity occurring in areas CA1 and CA3 of control and mutant hippocampal slices in response to the pattern of repeated tetanization used to induce L-LTP. Given the restricted hippocampal expression pattern of the dnMEK1 transgene, analysis of area CA3 of mutant slices provides an internal control for the specificity of transgene-dependent inhibition in area CA1. Metabolic pulse labeling in the presence of actinomycin-D revealed increased translation in both areas CA1 and CA3 of control slices following tetanization in the CA1 stratum radiatum, relative to slices that received only low-frequency stimulation (Figure 6A). In contrast, while the same tetanic stimulation induced ^{35}S-methionine incorporation in area CA3 of mutant slices, it failed to do so in area CA1. Electrophoretic separation revealed decreased labeling across the entire range of resolved molecular weights in mutant slices (data not shown). Stimulation of ERK phosphorylation following tetanization was also selectively abolished in area CA1 of mutant slices, indicating that the translational blockade in area CA1 of mutant slices correlates with a corresponding blockade of ERK activation (Figure 6B). The stimulation of ERK phosphorylation and translation observed in both areas CA1 and CA3 of control slices presumably reflects the ability of tetanic stimulation in the CA1 stratum radiatum to induce both SC-CA1 and C/A-CA3 LTP through activation of the Schaeffer collateral and commissural/associational projections of CA3 neurons, respectively (Chattarji et al., 1989; Williams and Johnston, 1996).

We next addressed whether neuronal activity-induced ERK-dependent protein synthesis in the context of L-LTP operates via regulation of the translation initiation process. Specific phosphorylation of the translation factors S6 and eIF4E in mutant slices following tetanic stimulation exhibited a similar selective pattern of inhibition; phosphorylation of both factors in mutant slices was stimulated to control levels in area CA3, but was not stimulated in area CA1 (Figures 6C–6D). These observations suggest that ERK signaling regulates the translational events required for long-lasting synaptic plasticity in the adult hippocampus and provide further evidence that a translational defect underlies the L-LTP deficit in dnMEK1 mice.

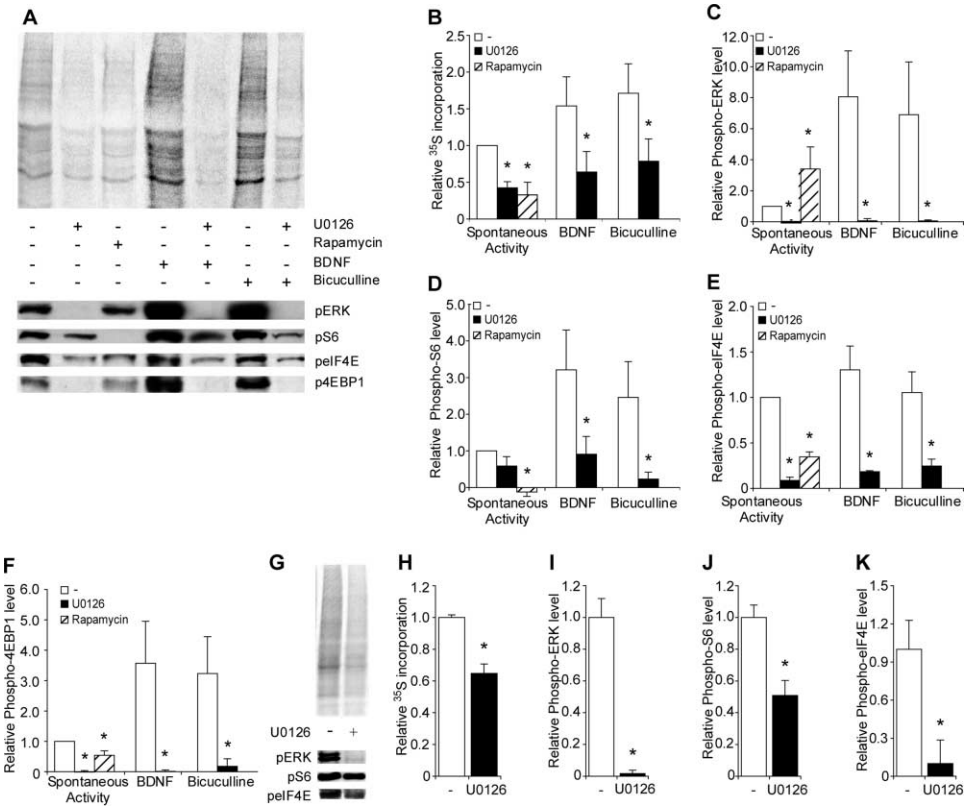

Figure 5. The ERK Signaling Pathway Regulates Neuronal Activity-Dependent Translation by Modulating the Phosphorylation State of Translation Initiation Factors

