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Autophosphorylated CaMKIIα Acts as a Scaffold to Recruit Proteasomes to Dendritic Spines

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SUMMARY

The molecular mechanisms regulating the ubiquitin proteasome system (UPS) at synapses are poorly understood. We report that CaMKIIα—an abundant postsynaptic protein kinase—mediates the activity-dependent recruitment of proteasomes to dendritic spines in hippocampal neurons. CaMKIIα is biochemically associated with proteasomes in the brain. CaMKIIα translocation to synapses is required for activity-induced proteasome accumulation in spines, and is sufficient to redistribute proteasomes to postsynaptic sites. CaMKIIα autophosphorylation enhances its binding to proteasomes and promotes proteasome recruitment to spines. In addition to this structural role, CaMKIIα stimulates proteasome activity by phosphorylating proteasome subunit Rpt6 on Serine 120. However, CaMKIIα translocation, but not its kinase activity, is required for activity-dependent degradation of polyubiquitinated proteins in spines. Our findings reveal a scaffolding role of postsynaptic CaMKIIα in activity-dependent proteasome redistribution, which is commensurate with the great abundance of CaMKIIα in synapses.

INTRODUCTION

Synapses between neurons can change functionally and structurally in response to activity – a process known as synaptic plasticity. Calcium/calmodulin-dependent protein kinase II (CaMKII) is a multi-functional enzyme of central importance for synaptic plasticity (Lisman et al., 2002), and the most abundant protein in the postsynaptic density (PSD) (Cheng et al., 2006). CaMKII becomes autophosphorylated on Thr-286 and constitutively active following synaptic excitation and it maintains its enzymatic activity even after calcium levels have returned to baseline levels, thus potentially functioning as a molecular memory trace in stimulated synapses. Activated CaMKII phosphorylates AMPA receptors and other PSD proteins, which likely contributes to enhancement of synaptic strength (Lisman et al., 2002). The predominant isoforms in neurons, CaMKIIα and -β, form either CaMKIIα homomultimers or CaMKIIα and -β heteromultimers (Brocke et al., 1999). CaMKIIβ predominates in early brain development and it promotes neurite extension (Fink et al., 2003) whereas CaMKIIα is expressed postnatally during brain maturation and stabilizes dendritic arbor structure (Wu and Cline, 1998).

CaMKIIα and -β are also regulated by neuronal activity with respect to their subcellular localization. Both isoforms show activity-dependent redistribution from the dendritic shaft to spines (Shen and Meyer, 1999). CaMKIIα accumulates in the PSD during LTP (Otmakhov et al., 2004) and ischemia (Kolb et al., 1995). The remarkable abundance of CaMKII at synapses (up to 8% of mass of PSD [Cheng et al., 2006]) strongly suggests a structural role for CaMKII at synapses. In line with this idea, CaMKIIβ isoform appears to directly bundle F-actin in spines, and is crucial for maintenance of dendritic spine morphology (Okamoto et al., 2007). A structural function has not been discovered for CaMKIIα.

The majority of protein degradation in cells occurs via the UPS, in which conjugation of a polyubiquitin chain via K48 linkage marks the protein substrate for degradation by the 26S proteasome. In response to altered cellular needs, proteasomes undergo dynamic changes in their subcellular localization and composition, and their function can be altered by posttranslational modifications and interacting proteins (Bingol and Schuman, 2006; Glickman and Raveh, 2005; Shen et al., 2007).

In neurons, several specific UPS substrates have been identified whose degradation affect synaptic development and plasticity (Bingol and Schuman, 2005). In a general sense, long-term potentiation (LTP), long-term depression (LTD), and memory rely on proteasomes to degrade proteins (Colledge et al., 2003; Fonseca et al., 2006; Lopez-Salon et al., 2001). Additionally, activity-dependent changes in PSD composition during long term homeostatic plasticity requires proteasome function (Ehlers, 2003). Following synaptic excitation, the proteasome redistributes rapidly to spines from the dendritic shaft and

