Disruption of Dendritic Translation of CaMKIIα Impairs Stabilization of Synaptic Plasticity and Memory Consolidation

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Summary

Local protein translation in dendrites could be a means for delivering synaptic proteins to their sites of action, perhaps in a spatially regulated fashion that could contribute to plasticity. To directly test the functional role of dendritic translation of calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) in vivo, we mutated the endogenous gene to disrupt the dendritic localization signal in the mRNA. In this mutant mouse, the protein-coding region of CaMKIIα is intact, but mRNA is restricted to the soma. Removal of dendritic mRNA produced a dramatic reduction of CaMKIIα in postsynaptic densities (PSDs), a reduction in late-phase long-term potentiation (LTP), and impairments in spatial memory, associative fear conditioning, and object recognition memory. These results demonstrate that local translation is important for synaptic delivery of the kinase and that local translation contributes to synaptic and behavioral plasticity.

Introduction

Most mammalian neurons receive hundreds to thousands of presynaptic inputs, indicating a remarkably complex ability to integrate incoming patterns of activity. A major tenet of theoretical (Hebb, 1949; McClelland, 1994) and experimental neurobiology (Bliss and Collingridge, 1993) is that coincident pre- and postsynaptic activity can produce long-term changes in synaptic strength of particular synapses independently of other synapses of the same cell, raising the question of how this specificity is achieved at a molecular level. Changes in synaptic strength are initially produced by posttranslational modification of existing synaptic proteins (e.g., phosphorylation, dephosphorylation), while long-lasting synaptic plasticity requires new protein synthesis (Davis and Squire, 1984; Huang et al., 1996). In the face of complex morphological specialization and a large number of synapses, how are new proteins localized to strengthened synapses without altering the function of all the synapses in the cell? One hypothesis is that synaptic plasticity is partially mediated via local production of new proteins only at specific subsets of synapses or individual spines (see Steward and Schuman, 2001, for a review). Ultrastructural studies identified polyribosome complexes and associated membranous cisterns in dendrites, where they appear to be selectively localized beneath synapses (Steward et al., 1996). Although most mRNAs are restricted to the neuronal soma, a limited number of mRNAs are also found in dendrites at appreciable levels, and previous studies indicate that dendrites may be one of the sites of translational changes underlying synaptic plasticity (Steward and Schuman, 2001). For example, a protein synthesis-dependent form of potentiation induced by BDNF at Schaffer-collateral synapses onto hippocampal CA1 pyramidal cells can still be produced when the pyramidal cell dendrites are mechanically isolated from the soma (Kang and Schuman, 1996). Protein synthesis-dependent long-term depression induced via metabotropic glutamate receptors also persists after similar isolation of dendrites (Huber et al., 2000). In a culture preparation of Aplysia sensory and motor neurons, a branch-specific form of protein synthesis-dependent long-term facilitation is induced by repeated local application of 5-HT (Martin et al., 1997). However, none of these studies in model systems have been able to address the functional significance of dendritic protein synthesis on behavior. Further, the use of protein synthesis inhibitors does not resolve which mRNA species are of particular importance for plasticity.

In addition to its abundant localization in neuronal somas, the mRNA for CaMKIIα is highly expressed in dendrites (Burgin et al., 1990), suggesting that dendritic synthesis of CaMKIIα may play a role in plasticity. Studying the endogenous CaMKIIα protein in hippocampal slices, Ouyang et al. (1999) found that tetanic stimulation can rapidly increase CaMKIIα levels in the stratum radiatum of CA1 in a manner sensitive to inhibition of translation. Previous knockout, transgenic overexpression, and inhibitor studies have demonstrated that CaMKIIα is essential for synaptic plasticity and learning (Lisman et al., 2002), but these studies have not addressed the differential role of translation from somatic versus dendritic mRNA. Further, it is not known what proportion of the CaMKIIα in dendrites is synthesized locally and what proportion is synthesized in the neuronal soma and subsequently transported into dendrites. Here we address two questions. First, what is the relative role of dendritic translation in providing CaMKIIα to synapses? Second, what are the functional consequences for synaptic plasticity and learning of preventing dendritic translation of CaMKIIα? To directly address these questions in an intact mammalian system, we used genetic techniques to disrupt dendritic targeting of the endogenous CaMKIIα mRNA in mice. A previous study using fusion genes between portions of CaMKIIα and a GFP/β-gal reporter demonstrated that the 3′ untranslated region (UTR) of CaMKIIα contains the targeting signal for the localization of CaMKIIα message to dendrites.
neviral cell body layers for the reduction in CaMKII localization was readily apparent even at low magnification is critical for supplying the bulk of CaMKII and 3 dendritically localized but which contains an identical enriched in PSDs (Kennedy et al., 1983), and these estimates of CaMKII levels from wild-type mice (Figure 3C). CaMKIIα is highly enriched in PSDs (Kennedy et al., 1983), and these estimates of CaMKIIα levels in unfractionated homogenates reflected a greater reduction in dendritic CaMKIIα. PSD fractions from 3'UTR mutant mice contained only 3.8% of the CaMKIIα mRNA, the β subunit mRNA is restricted to the cell soma (Burgin et al., 1990). There was no alteration in the soma-restricted pattern of hybridization signal of the β subunit mRNA in the 3'UTR mutants (Figures 2G and 2H), indicating there was no compensatory alteration of CaMKIIβ mRNA localization.