(A) Stimulation of hippocampal neurons enhances ^{35}S-methionine incorporation and phosphorylation of ERK, S6, eIF4E, and 4E-BP1 in an ERK-dependent manner. Representative autoradiogram shows that synthesis of all detectable protein species changes uniformly upon pharmacological treatment.

(B) Quantification of ^{35}S-methionine incorporation in hippocampal neurons upon pharmacological treatment.

(C–F) Quantification of normalized levels of phosphorylated ERK, S6, eIF4E, and 4E-BP1 in hippocampal neurons upon pharmacological treatment.

(G) Top, representative autoradiogram shows that the synthesis of all detectable protein species in synaptoneurosomes prepared from hippocampal neurons changes uniformly upon pharmacological treatment. Bottom, phosphorylation of ERK, S6 and eIF4E also occurs in an ERK-dependent manner.

(H) Quantification of ^{35}S-methionine incorporation into synaptoneurosomes upon pharmacological treatment.

(I–K) Quantification of normalized levels of phosphorylated ERK, S6 and eIF4E in synaptoneurosomes upon pharmacological treatment.

In (B–F) and (H–K), results are expressed relative to values obtained under conditions of spontaneous activity ($n > 6$ in each case). Statistically significant differences at 95% confidence levels are denoted by asterisks.

We next examined translational activity in the hippocampus of control and dnMEK1 mice during hippocampal memory formation. The results described above indicate that eIF4E and S6 phosphorylation provide a reliable index of translational efficiency. We therefore monitored the phosphorylation of ERK, eIF4E, and S6 in hippocampal homogenates prepared from control and dnMEK1 mice 30 min following either contextual fear conditioning or exposure to the experimental environment without conditioning. Increases in the specific phosphorylation of ERK, S6, and eIF4E were observed as a consequence of fear conditioning in control mice, and these increases were significantly reduced in dnMEK1 mice (Figures 6E–6G). These observations further support the hypothesis that a translational defect contributes to the selective impairment in memory consolidation in dnMEK1 mice.

Discussion

Specific Blockades of Memory Consolidation and L-LTP in dnMEK1 Mice

Employing a conditional genetic approach in mice, we have demonstrated an important and selective role for the ERK signaling pathway in L-LTP and memory consolidation. Previous studies have implicated the ERK signaling pathways in diverse aspects of synaptic function and plasticity (Sweatt, 2001). In our study, basal synaptic transmission, presynaptic function, and E-LTP were all normal, while L-LTP was selectively deficient. Several features of our approach may have contributed to our discernment of a selective role for ERK activation in hippocampal L-LTP. In contrast to the complete blockade of ERK activation in pharmacologic studies, basal levels of hippocampal ERK phosphorylation were pre-

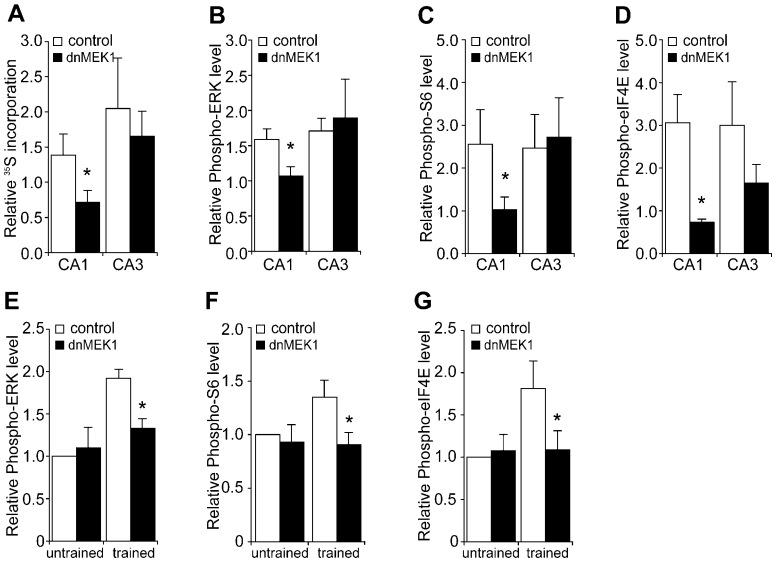

Figure 6. Stimulation of Translational Activity by L-LTP-Inducing Tetanization and Long-Term Memory Formation Is Impaired in dnMEK1 Mice