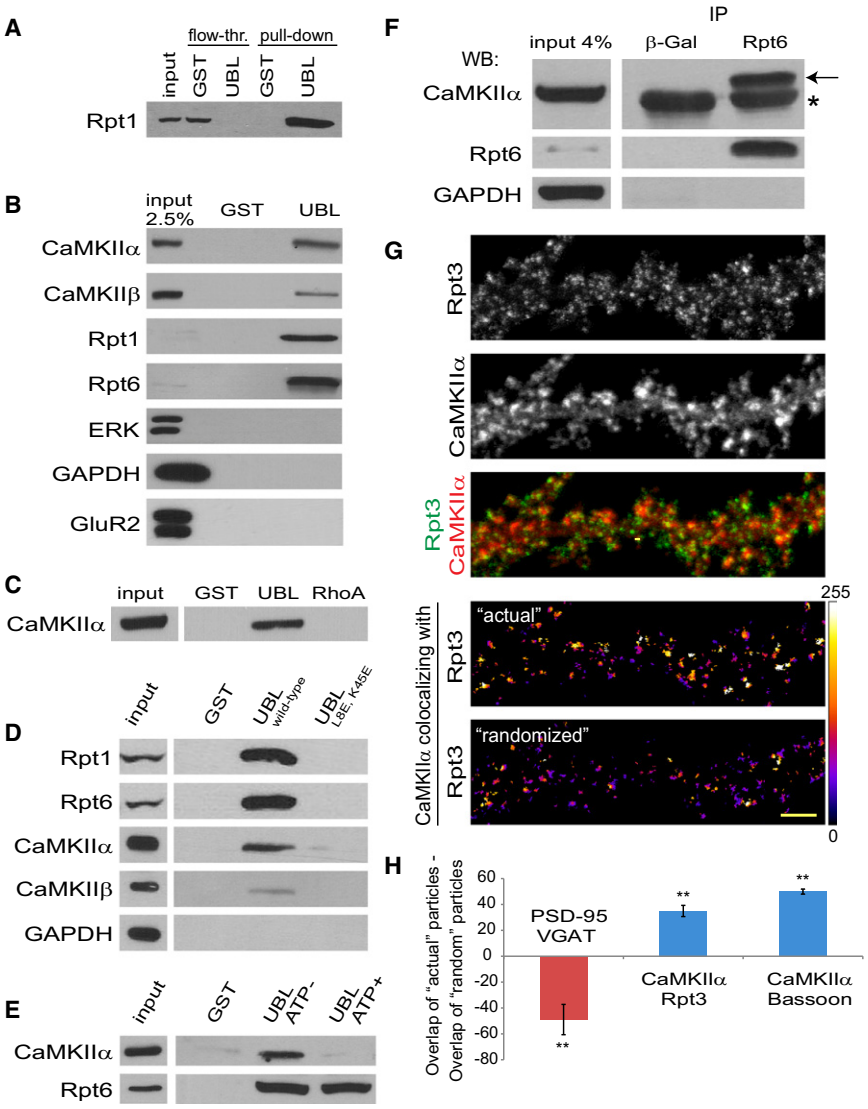

Figure 1. CaMKII and Proteasome are Associated in the Brain

(A) Immunoblot showing Rpt1 from rat brain P2 fraction binding to GST-UBL but not GST alone.

(B and C) Immunoblots showing precipitation of proteasome subunits (Rpt1 and Rpt6), CaMKIIα and CaMKIIβ, with GST-UBL beads, but not with GST-alone or GST-RhoA beads.

(D) Immunoblot showing that proteasome-binding deficient UBL mutant "L8E, K45E" does not precipitate CaMKIIα or CaMKIIβ.

(E) Immunoblotting of CaMKIIα and Rpt6 in GST-UBL precipitates purified in the absence or presence of ATP.

(F) Immunoblots showing coprecipitation of CaMKIIα (arrow), but not GAPDH, with proteasomes using anti-Rpt6 antibody. Anti-β-Gal antibody was used as control. (Asterisk indicates IgG heavy chain).

(G) Immunostaining of cultured hippocampal neurons for Rpt3 (green) and CaMKIIα (red). The overlap signal is shown in "heat" color scale (white as pixel value of 255 and blue as pixel value of 0, bottom panel). The scale bar represents 3 μm.