Alteration of CaMKIIα Protein Distribution
We examined CaMKIIα protein levels to determine if elimination of dendritic translation would reduce CaMKIIα protein. Indeed, immunohistochemistry demonstrated a large reduction in CaMKIIα immunoreactivity in dendritic layers of the hippocampus (Figures 3A and 3B). However, the dendritic labeling was not eliminated, indicating that in addition to localization of CaMKIIα protein through dendritic synthesis, CaMKIIα translated from mRNA in the cell soma is transported into dendrites. We measured the degree of reduction in CaMKIIα protein with quantitative immunoblots. Consistent with the reduced mRNA, immunoblots of unfractionated homogenates prepared from hippocampus and cortex of 3'UTR mutant mice contained 44% ± 3.8% of the CaMKIIα levels from wild-type mice (Figure 3C). CaMKIIα is highly enriched in PSDs (Kennedy et al., 1983), and these estimates of CaMKIIα levels in unfractionated homogenates reflected a greater reduction in dendritic CaMKIIα. PSD fractions from 3'UTR mutant mice contained only 17.1% ± 2.5% of the amount of CaMKIIα in PSDs from wild-type mice (Figure 3D), indicating that local translation is critical for supplying the bulk of CaMKIIα to postsynaptic sites. There was no significant compensation for the reduction in CaMKIIα by alteration in levels of CaMKIIβ in the mutants (Figures 3C and 3D). The levels 2A and 2B). In the hippocampus of the mutants, CaMKIIα hybridization signal was absent in the stratum radiatum, stratum oriens, and stratum molecule, while intense labeling remained over the CA1–CA3 pyramidal cell body layers and the dentate gyrus granule cell layer (Figures 2D and 2E). Even with prolonged overexposure, the hybridization signal in the neuropil regions did not rise significantly above background levels (data not shown), indicating that the disruption of dendritic mRNA targeting was complete. Semi-quantitative autoradiography (Figure 2F) was used to measure the reduction of CaMKIIα mRNA in the hippocampus of the mutants (48.1% ± 3% of wild-type) and to verify the differential effect on hybridization signals in dendrites (1.4% ± 1% of wild-type CA1 stratum radiatum) versus cell bodies (85.3% ± 2.5% of wild-type CA1 stratum pyramidal). Thus, the mutant has a specific deletion of the dendritically localized pool of CaMKIIα mRNA without a corresponding increase in somatic mRNA. The hybridization signal was also clearly reduced in the superficial neocortical layers, which are enriched in dendrites relative to the deeper layers, and the amygdala showed a modest reduction in hybridization. In addition to the high abundance of the α subunit of CaMKII, low levels of the β subunit are expressed in the forebrain (Miller and Kennedy, 1985). The holoenzyme exists predominantly as dodecomeric molecules, a large portion of which are heteromers comprised of α and β subunits (Bennett et al., 1983; Brocke et al., 1999). In contrast to the somato-dendritic localization of CaMKIIα mRNA, the β subunit mRNA is restricted to the cell soma (Burgin et al., 1990). There was no alteration in the soma-restricted pattern of hybridization signal of the β subunit mRNA in the 3'UTR mutants (Figures 2G and 2H), indicating there was no compensatory alteration of CaMKIIβ mRNA localization.

Results

Gene Targeting to Disrupt Dendritic CaMKIIα mRNA Localization
Targeted mutagenesis was used to disrupt the CaMKIIα 3'UTR (Figure 1), substituting sequences from the 3'UTR of bovine growth hormone mRNA, a message that is not dendritically localized but which contains an identical polyadenylation hexanucleotide as the endogenous CaMKIIα gene. In situ hybridization showed strong CaMKIIα mRNA expression in the forebrain of wild-type and 3'UTR mutant mice but with a marked disruption in dendritic localization in the mutants. The disrupted localization was readily apparent even at low magnification in the hippocampal formation due to its distinct laminar separation between neuronal cell body layers and neuropil regions containing few cell bodies (Figures A and B).
Figure 2. Dendritic Targeting of CaMKIIα mRNA Was Disrupted in the Mutants
(A and B) Film autoradiograms of coronal brain sections from wild-type (A) and 3′ UTR mutant (B) following in situ hybridization with a [35S]-labeled CaMKIIα oligonucleotide probe.
(C) Schematic illustration of the plane of section in (A) and (B). Abbreviations: A, amygdala; CP, caudate-putamen; Ctx, neocortex; DG, dentate gyrus; Hc, hippocampus; Hy, hypothalamus; Th, thalamus.
(D and E) Emulsion-dipped in situ hybridization sections show higher magnification of the region of hippocampus outlined in (C). Arrows indicate the position of the stratum radiatum and stratum moleculare, regions containing dendrites originating from the pyramidal cell somas (PC) and granule cell somas (GC), respectively.
(F) Semiquantitative autoradiography of sections processed in parallel with radioactivity standards confirmed the differential effect of the mutation on mRNA levels in CA1 cell bodies versus dendrites. Values are mean ± SEM, n = 4 mice per genotype. Abbreviations: S. Pyramidale, stratum pyramidale; S. Radiatum, stratum radiatum.
(G and H) Film autoradiograms of in situ hybridization for the α subunit of CaMKII in sections from wild-type (G) and mutant (H).
Scale bars: 1 mm in (B) and (H); 200 μm in (E).

of two other PSD proteins, GluR2 and PSD-95, were also unchanged (Figure 3D).
To verify the immunoblot results anatomically, immunolocalization with electron microscopy was used to qualitatively determine if low levels of CaMKIIα protein remain in postsynaptic spines even in the absence of dendritic mRNA. In CA1 stratum radiatum, there was strong immunoreaction product in the cytoplasm of spines and near or associated with the PSDs of wild-types, while weaker, but significant, labeling with a similar distribution was evident in spines of the 3′ UTR mutants (Figures 3E and 3F). These findings are consistent
with the immunoblots, indicating CaMKIIα protein is localized to dendrites by mechanisms in addition to local translation. In summary, the 3’UTR mutant mice have a deletion of dendritically localized CaMKIIα mRNA and a corresponding deletion of the dendritic CaMKIIα protein that is derived from local dendritic translation.