(A) L-LTP-inducing tetanization stimulates protein synthesis in area CA1 of control but not dnMEK1 hippocampal slices. In contrast, tetanization stimulates similar levels of translation in area CA3 of control and dnMEK1 slices. Levels of ^{35}S-methionine incorporation following L-LTP induction are normalized to the levels in untetanized slices in paired experiments (n = 6 each).

(B–D) L-LTP-inducing tetanization stimulates phosphorylation of ERK, S6, and eIF4E in area CA1 of control but not dnMEK1 hippocampal slices. In contrast, L-LTP stimulates similar levels of phosphorylation of the same proteins in area CA3 of both control and dnMEK1 mice. Normalized levels of the indicated phosphoproteins are expressed relative to the corresponding levels in untetanized slices (n = 6 each).

(E–G) Phosphorylation of ERK, S6, and eIF4E induced by contextual fear conditioning is inhibited in dnMEK1 mice. Normalized levels of the indicated phosphoproteins are expressed relative to the corresponding levels in untrained control animals. Statistically significant differences at 95% confidence levels are denoted by asterisks (n = 6 each).

served in dnMEK1 mice. In addition, the restricted inhibition of ERK activation in hippocampal area CA1 relative to area CA3 may have selectively targeted postsynaptic processes required for L-LTP at CA1 synapses while maintaining normal presynaptic function. The activation of ERK (ERK1/2) was specifically targeted by a conditional genetic strategy, obviating possible nonspecific effects associated with the use of pharmacologic agents or genetic manipulation of upstream signal transduction components with more pleiotropic effects. Our approach also reduces possible developmental or compensatory effects resulting from germline ablation of individual kinase isoforms. For example, recent analysis of ERK1 knockout mice has revealed significant upregulation of ERK2 activity (Mazzucchelli et al., 2002). Different approaches to perturbation of the MEK/ERK system may therefore elicit different patterns of impairment in synaptic and cognitive functions. Our results demonstrate that conditional inhibition of ERK activation can lead to selective impairments in the protein synthesis-dependent events underlying hippocampal L-LTP and long-term memory, with the early phases of LTP and memory remaining intact.

Impaired ERK-Dependent Translational Induction in dnMEK1 Mice

Selective deficits in L-LTP relative to E-LTP and memory impairments similar to those observed in this study have previously been reported with genetic manipulation of PKA (Abel et al., 1997), adenylyl cyclase (Wong et al., 1999), and CaMKIV (Kang et al., 2001). Consistent with prevailing models for memory consolidation, the phenotypes of these mutants were attributed to transcriptional defects. On the basis of studies with cultured cells, a role for ERK-dependent transcription in long-term synaptic plasticity and memory has been proposed, but such a role has not been directly demonstrated (Impey et al., 1999). In our current study, we demonstrated that ERK signaling regulates both neuron-wide and local protein synthesis in response to multiple forms of neuronal activity in hippocampal neurons. We also showed that ERK activation was required for specific phosphorylation of multiple components of the translation machinery in response to neuronal activity, providing a molecular mechanism for the observed dependence of translational induction on ERK activation. Furthermore, we showed that translational induction and translation factor phosphorylation were diminished in the hippocampus of dnMEK1 mice in response to L-LTP-inducing tetanization. We did not investigate whether neuronal activity-induced transcription is impaired in dnMEK1 mice. However, the time course of L-LTP inhibition in dnMEK1 mice is incompatible with the notion that impaired regulation at the transcriptional level is the major cause of the observed L-LTP impairment. On the other hand, impaired translational regulation can account entirely for the L-LTP time course in dnMEK1 mice. It is still possible that ERK-dependent transcriptional activation of a certain set of genes contributes to L-LTP in its later phase (later than 60–90 min following tetanization). Nevertheless, as far as the ERK pathway is concerned, impaired translational regulation could account for the observed L-LTP deficit throughout its entire time course.