(H) Quantification of the difference between actual and random overlap for the indicated protein pairs. Higher overlap of actual particles than randomized indicates real colocalization of the two signals; negative overlap indicates segregation (n = 10 dendrites. **p < 0.01 compared to 0; t test. Error bars denote SEM). See also Figure S1.

RESULTS

CaMKII and Proteasome Are Associated in the Brain

To obtain insight into molecular mechanisms of proteasome regulation in neurons, we searched for proteasome-associated proteins in brain by mass spectrometry (MS) analysis of purified proteasome complexes. Proteasomes were affinity-purified from synaptosome-enriched fraction of rat forebrain (at almost 100% efficiency) using the ubiquitin-like (UBL) domain of Rad23, which binds to the 19S subunit of the proteasome (Schauber et al., 1998). Control columns charged with GST alone failed to precipitate the proteasome (Figure 1A, Figures S1A and S1B). Proteins were eluted with 2M NaCl/2% Triton-X, and examined by MS (Choe et al., 2007). In GST-UBL eluates, the highest number of peptides identified by MS corresponded to all 6 ATPase subunits and 11 of 14 non-ATPase subunits of the 19S proteasome (Table S1). No proteasome peptides were identified in eluates from control GST columns. Consistent with the known interactions between the ubiquitin conjugation/removal machinery and the proteasome (Glickman et al., 1998), ubiquitin-ligases (E3s) and deubiquitinating enzymes (DUBs) were also identified in GST-UBL eluates (Kcmf1, Rnf43 and Rab40C E3s, and Usp5, −14, −52 and −53 DUBs). Notably, some proteins identified in GST-UBL eluates confirmed previously identified proteasome interacting proteins

becomes more proteolytically active (Bingol and Schuman, 2006).

What are the molecular mechanisms underlying the redistribution and activation of proteasomes in response to neuronal activity in neurons? Here we report that CaMKIIα, a well-studied enzyme critical for neuronal plasticity, can act as a scaffold for proteasomes at synapses. We present evidence that activated, autophosphorylated CaMKIIα–independent of its kinase activity toward heterologous substrates–mediates postsynaptic recruitment of proteasomes. CaMKIIα binds to proteasomes, and its translocation to synapses is both necessary and sufficient for proteasome redistribution to spines. Additionally, CaMKIIα, functioning as a kinase, enhances proteasome proteolytic activity by phosphorylating Serine-120 of Rpt6 subunit of 19S proteasome. However, it is CaMKIIα redistribution, rather than its kinase activity, that is required for the activity-dependent degradation of polyubiquitinated proteins at postsynaptic sites.

in non-neuronal cells (heat shock protein 8, elongation factor Tu, ribosomal protein L10, and Usp5 and −14 DUBs (Bousquet-Dubouch et al., 2009; Scanlon et al., 2009; Wang and Huang, 2008). Thus, UBL-affinity chromatography successfully isolated proteasome-related protein complexes from the brain.

Several other proteins were also identified in GST-UBL precipitates with multiple peptide "hits" (Table S1). Among these we were particularly interested in CaMKIIα, an activity-regulated protein kinase that, like the proteasome, redistributes to synapses with synaptic stimulation (Bingol and Schuman, 2006; Shen and Meyer, 1999). We confirmed that CaMKII was copurified with the proteasome by immunoblotting. CaMKIIα was consistently present in the GST-UBL precipitate, whereas CaMKIIβ was detectable in half of the experiments (Figure 1B; 3.9 ± 0.5% of total input CaMKIIα was precipitated by GST-UBL beads, versus 90.0 ± 4.0% of proteasome subunit Rpt1 and 95.2 ± 3.5% of Rpt6; n = 8 experiments). Columns charged with GST alone or with GST-RhoA failed to precipitate CaMKIIα (Figures 1B and 1C). Accordingly, neither CaMKIIα nor β was detected by MS in eluates from GST-alone beads. Other abundant cytoplasmic enzymes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Extracellular Signal-Regulated kinase (ERK), and membrane proteins such as AMPA receptor subunit GluR2, were not detected in GST-UBL precipitates (Figure 1B), indicating some specificity in the association of CaMKII with UBL. Two amino acid substitutions in UBL that render it unable to bind to the 19S proteasome (L8E, K45E (Goh et al., 2008)) also failed to precipitate CaMKIIα or β, further supporting that CaMKII associates specifically with the proteasome (Figure 1D). Similar to other proteasome interacting proteins, CaMKIIα and proteasome association was inhibited by addition of ATP, confirming the interaction between CaMKIIα and the proteasome is real and not due to non-specific binding of CaMKIIα to GST-UBL beads. (Verma et al., 2000) (Figure 1E). Furthermore, immunoprecipitation (IP) of proteasomes from P2 lysates with Rpt6 antibody co-IPed CaMKIIα, but not GAPDH (Figure 1F). CaMKIIβ was not detectably coprecipitated with Rpt6 antibodies (data not shown). Thus, these data collectively indicate that a subset of CaMKIIα is specifically complexed with proteasomes in the brain.