Alterations in Synaptic Plasticity

Electrophysiological measurements of synaptic plasticity were performed with stimulation of the Schaffer collateral pathway and extracellular field recordings in the CA1 region of hippocampal slices. Synaptic efficacy did not appear to be altered in the 3’UTR mutants; for example, the ratio of the presynaptic fiber volley amplitude to the excitatory postsynaptic potential slope was not altered between slices prepared from wild-type and 3’UTR mutant mice (3.8 ± 0.4 versus 4.4 ± 0.5, p > 0.4; Figure 4A). In addition, paired-pulse facilitation, a presynaptic form of short-term plasticity, was not significantly altered in the mutant (Figure 4B). The effect of the 3’UTR mutation was examined on both an early form of LTP and on stable, late-phase LTP. Early LTP is decremental and depends on posttranslational modification of existing synaptic proteins, while stable late-phase LTP requires new protein synthesis (Huang et al., 1996). A tetanus protocol for early LTP (one burst of 100 Hz stimulation, 1 s in duration) produced potentiation of similar amplitude and a similar decremental time course in slices from wild-type and 3’UTR mutant mice (Figure 4C). A more robust tetanizing protocol (four 100 Hz bursts of 100 Hz stimulation, 1 s in duration) produced potentiation that was significantly greater in wild-type than in 3’UTR mutant mice (Figure 4D).
Figure 4. Hippocampal Field Potential Recordings Show Normal Synaptic Function, Paired-Pulse Facilitation, and Early LTP but Impaired Late-Phase LTP

(A) Scatterplot showing the presynaptic fiber volley (PSFV) and field excitatory postsynaptic potential (fEPSP) of each slice at baseline. The ratio of PSFV to fEPSP slope was not different between slices prepared from wild-type (mean 3.8 ± 0.4, 47 slices from 29 animals) and 3'UTR−/− mice (mean 4.4 ± 0.5, 45 slices from 29 animals; t test, p > 0.4).

(B) Paired-pulse facilitation was not significantly different between slices from wild-type (18 slices from 12 animals) and 3'UTR mutant mice (3'UTR−/−, 17 slices from 14 animals), as indicated by a lack of genotype × interpulse interval interaction (ANOVA F[4,96] = 1.9, p > 0.1). Planned comparisons revealed that the tendency for enhanced paired-pulse facilitation at the individual short intervals was also not significantly different (50 ms, p = 0.25; 100 ms, p = 0.14).

(C) Early LTP was not different between wild-type slices (17 slices from 10 animals) and 3'UTR mutant slices (17 slices from 9 animals; genotype × time interaction, ANOVA F[11,187] = 0.4, p > 0.95).

(D) Late-phase LTP was reduced in 3'UTR mutants versus wild-type controls (9 slices from 9 animals for each genotype), as indicated by a strong genotype × time interaction (ANOVA F[47,752] = 2.4, p < 0.001). Planned comparisons revealed that potentiation was significantly reduced in the mutant slices starting at 2.5 hr after tetanus (p < 0.05). Insets in (A) and (B) show examples of LTP from a representative slice (WT, top; mutant, bottom). These superimposed EPSPs are each averages of five traces recorded just before tetanus and after 45 min ([A], early LTP) or 4 hr ([B], late-phase LTP). Scale bars: 1mV, 5 ms. fEPSP, field excitatory postsynaptic potential. Values in (B)–(D) are expressed as mean ± SEM.

Impaired Spatial Memory

The effect of the 3'UTR mutation on hippocampal-dependent spatial memory was examined in the water maze (Morris et al., 1982) in which mice are released into a pool of cloudy water and must learn the location of a hidden escape platform by learning the spatial relationships between distal visual cues in the room and the location of the platform. Wild-type and mutant littermates exhibited a normal reduction in latency to mount the escape platform during the acquisition phase (Figure 5A). Spatial memory was then assessed in a probe trial with the platform removed by measuring the amount of time spent searching the quadrant of the maze that previously held the platform. The 3'UTR mutants were impaired in the probe trial, failing to search the target quadrant with a significant selectivity relative to the other quadrants (Figure 5B; ANOVA F[3,68] = 7.6, p < 0.001), spending more time searching in the target quadrant that previously held the platform than in each of the other quadrants. Average proximity analysis

Hz bursts, 1 s in duration, 5 min inter-burst interval) produces a long-lasting form of LTP that can be blocked by inhibiting translation with anisomycin (Frey et al., 1988). In slices from wild-type mice, this type of stimulation produced a large and persistent potentiation (107% ± 15% potentiation at 2.5 hr, Figure 4D). A similarly large potentiation was initially produced in slices from the 3'UTR mutants; however, the potentiation was decremental, significantly diverging from that of wild-type slices by 2.5 hr (67% ± 15% potentiation, p < 0.05; Figure 4D).

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Figure 5. Effect of the CaMKII 3’ UTR Mutation on Memory in a 129/BL6 Hybrid Genetic Background

(A) Wild-types (n = 17) and 3’ UTR mutant mice (n = 18) acquired the hidden platform version of the Morris water maze normally with one trial/day training.

(B) In a probe trial with the target platform removed, the 3’ UTR mutants were impaired on spatial memory. For wild-types, ANOVA showed a strong effect of quadrant (ANOVA F3,64 = 7.6, p < 0.001) and post hoc analysis showed that the wild-type spent more time searching in the target quadrant that previously held the platform than in each of the other quadrants (*p < 0.001). In contrast, the 3’ UTR mutants failed to search any quadrant selectively (ANOVA F3,68 = 2.1, p > 0.1). The dashed line indicates the level of a random search strategy.

(C) In the probe trial, the mutants searched closer to the platform location than to the equivalent location in the opposite quadrant (paired t-test, p < 0.01), while the mutants did not (p > 0.1).

(D) Fear Conditioning: freezing during the training session and after 30 min was normal (t tests, p > 0.5) in the 3’ UTR mutants for both contextual (n = 15/genotype) and cued (n = 8/genotype) conditioning. However, 24 hr after training, the 3’ UTR mutants (n = 24) displayed reduced freezing compared to wild-types (n = 25) on both the contextual and cued versions (t tests, p < 0.05). All values are expressed as mean ± SEM.