In this study, we did not directly investigate whether ERK-dependent translational regulation is essential for long-term memory. However, given the large body of evidence favoring a mechanistic relationship between L-LTP and long-term memory (Frey, 2001), our results implicating ERK-dependent translational regulation in L-LTP suggest that it is also relevant to long-term memory. Consistent with this argument, we identified similar ERK-dependent changes in translation factor phosphorylation occurring during L-LTP induction and long-term memory formation.

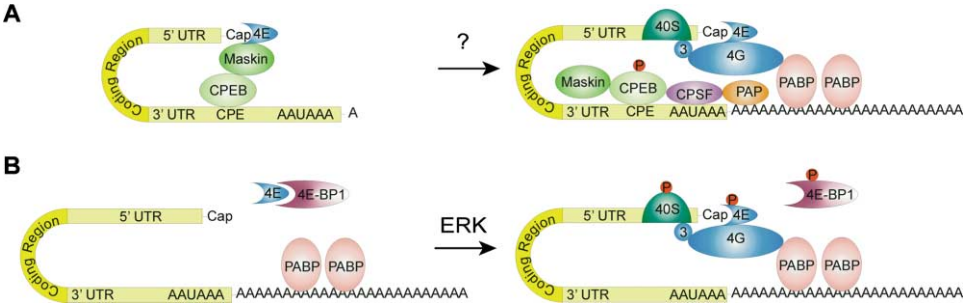

Figure 7. The Molecular Mechanism of Translational Regulation by the ERK Signaling Pathway

The present study addresses two possible mechanisms by which ERK signaling may regulate neuronal activity-dependent translation.

(A) Inducible cytoplasmic polyadenylation has been proposed as a mechanism for stimulation of translational efficiency in response to neuronal activity (Richter and Lorenz, 2002). Cytoplasmic polyadenylation elements (CPE) in the distal 3′ UTRs of specific mRNAs (e.g., α*CaMKII*) are recognized by a specific binding protein, CPEB. CPEB phosphorylation in response to neuronal activity is proposed to result in polyadenylation, displacement of Maskin and poly(A) binding protein (PABP)-mediated recruitment of eIF4G. In our study, neuronal activity-induced translation was strongly ERK-dependent in both the presence and absence of functional CPE (and hexamer) sequences, indicating that translational regulation by the ERK pathway does not require cytoplasmic polyadenylation.

(B) Recognition of the mRNA 5′ cap by eIF4E and subsequent recruitment of the 40S ribosomal subunit by eIF4G are key steps in the initiation of translation. Phosphorylation of eIF4E and its inhibitor, 4E-BP1, regulates the activity and availability of eIF4E for cap recognition and interaction with eIF4G. Increasing poly(A) tail lengths are thought to stimulate translation (see Figure 4A) through an interaction of PABP with eIF4G. Our results demonstrate a general requirement for ERK signaling in neuronal activity-dependent translation. Consistent with these findings, phosphorylation of eIF4E, 4E-BP1, and S6 was stimulated by neuronal activity in a highly ERK-dependent manner. A similar ERK requirement for eIF4E and S6 phosphorylation was observed during hippocampal L-LTP and memory formation. Thus, the ERK pathway plays an important role in neuronal activity-dependent regulation of translation initiation.

Mechanisms of ERK-Dependent Translational Regulation

We found that activity-dependent translation of reporter mRNAs lacking known *cis*-acting regulatory elements remained dependent on ERK activation (Figure 4). Similarly, metabolic labeling studies in cultured neurons, synaptoneurosomes, and hippocampal slices revealed a general dependence of neuronal translation on ERK signaling (Figures 5–6). These findings imply that the translation-enhancing activity of the ERK pathway applies to most or all neuronal mRNA species rather than a restricted subset carrying a particular *cis*-acting element. The target of the ERK signaling pathway thus appears to reside in the general translational machinery (see model, Figure 7). In support of this interpretation, we found that ERK activation was required for inducible phosphorylation of multiple factors that play central roles in the process of translation initiation. Specific phosphorylation of ribosomal protein S6, eIF4E, and 4E-BP1 has been linked mechanistically to increases in translational efficiency in response to a variety of growth-inducing stimuli (Raught et al., 2000). Our observations demonstrate that these phosphorylation events are regulated coordinately with translational induction by the ERK pathway in response to multiple forms of neuronal activity. The relevance of these findings to long-term synaptic plasticity and memory is supported by our demonstration of similar ERK-dependent phosphorylation of S6 and eIF4E phosphorylation in response to tetanic stimulation applied to hippocampal slices and hippocampus-dependent conditioning applied to the intact animal.