If CaMKIIα interacts with proteasomes, they should colocalize at least in part within neurons. We immunostained cultured hippocampal neurons, using anti-Rpt3 (19S proteasome) and anti-CaMKIIα antibodies (Bingol and Schuman, 2006). Both proteins showed a widespread punctate distribution in dendrites, and we observed that most CaMKIIα puncta overlapped with Rpt3, and vice versa (Figure 1G). To confirm that the observed overlap of CaMKIIα and proteasome staining reflected a real colocalization, rather than a random coincidence of these two signals, we "randomized" the distribution of proteasome particles over the dendritic area while keeping the CaMKIIα signal fixed (Figure S1C, D). The actual observed colocalization was significantly higher than when the proteasome was randomly distributed along the same dendritic segment (Figure 1G, overlap signal shown in "heat" color scale). The difference between actual overlap and random overlap was +35 ± 4% for Rpt3 and CaMKIIα (compared to +50 ± 2% for Bassoon and CaMKIIα, which are both known to concentrate at excitatory synapses) versus −49 ± 11% for PSD-95 and VGAT (which are segregated at excitatory and inhibitory synapses, respectively) (Figure 1H, Figures S1E and S1F), confirming the validity of this method. Thus, CaMKIIα shows real and significant colocalization with proteasomes in neuronal dendrites, consistent with possible interaction in the postsynaptic compartment.

CaMKIIα Regulates Activity-Induced Proteasome Redistribution in Neurons

To examine whether CaMKII regulates the activity-dependent proteasome localization to spines, we monitored the distribution of monomeric green fluorescent protein (mGFP)-tagged 19S proteasome subunit Rpt1 (Rpt1-GFP) by time-lapse imaging in cultured hippocampal neurons transfected additionally with various CaMKII expression plasmids (Bingol and Schuman, 2006). Under basal conditions, Rpt1-GFP was distributed approximately uniformly in dendritic shaft and spines. In control neurons (transfected with empty vector or β-Gal), NMDA stimulation (30 μM, 3 min) induced a rapid and persistent Rpt1-GFP redistribution from dendritic shaft into spines (Figure 2). Compared to control cells, Rpt1-GFP in CaMKIIα- or CaMKIIβ-transfected neurons exhibited a significantly greater degree of accumulation in spines in response to NMDA stimulation at all time points examined (Figure 2). Thus overexpression of CaMKII enhances activity-dependent recruitment of proteasomes to spines.