(Gallagher et al., 1993), a more sensitive measure of search strategy than time in quadrant, also demonstrated that the wild-types, but not the 3’ UTR mutants, searched significantly closer to the exact platform location than to the equivalent location in the opposite quadrant (Figure 5C; wild-type, p < 0.01 3’ UTR mutant, p > 0.1). This probe trial deficit was not due to an alteration in swimming ability or an increase in floating behavior as the wild-types and mutants swam a similar distance during the fixed interval of the probe trial (wild-types, 6.78 ± 2.1 min versus mutants 6.84 ± 3.3 min). Moreover, the mutants exhibited normal acquisition of both the hidden platform (Figure 5A) and visible platform versions (data not shown) of the task, further indicating that the mice have normal motor coordination, swimming ability, motivation to escape the water, and visual acuity to perform the task.

Impaired Associative Fear Conditioning
We also examined the effect of the 3’ UTR mutation on contextual and cued fear conditioning, tasks more amenable than the water maze to experimental separation of short- and long-term phases of memory. Mice were given pairing of a tone to a mild foot-shock, and learning was subsequently evaluated as the amount of time spent freezing in response to presentation of either the same training chamber (contextual memory) or the same tone presented in a different testing apparatus (cued memory). Mutants did not differ from wild-types in the amount of freezing during the baseline period or the tone presentation, and they showed a normal increase in freezing during the immediate postshock period (Figure 5D). When contextual memory was tested 30 min after training, mutants and wild-types displayed similar amounts of freezing; however, 24 hr after training, the 3’ UTR mutants froze less than the wild-types (p < 0.05). In the cued version of testing, both genotypes again froze the same amount at 30 min, but the mutants froze less at 24 hr (Figure 5D, p < 0.05). Thus, the initial memory trace for fear conditioning in the 3’ UTR mutants appeared normal, but its consolidation into a long-term memory store was impaired. Performance in fear conditioning could be affected by differences in shock perception, altered locomotor activity levels, or generalized alterations in fear behavior. However, the current threshold to produce a flinch was the same for wild-types.
Persistence of Behavioral Phenotype in a Homogeneous Genetic Background

As is customary in gene targeting studies, the initial characterization of the 3′UTR mutation was done on an F2 129/BL6 hybrid genetic background. However, behavioral analysis of targeted mutants in a hybrid background is susceptible to potential confounds due to genetic variability and potential allelic differences in linked genes, which may affect behavior (as reviewed in Gerlai, 2001). For these reasons, the behavioral phenotype of the 3′UTR mutation was also analyzed in a C57BL/6 (BL6) genetic background. Heterozygous mutants were backcrossed eight generations into the BL6 strain, producing mice with an average of 99.6% BL6 alleles. These 3′UTR/BL6 mutants were not significantly different from their wild-type littermates during acquisition of the hidden platform version of the Morris water maze (Figure 6A). However, in the probe trial, 3′UTR/BL6 mutants showed no preference for the target quadrant (ANOVA F_{3,32} = 0.9, p > 0.4) or platform location, while the wild-types displayed a highly selective search strategy (F_{3,32} = 10.1, p < 0.0001, Figures 6B and 6C). In fear conditioning, the 3′UTR/BL6 mutants showed no significant differences in freezing during training or at the 30 min retention interval but showed markedly reduced freezing at 24 hr for both the contextual and cued versions of the task (Figure 6D, p < 0.001). The amount of cued conditioning produced for both genotypes was less in the BL6 background compared to the 129/BL6 hybrid background (Figure 5D versus 6D), a discrepancy that may be related to the auditory deficits in BL6 mice (Willott, 1986). Multiple tone-shock pairings (three repetitions with a 1 min inter-shock interval) produced greater freezing in the BL6 wild-types; however, freezing in their mutant littermates was still markedly impared by comparison (wild-type, 64.7% ± 8%, versus mutants, 32.0% ± 10% freezing, p < 0.01). Again, these behavioral abnormalities could not be explained by motor or sensory alterations because the mutants displayed normal swimming ability and visible platform learning in the water maze, normal locomotor activity and thigmotaxis in the open field, and unaltered nociception thresholds (data not shown).

In addition to replicating the water maze and fear conditioning phenotypes in the 3′UTR/BL6 mice, we examined the temporal gradient of the memory deficit in object recognition memory, a nonspatial, hippocampal-dependent task (Clark et al., 2000). The object recognition test takes advantage of the innate curiosity of rodents to explore novel objects. In the training session, mice were exposed to an object and allowed to explore it; after a variable retention interval, the mice were exposed to the same object again, this time together with a novel object. The relative preference in exploration time for the novel object over the familiar object is taken as an indicator of memory for the familiar object. The 3′UTR/BL6 mutants displayed normal performance at 1 hr; however at 24 hr, they had impaired object recognition memory, spending equal time exploring the novel object and the object to which they were previously exposed (Figure 6E, p < 0.05).

In general, the memory deficits appeared to be more severe in the homogeneous BL6 genetic background compared to the original 129/BL6 hybrid background. In the water maze, the 3′UTR/BL6 mutants showed no tendency to search the target quadrant (Figure 6B), while the 129/BL6 mutants had a statistically insignificant trend to do so (Figure 5B). The 24 hr fear conditioning deficit also appeared to be greater in the 3′UTR/BL6 mutant (Figure 5D) than in the 129/BL6 hybrid background (Figure 5D). Preliminary results indicate that the phenotype severity was also different in the two genetic backgrounds following more intensive training protocols (data not shown). 3′UTR/BL6 mutants were impaired on the probe trial even following intensive water maze training (six trials per day with a 30–40 min inter-trial interval), while this type of training produced normal learning in the mutants in the hybrid background. Intensive training on fear conditioning (three tone-shock pairings with a stronger shock, 0.75 mA) produced impaired cued conditioning in the 3′UTR/BL6 mutants but normal conditioning in the mutants in the hybrid background.