Prior work in mitotic cells has identified the ERK-dependent kinase Mnk1 as the major eIF4E kinase, indicating a dominant role for ERK signaling in eIF4E phosphorylation (Wang et al., 1998; Waskiewicz et al., 1997). The complex pattern of inducible 4E-BP1 hyperphosphorylation appears to be mediated primarily by rapamycin-sensitive mTOR-dependent pathways, while some evidence has suggested ERK-dependent modulation of Ser65 phosphorylation (Gingras et al., 2001; Herbert et al., 2002). Studies on the mitogen-induced hyperphosphorylation of S6 have delineated a central role for mTOR-dependent activation of S6 kinase. Our findings demonstrate a major role for the ERK pathway in the neuronal activity-induced phosphorylation of S6, eIF4E, and 4E-BP1, with consistently greater effects on eIF4E relative to S6 across all levels of analysis. Interestingly, we also found the phosphorylation of all three factors to be highly sensitive to rapamycin, with the greatest effect on S6. These observations suggest that the ERK and mTOR pathways cooperate in the coordinate regulation of cap-dependent and 5′ TOP-dependent translation. Hippocampal L-LTP and serotonin-induced LTF in *Aplysia* have been shown to be sensitive to rapamycin, implicating mTOR-dependent translation in these processes (Casadio et al., 1999; Tang et al., 2002). Translational efficiency during the establishment of long-term synaptic plasticity and memory may therefore be determined through the functional interplay of ERK- and mTOR-dependent signaling mechanisms. General translational induction of a broad range of neuronal mRNAs by such activity-dependent mechanisms may provide the protein products required for the input-specific "capture" of long-term synaptic plasticity by "tagged" synapses (Frey and Morris, 1997).

Experimental Procedures

Plasmid Constructions

The conditional transgene vector pCLSL contains a floxed transcriptional and translational "stop" cassette (Lakso et al., 1992) inserted downstream of promoter sequences derived from the chicken

β-actin gene. A dominant-negative MEK1 cDNA bearing a K97M mutation and the SV40 late polyadenylation signal was derived from pMCL-dnMEK1 (gift of N. Ahn) and inserted downstream of the stop cassette in pCLSL to generate pCLSL-dnMEK1. pCMV-EGFP-CKUTR was derived from pEGFP-N1 by deletion of the SV40 polyadenylation signal and insertion of a 160-bp PCR fragment encoding the distal sequences of the α*CaMKII* 3′ UTR (including both CPEs and the hexamer sequence; Wu et al., 1998). The α*CaMKII* 3′ UTR fragment was amplified from a rat brain Marathon cDNA library (Clontech). CPE mutations were introduced using previously described primers (Wu et al., 1998). The hexamer mutation AAGAAA was similarly introduced by PCR.

In Situ Hybridization

Transgene expression was analyzed using a ^{33}P-labeled RNA oligonucleotide specific for the transgene 5′ UTR. In situ hybridization was performed on sagittal cryosections as described (Zeng et al., 2001). Fluorescent in situ hybridization of hippocampal neurons was performed with a digoxigenin-labeled cRNA probe derived from the EGFP coding region. Fluorescence intensities were quantified with ImageJ (NIH). Relative mRNA levels were expressed as the mean fluorescence intensity of randomly selected neurons.

Mouse Behavioral Studies

The Morris water maze and fear conditioning tasks were conducted essentially as described (Tsien et al., 1996; Zeng et al., 2001). The training phase for the hidden platform task of the Morris water maze consisted of two blocks of four 60 s trials per day for a total of five consecutive days. Probe trials (60 s) were administered following the completion of training. The training sessions for contextual and cued fear conditioning consisted of a 3 min exploration period followed by three CS-US pairings separated by 1 min each (foot-shock intensity 0.75 mA, duration 0.5 s; tone 75 db white noise, 30 s duration.). Context tests were performed in the training chamber after retention delays of 60 min and 24 hr. Tone tests were performed in a distinct chamber located in a different room; baseline freezing was monitored (2 min) prior to phasic presentation of the tone (75 db white noise, 3 min duration). Control groups contained equivalent numbers of single transgenic "floxed" and α*CaMKII-Cre* mice. Control and mutant groups consisted of age-matched littermates (12–20 weeks of age) for each analysis.