To test if CaMKII is required for activity-dependent proteasome distribution to spines, we used pSuper-based RNA interference (RNAi) to express small hairpin RNAs (shRNAs) targeting CaMKIIα or CaMKIIβ (Okamoto et al., 2007). In neurons, these RNAi constructs knocked down the respective CaMKII isoforms by ~70%, based on immunostaining (Figure S2A, B). In neurons transfected with a control RNAi construct targeting luciferase (luc-RNAi) (Seeburg et al., 2008), Rpt1-GFP showed normal redistribution from shaft to spines in response to NMDA, quantitatively similar to untransfected neurons (Figures 3A and 3C). Neurons transfected with CaMKIIα-RNAi showed significantly impaired Rpt1-GFP accumulation, whereas knockdown of CaMKIIβ had no effect (Figures 3A and 3C). Cotransfection of an RNAi-resistant CaMKIIα construct (CaMKIIα*), but not an unrelated protein, β-Gal, rescued the Rpt1-GFP redistribution defect caused by CaMKIIα-RNAi (Figure 3A, C). In fact, neurons cotransfected with CaMKIIα* plus CaMKIIα-RNAi showed higher levels of proteasome accumulation than control neurons, similar to neurons overexpressing wild-type CaMKIIα in the absence of CaMKIIα-RNAi (Figure 2). The crucial role of CaMKIIα in proteasome redistribution was also confirmed using a "chemical LTP" protocol to stimulate synapses (Lu et al., 2001) (Figures S2C and S2D).

As previously reported, shRNA suppression of CaMKIIβ made dendritic spines longer and thinner (Fink et al., 2003; Okamoto et al., 2007). Nevertheless, in CaMKIIβ-RNAi transfected neurons, proteasomes still accumulated in these thin spines to the same extent as in luc-RNAi control neurons (Figures 3A and 3C). Reduced spine size could not explain the absence of CaMKIIβ-RNAi effect on proteasome redistribution because in our analysis the Rpt1-GFP intensity in each spine is normalized to its own baseline to quantify the temporal change. Thus, these data collectively indicate that CaMKIIα not only promotes, but

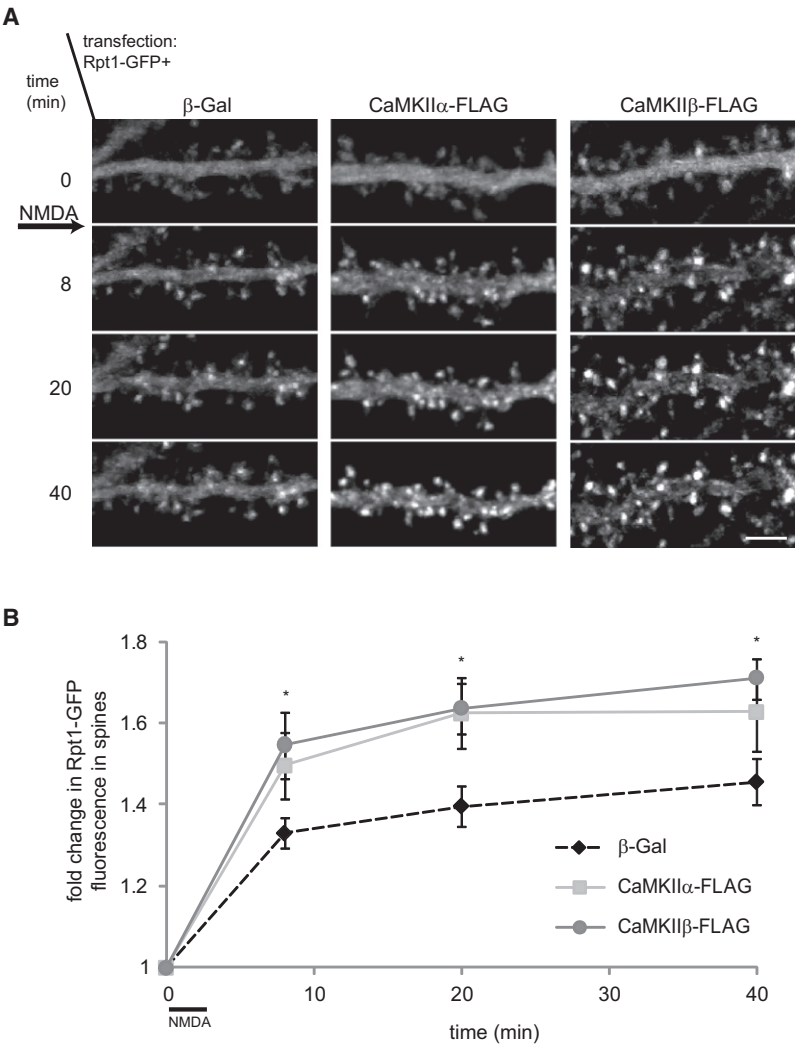

Figure 2. CaMKII Enhances NMDA-Induced Proteasome Redistribution into Spines

(A) Time-lapse imaging of proteasome (Rpt1-GFP) localization in live neurons. CaMKIIα or CaMKIIβ, but not β-Gal, overexpression enhances Rpt1-GFP redistribution induced by NMDA (30 μM, 3 min - arrow). The scale bar represents 3 μm.