Discussion

Gene Targeting and Disrupted mRNA Distribution

The addition of the 3′UTR of CaMKIIα to a chimeric transgene is sufficient to confer a dendritic mRNA localization pattern to a reporter mRNA (Mayford et al., 1996). Disruption of this portion of the endogenous mRNA sequence in the current study confirms it is necessary for targeting of the endogenous CaMKIIα mRNA in vivo. The question remains whether a discrete targeting element exists within the CaMKIIα 3′UTR, or whether targeting is mediated by the tertiary structure of a large portion of the 3′UTR or by the contribution of multiple dispersed elements in the 3′UTR. Two recent cell culture studies (Mori et al., 2000; Blichenberg et al., 2001) identified differing regions in the 3′UTR that can produce dendritic localization of a reporter mRNA in 40%–60% of transfected hippocampal neurons. Mori et al. (2000) reported that a sequence within the first 94 bp of the 3′UTR could produce such localization; however, this portion of the CaMKIIα 3′UTR was left intact in the present study, indicating that this region alone is not sufficient to produce dendritic targeting in the intact brain. Blichenberg et al. (2001) found targeting was optimally produced with a 1.2 kb portion of the 3′UTR that was removed in (0.038 ± 0.004 mA) and 3′UTR mutants (0.037 ± 0.003 mA) as was the threshold to produce a run/jump response (0.120 ± 0.008 mA in wild-types versus 0.124 ± 0.012 mA in mutants, p > 0.8). Measurement of locomotor activity in an open field also showed no difference in distance traveled (wild-types 34.2 ± 1.4 min versus mutants 35.6 ± 1.4 min, p > 0.3). Thigmotaxis, an innate fear-related behavior, is the natural tendency of mice to stay near the perimeter of an open field, avoiding the center area. Percentage of time spent in the center of the field was also unaltered in the mutants (wild-types, 8.1% ± 1.3%, versus mutants, 6.4% ± 0.7%, p > 0.3). These findings, and more significantly, the normal freezing response immediately after the shock and at 30 min, indicate that the deficits in performance at 24 hr were not due to nonspecific motor or sensory alterations that might disrupt performance.
Memory Impairments in the 3'UTR Mutants in a BL6 Genetic Background

(A) Wild-types (n = 12) and 3'UTR mutant mice (n = 9) were not significantly different in the acquisition of the water maze. ANOVA showed an effect of training day (F_{13, 247} = 2.8, p < 0.001), no effect of genotype (F_{1,19} = 3.1, p = 0.1), and no genotype × training day interaction (F_{13, 247} = 0.9, p = 0.5).

(B) In the probe trial, wild-types showed a highly selective search pattern in the quadrant that previously held the platform (ANOVA F_{3,44} = 10.1, p < 0.0001), while the mutants did not (ANOVA F_{3,32} = 0.9, p > 0.4). Post hoc analysis confirmed that the wild-types spent more time searching in the target quadrant than in each of the other quadrants (*p < 0.01).

(C) In the probe trial, the wild-types searched closer to the platform location than to the equivalent location in the opposite quadrant (paired t test, **p < 0.001), while the mutants showed no search selectivity between the two locations (p > 0.3).

(D) In fear conditioning, freezing during the training session was not significantly different between wild-types and mutants (p > 0.2). For both context and cued testing, freezing was normal 30 min following training (p > 0.5, wild-types, n = 16; mutants, n = 11). However, after a 24 hr retention interval, freezing in both versions was significantly reduced (**p < 0.001) in mutants (n = 16) versus wild-types (n = 18).

(E) In the object recognition task, mutants (n = 14) were not significantly different from wild-types (n = 17) at 1 hr (p = 0.4) but showed reduced memory for the familiar object at 24 hr (*p < 0.05). The dashed line indicates the level of equal exploration of both objects. All values are expressed as mean ± SEM.

the deletion made in the present study. Due to the difficulty of extrapolating from culture to the in vivo situation, we initially chose to delete a large portion of the 3'UTR to be sure of abolishing dendritic targeting.

While it is clear that all translation from the dendritic CaMKIIα mRNA pool is eliminated in our mutants, we cannot exclude the possibility that removal of other potential translational regulatory elements in the 3'UTR alters expression of the somatic message and contributes to the observed phenotype. For example, it has been reported that two cytoplasmic polyadenylation elements (CPEs) in the 3'UTR regulate CaMKIIα translation of a reporter gene in cultured embryonic neurons (Wells et al., 2001), and these elements were disrupted in the 3'UTR mutant mice. The role of CPEs is particularly well characterized in oocyte maturation and early embryogenesis, where they suppress polyadenylation and thus translation of several dormant mRNAs (Mendez and Richter, 2001). Disruption of these elements in the CaMKIIα 3'UTR mutant mice might thus be expected to alter translation; however, we found that the adult steady-state ratio of protein to RNA is not different in the mutants compared to wild-types (44% ± 3% of wild-type protein versus 48% ± 3% of wild-type RNA; Figures 2F and 3C), suggesting that these elements may not function in the adult hippocampus as they do in oocytes or cultured embryonic neurons.

Disruption of Protein Localization

Elimination of CaMKIIα mRNA from dendrites led to a large reduction in CaMKIIα protein in PSDs. This demonstrates that one function of dendritic mRNA transport is to deliver the cognate protein to one of its sites of action. Whether the local translation is constitutive or dependent on neuronal activity is not addressed by the current results. However, previous studies using synaptosomes, cultured neurons, or hippocampal slices indicate that translation of dendritic CaMKIIα mRNA is inducible. For example, Wu et al. (1998) and Wells et al. (2001) demonstrated an increase in CaMKIIα protein in...
synaptosomes prepared from visual cortex when dark-reared rat pups were transferred to light. This increase could be blocked with inhibitors of translation, NMDA receptor activation, or polyadenylation. Similarly, NMDA stimulation rapidly increased CaMKII levels in synaptosomes prepared from superior colliculi of rat pups (Scheetz et al., 2000). Aakalu et al. (2001) transfected cultured neurons with a reporter construct containing the 5' UTR and 3' UTR of CaMKII to confer translational regulation and dendritic localization. They demonstrated that BDNF increased translation of the reporter at hotspots that were stable over time and colocalized with ribosomal and synapptic markers. Studying the endogenous CaMKIIα protein in hippocampal slices, Ouyang et al. (1999) found that tetanic stimulation can rapidly increase CaMKIIα levels in the stratum radiatum of CA1 in a manner sensitive to inhibition of translation. These findings, together with the current results, suggest that the bulk of CaMKIIα present in the PSDs arises from activity-dependent translation from the dendritic mRNA pool.