Electrophysiology

Transverse hippocampal slices were prepared from age-matched littermates (8–16 weeks of age) and maintained in an immersion chamber perfused with oxygenated artificial cerebrospinal fluid (Kang et al., 2001). Extracellular fEPSPs were evoked by stimulation of the Schaeffer collateral pathway afferents and were recorded in the CA1 stratum radiatum. For LTP studies, stimulation was applied at 0.033 Hz using an intensity that produced ~35% of the maximal fEPSP slope. Tetanic stimulation was delivered in 1 s trains at 100 Hz, with 2 trains separated by 30 s used to induce E-LTP, and 4 trains separated by 5 min each used to induce L-LTP. Anisomycin (40 μM) and actinomycin-D (40 μM) were added to the perfusate 30 min prior to tetanization.

Primary Hippocampal Neuronal Culture and Reporter mRNA Transfection

High-density hippocampal pyramidal cell (CA1-CA3) cultures were prepared from P1-P2 rat pups as previously described (Liu and Tsien, 1995). Capped reporter mRNAs were synthesized in vitro using T7 Message Machine (Ambion). Templates were generated by PCR amplification of EGFP-αCaMKII 3′ UTR fragments from pCMV-EGFP-CKUTR using primers encoding an upstream T7 promoter and downstream oligo(dT) stretches of specific lengths. Reporter mRNA generated by enzymatic tailing contained >150 poly(A) residues. Reporter mRNAs were transfected on DIV 8 (TransMessenger reagent, Qiagen). Neurons were pretreated with pharmacological inhibitors (1 μM tetrodotoxin, 100 μM AP5, 10 μM DNQX, 20 μM U0126) for 12 hr prior to transfection. Stimulations (100 ng/mL BDNF for 4 hr; 40 μM bicuculline for 8 min.; 90 mM KCl for 3 min four times spaced by 10 min) were applied immediately following transfection. Coverslips were fixed for analysis 4 hr following transfection. Reporter translation was quantified as the total number of EGFP-positive neurons.

Synaptoneurosomes

Synaptoneurosomes were prepared from cultured hippocampal neurons by sequential passage through PTFE filters (Millipore) of decreasing pore size, as previously described (Scheetz et al., 2000).

Western Analysis

Homogenates were prepared in cold RIPA buffer containing protease inhibitors and phosphatase inhibitors. Hippocampal neurons were homogenized 8 min after stimulation. Hippocampal slices were frozen on dry ice 10 min after tetanization, and the CA1 and CA3 fields were microdissected and homogenized. Western analysis was performed with rabbit polyclonal antisera against dually phosphorylated ERK1/2, phospho-S6 (S235/S236), phospho-eIF4E (S209), and phospho-4E-BP1 (S65) (Cell Signaling). Blots were stripped and reprobed with antisera directed against total ERK1/2, S6, eIF4E, and 4E-BP1 (Cell Signaling). Results were quantified with ImageJ (NIH), calculated as the ratio of phosphorylated species to total ERK, and then normalized to the untreated control condition. Staining of total protein with Ponceau-S confirmed equal loading.

Metabolic Pulse Labeling

Hippocampal neurons (DIV8) were preincubated in sulfur-free MEM for 1 hr prior to stimulation. ^{35}S-methionine (0.2 mCi/mL) was added to the culture medium at the onset of stimulation. Synaptoneurosomes were incubated for 30 min at 37°C in Tyrode solution supplemented with ^{35}S-methionine (0.2 mCi/mL), protease inhibitors and RNase inhibitor. Hippocampal slices were perfused with aCSF supplemented with ^{35}S-methionine (1 mCi/ml) for 30 min after delivery of the last tetanus. Pulse labeling was conducted in the presence of actinomycin-D (Calbiochem, 40 μM) and chloramphenicol (Sigma, 200 μg/ml). Equal amounts of protein from each sample were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Autoradiography was performed on the dried membranes. Staining with Ponceau-S confirmed equal loading.

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References

Abel, T., Nguyen, P.V., Barad, M., Deuel, T.A., Kandel, E.R., and Bourtchouladze, R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell *88*, 615–626.

Antoniadis, E.A., and McDonald, R.J. (2000). Amygdala, hippocampus and discriminative fear conditioning to context. Behav. Brain Res. *108*, 1–19.

Barco, A., Alarcon, J.M., and Kandel, E.R. (2002). Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell *108*, 689–703.