In the absence of dendritic translation in the 3' UTR mutant mice, a minor fraction of PSD CaMKIIα remains, and these α subunits may be localized through transport from the soma as part of a preassembled holoenzyme. It has been proposed that CaMKII holoenzymes are assembled shortly after translation of their subunits and that the β subunit mediates association of the holoenzyme with the actin cytoskeleton and the resultant transport to synapses (Shen et al., 1998). Since the mRNA encoding the β subunit is located only in the soma (Burgin et al., 1990), it seems likely that some CaMKIIα translated in the soma is transported into dendrites as part of the preassembled holoenzyme.

Stabilization of Synaptic Plasticity Is Impaired in the 3'UTR Mutants

A key question in LTP is whether postsynaptic CaMKII is required for LTP induction. Postsynaptic application of peptide inhibitors of CaMKII block the induction of LTP (Chen et al., 2001; Malinow et al., 1989; Otmakhov et al., 1997); however, these inhibitors may not be specific for CaMKII, also inhibiting CaM kinases I, IV, and protein kinase C (Hvalby et al., 1994). In a null mutant with complete elimination of CaMKIIα, the impairment in LTP was either marked (Silva et al., 1992b) or moderate (Hinds et al., 1998), but LTP was not eliminated, indicating that other CaMK subunits and signaling pathways contribute to the formation of LTP. Unlike the inhibitor studies, experiments with the null mutant cannot distinguish a pre- from a postsynaptic site of action. In fact, the null mutants also showed a reduction in paired-pulse facilitation (Chapman et al., 1995), a short-lasting, presynaptically mediated form of plasticity that was not reduced in the 3'UTR mutants. In the current study, mutants with a large reduction in CaMKIIα in the PSD show normal LTP induction and maintenance up to 1 hr posttetanus. This indicates that the absolute levels of CaMKIIα in the PSD are not limiting for the induction of LTP.

While reduced levels of dendritic CaMKIIα may be sufficient for normal early LTP, our results suggest that the maintenance of stable late-phase LTP is at least partially dependent on CaMKIIα arising from local dendritic translation. What might be the mechanism for the selective impairment of late-phase LTP? During the induction of LTP, transient calcium entry through NMDA channels stimulates Thr286 autophosphorylation and activation of CaMKIIα, resulting in its translocation to PSDs (as reviewed in Lisman et al., 2002). Following induction, two processes oppose the continued activation of CaMKII: protein phosphatase-mediated dephosphorylation and degradation of activated holoenzyme. Even after calcium returns to basal levels, Lisman and Zhabotinsky (2001) propose that the CaMKII in the PSD can remain fully autophosphorylated due to saturation of its opposing phosphatase, protein phosphatase 1 (PP1). As existing proteins turn over, newly synthesized holoenzymes are inserted into the PSD, and they too can become activated at resting calcium if PP1 remains saturated. In the mutant, the calcium entry through NMDA channels during LTP induction should catalyze the maximal autophosphorylation of all the available holoenzyme, resulting in translocation to the PSD and phosphorylation of the same substrates as in wild-type (Figure 7). These levels of activated enzyme are apparently sufficient to induce and maintain early LTP. However, dephosphorylation-dependent decay may be more rapid than in wild-types because the CaMKII levels in the mutants would not be sufficient to saturate the phosphatase. Degradation would also have a greater effect because it would not be counteracted by dendritic translation to rapidly deliver kinase to the potentiated synapses (Figure 7). Consistent with this interpretation, early LTP is relatively insensitive to reduction in CaMKII activity by application of CaMKII inhibitors applied after induction of LTP (Chen et al., 2001; Malinow et al., 1989; Otmakhov et al., 1997); however, similar treatments do block maintenance of late-phase LTP (Feng, 1995). Interestingly the decay in LTP observed in 3'UTR mutant mice also parallels the time course of LTP decay reported with inhibitors of protein synthesis (Frey et al., 1988; Huang et al., 1996). This suggests that one component of this protein synthesis-dependent effect is a requirement for local CaMKIIα synthesis to maintain elevated levels of the kinase at the activated synapses. It has also been proposed that CaMKII can maintain potentiation via a structural role in which autophosphorylated CaMKII binds tightly to the NMDA receptor and acts as a part of a molecular scaffold, which provides additional anchoring sites for AMPA receptors (Lisman and Zhabotinsky, 2001). If local dendritic translation was the source of the CaMKIIα involved in this scaffolding, perhaps disruption of the formation of these new anchoring sites could account for the deficit in late-phase LTP in the 3'UTR mutants.

Memory Impairments

Null mutations of CaMKIIα produce severe behavioral abnormalities. Homozygous CaMKIIα nulls perform poorly in the water maze and on fear conditioning even with robust training protocols (Chen et al., 1994; Silva et al., 1992a). Studies of the heterozygous nulls have shown memory at 24 hr can be either impaired (Chen et al., 1994; Silva et al., 1996) or relatively normal (Frankland et al., 2001), with the differing results apparently depending upon the intensity of training and the
Under normal conditions, CaMKII can be supplied to the PSD via transport both from the neuronal soma and through local synthesis. At resting calcium levels, the amount of activated CaMKII is determined by the balance of activity between intersubunit autophosphorylation and phosphatase activity. During induction of LTP, calcium flows in through the NMDA receptor, CaMKII is activated by binding calcium/calmodulin, and an autophosphorylation reaction propagates between adjacent subunits of the holoenzyme. At least two potentially interactive mechanisms can convert this initial activation to a stable change in wild-type mice. Lisman and Zhabotinsky (2001) propose that as activated CaMKII translocates to the PSD microenvironment, the PSD phosphatase, PP1, becomes saturated, and the kinase autophosphorylation reaction outpaces the dephosphorylation reaction, even when calcium returns to resting levels. Additionally, Ouyang et al. (1999) demonstrated that LTP-inducing stimuli produce a rapid upregulation of local dendritic synthesis of CaMKII. Such an increase in locally available enzyme could provide subunits for assembly of new holoenzymes and provide replacement enzyme for degradation of previously activated holoenzymes. The autophosphorylation reaction could propagate in the newly synthesized holoenzyme even at resting calcium because of the saturation of PP1. In the 3'UTR mutant, the CaMKIItranslated and assembled in the soma will still be transported into spines, but the total pool of available CaMKII will be less than in wild-type. Following LTP induction, the existing CaMKII will be activated and translocate to the PSD. This autophosphorylated enzyme will phosphorylate the same substrates as in wild-type (including AMPA receptors); however, the activated state will decay more quickly than in wild-type because PP1 will not be saturated to the same degree and because no additional enzyme can be locally synthesized for immediate delivery to the PSD.

Figure 7. Proposed Mechanism for Transient Plasticity in the 3’UTR Mutants

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genetic background used. Similarly, in our mutant, we found differences in the severity of the behavioral phenotype, depending on the intensity of the training protocol and on the genetic background. One clear behavioral difference between the 3’UTR mutants and the CaMKIIα null homozygous and heterozygous mice is that the 3’UTR mutants do not display abnormal aggression or decreased fear behaviors in the open field (Chen et al., 1994). This suggests that at least some of the behavioral deficits seen in the null mutant mice are due to disruption of CaMKIIα signaling outside of the PSD. For example, Silva and colleagues (Chapman et al., 1995; Silva et al., 1996), demonstrated a correlation between the alterations in short-term, presynaptically mediated plasticity
in null mutant heterozygous mice and their memory impairments.

The 3' UTR mutants had impaired memory in three different hippocampal-dependent tasks: the water maze spatial task, object recognition, and contextual fear conditioning (Clark et al., 2000; Morris et al., 1982; Phillips and Le Doux, 1992). Cued fear conditioning, an amygdala-dependent task (Phillips and Le Doux, 1992), was also impaired. These behavioral deficits are anatomically consistent with the high levels of CaMkIIα expressed in both hippocampus and amygdala and with the altered mRNA localization in the mutant. In contrast to their impaired memory at 24 hr retention intervals, the mutants were normal on a variety of sensory and motor measures. These results and the normal memory at short retention intervals indicate that the deficits at longer intervals were not due to nonspecific motor or sensory alterations that might disrupt performance. Further, because the water maze, fear conditioning, and object recognition have different motivational, perceptual, and motor requirements, the demonstration of memory deficits on these tasks is indicative of cognitive alterations rather than performance factors.

An important finding of the present study is that disrupting dendritic localization and translation of CaMkIIα mRNA disrupted long-term memory without disrupting short-term memory formation. Based on administration of protein synthesis inhibitors either systemically or to specific brain regions, it is well established that new protein synthesis is required for the consolidation of short-term into long-term memory in diverse species and for a variety of learning tasks (Davis and Squire, 1984). The present results suggest that synthesis of CaMkIIα in dendrites may be a component of the translational requirement for memory consolidation. Locally synthesized CaMkIIα may mediate facilitated transmission by regulatory phosphorylation of AMPA receptors (Barria et al., 1997) or by facilitating the insertion of additional AMPA receptors into the synapse (Hayashi et al., 2000). Additionally, CaMkIIα has been implicated in stabilization of dendritic arbors and regulating synapse shape and density (Koh et al., 1999; Rong and Kaplan, 1999; Wu and Cline, 1998). These long-lasting modifications may require ongoing dendritic translation of CaMkIIα to contribute to structural alterations that have been shown to occur with plasticity (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Martin et al., 1997; Toni et al., 1999).

The temporal pattern of the deficits in fear conditioning and object recognition memory parallel the findings with LTP in which an early phase of LTP was intact, but late-phase LTP was diminished, indicating a similar susceptibility of LTP maintenance and memory consolidation to the elimination of CaMkIIα mRNA from the dendrite. The current results do not distinguish whether this susceptibility was produced by elimination of translation at resting activity levels or by blocking increases in dendritic translation following tetanic stimulation or neuronal activity during a learning trial. However, these findings are consistent with a model of synaptic and behavioral plasticity in which synaptic activity triggers local dendritic translation of specific proteins, and the newly accumulating proteins act to stabilize and maintain long-term plastic changes.

### Experimental Procedures

#### Gene Targeting

A 17 kb genomic clone containing the 3' UTR of CaMkIIα was isolated and mapped. The targeting vector included a segment from the 3' UTR/polyadenylation signal from bGH (512 bp Xba/Xho fragment of pRC/CMV, Invitrogen) and a pk-neo gene inserted at a blunted EcoRI site 97 bp downstream of the CaMkIIα stop codon and flanked with 1.7 and 2.8 kb regions of genomic homology. The targeting vector was electroporated into RI embryonic stem cells (derived from 129/Sv × 129/SvJ mice) propagated under genetic selection. Homologous recombination was examined by Southern blotting. Chimeras were generated by injection of a properly recombined clone into blastocysts from BL6 mice and were bred to BL6 females to produce F1 heterozygotes. Except as otherwise noted, mice used in this study were generated from heterozygote crossings to produce F2 mice homozygous for the mutation as well as wild-type littermate controls; these mice have a hybrid 129/BL6 genetic background. Behavioral studies were replicated in a nearly pure genetic background by backcrossing heterozygotes for eight generations into BL6.