Casadio, A., Martin, K.C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C.H., and Kandel, E.R. (1999). A transient neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell *99*, 221–237.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. Nature *410*, 37–40.

Chattarji, S., Stanton, P.K., and Sejnowski, T.J. (1989). Commissural synapses, but not mossy fiber synapses, in hippocampal field CA3 exhibit associative long-term potentiation and depression. Brain Res. *495*, 145–150.

Davis, H.P., and Squire, L.R. (1984). Protein synthesis and memory: a review. Psychol. Bull *96*, 518–559.

Frankland, P.W., Cestari, V., Filipkowski, R.K., McDonald, R.J., and Silva, A.J. (1998). The dorsal hippocampus is essential for context discrimination but not for contextual conditioning. Behav. Neurosci. *112*, 863–874.

Frey, J.U. (2001). Long-lasting hippocampal plasticity: cellular model for memory consolidation? Results Probl. Cell Differ. *34*, 27–40.

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

Frey, U., Frey, S., Schollmeier, F., and Krug, M. (1996). Influence of actinomycin D, a RNA synthesis inhibitor, on long-term potentiation in rat hippocampal neurons in vivo and in vitro. J. Physiol. *490*, 703–711.

Frey, U., Krug, M., Reymann, K.G., and Matthies, H. (1988). Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA region *in vitro*. Brain Res. *452*, 57–65.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev. *15*, 807–826.

Godsil, B.P., Quinn, J.J., and Fanselow, M.S. (2000). Body temperature as a conditional response measure for pavlovian fear conditioning. Learn. Mem. *7*, 353–356.

Herbert, T.P., Tee, A.R., and Proud, C.G. (2002). The extracellular signal-regulated kinase pathway regulates the phosphorylation of 4E–BP1 at multiple sites. J. Biol. Chem. *277*, 11591–11596.

Huang, Y.Y., and Kandel, E.R. (1994). Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. Learn. Mem. *1*, 74–82.

Impey, S., Obrietan, K., and Storm, D.R. (1999). Making new connections: role of ERK/MAP kinase signaling in neuronal plasticity. Neuron *23*, 11–14.

Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030–1038.

Kang, H., Sun, L.D., Atkins, C.M., Soderling, T.R., Wilson, M.A., and Tonegawa, S. (2001). An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. Cell *106*, 771–783.

Kim, J.J., and Fanselow, M.S. (1992). Modality-specific retrograde amnesia of fear. Science *256*, 675–677.

Lakso, M., Sauer, B., Mosinger, B., Jr., Lee, E.J., Manning, R.W., Yu, S.H., Mulder, K.L., and Westphal, H. (1992). Targeted oncogene activation by site-specific recombination in transgenic mice. Proc. Natl. Acad. Sci. USA *89*, 6232–6236.

Liu, G., and Tsien, R.W. (1995). Synaptic transmission at single visualized hippocampal boutons. Neuropharmacology *34*, 1407–1421.

Mansour, S.J., Matten, W.T., Hermann, A.S., Candia, J.M., Rong, S., Fukasawa, K., Vande Woude, G.F., and Ahn, N.G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. Science *265*, 966–970.

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.

Mathews, M.B., Sonenberg, N., and Hershey, J.W.B. (2000). Origins and principles of translational control. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY, Cold Spring Harbor Press), pp. 1–31.

Mazzucchelli, C., and Brambilla, R. (2000). Ras-related and MAPK signalling in neuronal plasticity and memory formation. Cell. Mol. Life Sci. *57*, 604–611.

Mazzucchelli, C., Vantaggiato, C., Ciamei, A., Fasano, S., Pakhotin, P., Krezel, W., Welzl, H., Wolfer, D.P., Pages, G., Valverde, O., et al. (2002). Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. Neuron *34*, 807–820.

McAllister, A.K., Katz, L.C., and Lo, D.C. (1999). Neurotrophins and synaptic plasticity. Annu. Rev. Neurosci. *22*, 295–318.

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

Mendez, R., and Richter, J.D. (2001). Translational control by CPEB: a means to the end. Nat. Rev. Mol. Cell Biol. *2*, 521–529.

Meyuhas, O. (2000). Synthesis of the translational apparatus is regulated at the translational level. Eur. J. Biochem. *267*, 6321–6330.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P., and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. Nature *297*, 681–683.