#### mRNA and Protein

For Northern blots, 5 μg of hippocampal tissue was blotted on nylon and hybridized with a [32P]-labeled oligonucleotide specific to a coding region of either CaMkIIα or CaMkIIβ. For quantitative analysis (Figure 2F), four to six sections/mouse from each of four mice/genotype were processed together. Each slide contained sections from mice of both genotypes. Sections were exposed on the same film with [35S]-labeled microscale standards (Amersham) containing known amounts of radioactivity, and the resulting autoradiograms were digitized with a high-resolution flatbed scanner. Images were analyzed with Scion Image, and polynomial calibration curves were generated from the standards for the relationship between nanocurie per gram of tissue and relative optical density. Multiple determinations were made in stratum pyramidale and stratum radiatum of each section, using square sample-field templates kept at a constant size between sections. Mean hippocampal measurements were made by outlining the entire hippocampus in each section. Slides were also processed for emulsion autoradiography to produce higher resolution images (Figures 2D and 2E).

For immunohistochemistry, mice were transcardially perfused with 4% paraformaldehyde/0.1% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2). For confocal microscopy, 50 μm vibratome sections from four mice of each genotype were reacted with an anti-CaMkIIα monoclonal antibody (6G9, Chemicon) and a FITC-conjugated secondary antibody. Images were obtained from the center plane of each section with constant settings between sections. For ultrastructural localization, 6G9-labeled sections were incubated with biotinylated secondary antibody and reacted with streptavidin-horseradish peroxidase and diaminobenzidine (10 min). Three blocks per animal were used from each of three mutants and two wild-types. Five ultrathin sections (80 nm) were cut from each block and comparisons were made of sections from similar depth of tissue and processed in parallel. Each section contained numerous synapses in a variety of different planes of section; representative images are shown.

Immunoblot samples were prepared by homogenizing hippocampi and cortex together from individual mice. Synaptosomes were isolated from these homogenates with discontinuous sucrose density gradients followed by two extractions with Triton X-100 to yield the PSD fraction (Cho et al., 1992). Each sample was analyzed in duplicate on each of two separate SDS-PAGE gels. Five dilutions of a control sample were run on each gel for preparation of standard curves. Membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibody and an enhanced chemiluminescence substrate (Amersham), scanned with a Molecular Dynamics Typhoon, and analyzed with
ImageQuant software. Amounts of CaMKIIα, III, and β actin present were extrapolated from the standard curves and CaMKIIα and III levels were normalized to β actin levels as a loading control. Blots were probed with antibodies recognizing both CaMKIIα and III (H-300, Santa Cruz Biotechnology), CaMKIIα only (6G9), CaMKIIIII only (CBI-1, Zymed), β actin (AC15, Sigma), PSD-95 (clone 16, BD Transduction), or GluR2 (#1768, Chemicon). Similar results were obtained analyzing CaMKIIIII subunits with either H-300 (n = 6–9, data not shown), or CBI-1 (n = 3, data not shown).

Slic Electrophysiology

Transverse sections (400 μm) were incubated in an interface chamber at 28°C in oxygenated ACSF composed of 124 mM NaCl, 4.4 mM KCl, 26 mM NaHCO3, 1.3 mM MgSO4, 10 mM glucose, and 2.5 mM CaCl2. Extracellular field potentials were recorded in stratum radiatum of the hippocampal CA1 region with ACSF-filled glass pipets in response to stimulation of the Schaffer collaterals with bipolar tungsten wire electrodes. Stimulus intensity was set to a level that elicited an EPSP amplitude that was 35%–40% of maximum; pulse width was 50 μs. Stimulation was once every 60 s. Slices were prepared from mice that were 2–4 months old with the experimenter blind to the genotype. When more than one slice was used per mouse, the data were averaged between the slices; thus the n represents the number of mice, not the number of slices. To compare LTP between genotypes, responses were averaged for each 5 min interval after tetanus and analyzed by two-way ANOVA with time as a repeated measure.

Behavioral Analysis

Mice used for behavior were 2.5–5.5 months old, and the experimenter was blind to the genotype of the subjects. The water maze tank (1.2 min in diameter) was filled with water made opaque by the addition of tempura paint and maintained at 24°C ± 0.5°C. The hidden escape platform was submerged 1 cm below the surface of the water and kept in a constant location for each mouse, while the release position was varied pseudorandomly. Mice had one trial per day for 14 days with a maximum trial length of 90 s, after which time mice that had not yet found the platform were led to it. At the end of each trial, mice were left on the platform for 30 s before being returned to the home cage. The probe trial was on day 15, when the search pattern of the mouse was recorded for 30 s in the absence of the escape platform. Data was acquired and analyzed with digital video tracking and San Diego Instruments Polytrack or Actimetrics WaterMaze software.

The fear conditioning training session consisted of a 3 min baseline equilibration to the training chamber and one foot shock/tone pairing (20 s tone with a 1 s 0.5 mA foot shock during the final 1 s of tone) followed by a 30 s postshock rest period in the chamber. After the specified intervals, contextual memory was measured as freezing in the training chamber for 3 min. For cued memory, freezing was measured in different chambers in response to three tones of 20 s each, separated by 1 min intervals. Freezing was scored with automated detection of either infrared beam breaks (San Diego Instruments) or pixel differences (FreezeFrame, Actimetrics) with similar results.

For object recognition, mice were given a 1 hr exposure in their home cage to the first object in an object pair. Following a 1 hr or 24 hr retention interval, mice were reexposed to this familiar object along with a novel object, and the time spent exploring each object was recorded for 2 min. Objects were thoroughly washed between presentations to remove odor cues. Exploration scoring included orientation toward the object with the mouse’s nose within 1–2 cm of the object and touching or chewing the object, but did not include standing or sitting on the object, propping up on the object to look out of the cage, or incidental touching of the object while digging through the bedding. Objects were plastic and were matched approximately for size, weight, and innate interest level based on preliminary tests with control mice. For each retention interval, the training and testing were done twice, using different pairs of objects, and the scores were averaged.

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