Nguyen, P.V., Abel, T., and Kandel, E.R. (1994). Requirement of a critical period of transcription for induction of a late phase of LTP. Science *265*, 1104–1107.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. *22*, 153–183.

Phillips, A.G., and LeDoux, J.E. (1992). Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav. Neurosci. *106*, 274–285.

Raught, B., Gingras, A.-C., and Sonenberg, N. (2000). Regulation of ribosomal recruitment in eukaryotes. In Translational Control of Gene Expression, N. Sonenberg, J.W.B. Hershey, and M.B. Mathews, eds. (Cold Spring Harbor, NY, Cold Spring Harbor Press), pp. 245–293.

Richter, J.D., and Lorenz, L.J. (2002). Selective translation of mRNAs at synapses. Curr. Opin. Neurobiol. *12*, 300–304.

Schafe, G.E., Nadel, N.V., Sullivan, G.M., Harris, A., and LeDoux, J.E. (1999). Memory consolidation for contextual and auditory fear conditioning is dependent on protein synthesis, PKA, and MAP kinase. Learn. Mem. *6*, 97–110.

Scheetz, A.J., Nairn, A.C., and Constantine-Paton, M. (2000). NMDA receptor-mediated control of protein synthesis at developing synapses. Nat. Neurosci. *3*, 211–216.

Soderling, T.R. (2000). CaM-kinases: modulators of synaptic plasticity. Curr. Opin. Neurobiol. *10*, 375–380.

Steward, O., and Schuman, E.M. (2001). Protein synthesis at synaptic sites on dendrites. Annu. Rev. Neurosci. *24*, 299–325.

Sweatt, J.D. (2001). The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J. Neurochem. *76*, 1–10.

Tang, S.J., Reis, G., Kang, H., Gingras, A.C., Sonenberg, N., and Schuman, E.M. (2002). A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc. Natl. Acad. Sci. USA *99*, 467–472.

Treisman, R. (1996). Regulation of transcription by MAP kinase cascades. Curr. Opin. Cell Biol. *8*, 205–215.

Tsien, J.Z., Chen, D.F., Gerber, D., Tom, C., Mercer, E.H., Anderson, D.J., Mayford, M., Kandel, E.R., and Tonegawa, S. (1996). Subregion- and cell type-restricted gene knockout in mouse brain. Cell *87*, 1317–1326.

Tyler, W.J., Alonso, M., Bramham, C.R., and Pozzo-Miller, L.D. (2002). From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learn. Mem. *9*, 224–237.

Wang, X., Flynn, A., Waskiewicz, A.J., Webb, B.L., Vries, R.G., Baines, I.A., Cooper, J.A., and Proud, C.G. (1998). The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J. Biol. Chem. *273*, 9373–9377.

Waskiewicz, A.J., Flynn, A., Proud, C.G., and Cooper, J.A. (1997). Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. *16*, 1909–1920.

West, A.E., Chen, W.G., Dalva, M.B., Dolmetsch, R.E., Kornhauser, J.M., Shaywitz, A.J., Takasu, M.A., Tao, X., and Greenberg, M.E. (2001). Calcium regulation of neuronal gene expression. Proc. Natl. Acad. Sci. USA *98*, 11024–11031.

Williams, S.H., and Johnston, D. (1996). Actions of endogenous opioids on NMDA receptor-independent long-term potentiation in area CA3 of the hippocampus. J. Neurosci. *16*, 3652–3660.

Winder, D.G., Mansuy, I.M., Osman, M., Moallem, T.M., and Kandel, E.R. (1998). Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. Cell *92*, 25–37.

Wong, S.T., Athos, J., Figueroa, X.A., Pineda, V.V., Schaefer, M.L., Chavkin, C.C., Muglia, L.J., and Storm, D.R. (1999). Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron *23*, 787–798.

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 alpha-CaMKII mRNA at synapses. Neuron *21*, 1129–1139.

Wu, G.Y., Deisseroth, K., and Tsien, R.W. (2001). Spaced stimuli stabilize MAPK pathway activation and its effects on dendritic morphology. Nat. Neurosci. *4*, 151–158.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R.J., and Greenberg, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science *270*, 1326–1331.

Zeng, H., Chattarji, S., Barbarosie, M., Rondi-Reig, L., Philpot, B.D., Miyakawa, T., Bear, M.F., and Tonegawa, S. (2001). Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory. Cell *107*, 617–629.