(B) Time course of mean spine Rpt1-GFP fluorescence intensity (n = 16-24 dendrites; *p < 0.05 compared to β-Gal control; two-way ANOVA. Error bars denote SEM).

also is required, for activity-dependent proteasome accumulation in spines, with little contribution from CaMKIIβ.

How does CaMKIIα lead to proteasome accumulation in spines? Following stimulation, CaMKIIα redistribution to spines occurs within tens of seconds of stimulation onset (Shen and Meyer, 1999). However, the increase in the proteasome accumulation in spines occurs more slowly, within minutes (Bingol and Schuman, 2006). We confirmed the temporal difference in CaMKIIα and proteasome redistribution by monitoring the dynamics of Rpt1-GFP and mCherry-tagged CaMKIIα in the same neurons (data not shown). Because CaMKIIα redistribution occurred more rapidly than proteasome accumulation, we hypothesized that CaMKIIα does not "convey" proteasomes directly to postsynaptic sites, but rather that translocated CaMKIIα provides a postsynaptic scaffold for binding of proteasomes, leading to local accumulation. To examine directly whether CaMKIIα can stabilize proteasomes in spines following activity, we measured Rpt1-GFP fluorescence recovery after photobleaching (FRAP) in spines of neurons expressing CaMKIIα-RNAi or control luciferase-RNAi (Figure S2E and S2F). In luciferase-RNAi expressing neurons, NMDA stimulation decreased the mobile fraction of the proteasomes from ~70% to ~15%, indicating strong stabilization of proteasomes in spines by NMDA. This NMDA-induced proteasome immobilization was blocked by knockdown of CaMKIIα and rescued by coexpressing CaMKIIα* (but not β-Gal) with CaMKIIα-RNAi (Figures S2E and S2F). Thus CaMKIIα stabilizes proteasomes in spines, leading to proteasome accumulation following NMDA stimulation.

To test whether normal CaMKIIα translocation to synapses is important for proteasome redistribution, we used a "molecular replacement" strategy to suppress endogenous wild-type CaMKIIα using RNAi, while replacing it with specific CaMKIIα mutants that affect its translocation (by cotransfection of RNAi-resistant cDNAs). Proteasome redistribution in these neurons was then measured by quantitative time-lapse imaging of Rpt1-GFP. Importantly, these CaMKIIα mutants had similar expression levels when cotransfected with CaMKIIα-RNAi (Figures S3A and S3B). CaMKIIα-RNAi reduced NMDA-induced Rpt1-GFP redistribution by ~55% compared to luc-RNAi transfected neurons (Figures 3B and 3D). In contrast to wild-type CaMKIIα*, molecular replacement with two distinct CaMKIIα mutants that are deficient in activity-dependent translocation failed to restore proteasome redistribution (Figure S3C and S3D, and Figures 3B and 3D; calmodulin binding deficient CaMKIIα*-T305D/T306D, and NR2B-binding deficient CaMKIIα*-I205K [Bayer et al., 2001; Shen and Meyer, 1999]). With the T305D/T306D mutant, proteasome recruitment to spines was even less than with CaMKIIα-RNAi alone, possibly due to dominant negative nature of this mutation (Elgersma et al., 2002) (Figures 3B and 3D). Thus, not only the presence of CaMKIIα, but also its ability to translocate to synapses, is required to support activity-induced proteasome redistribution.

CaMKIIα Translocation Is Sufficient to Recruit Proteasomes to Spines

We hypothesized that CaMKIIα translocation mediates recruitment of proteasomes via a physical interaction of CaMKIIα with proteasomes. To "move" CaMKIIα to synapses directly and specifically – without stimulating the neurons and activating

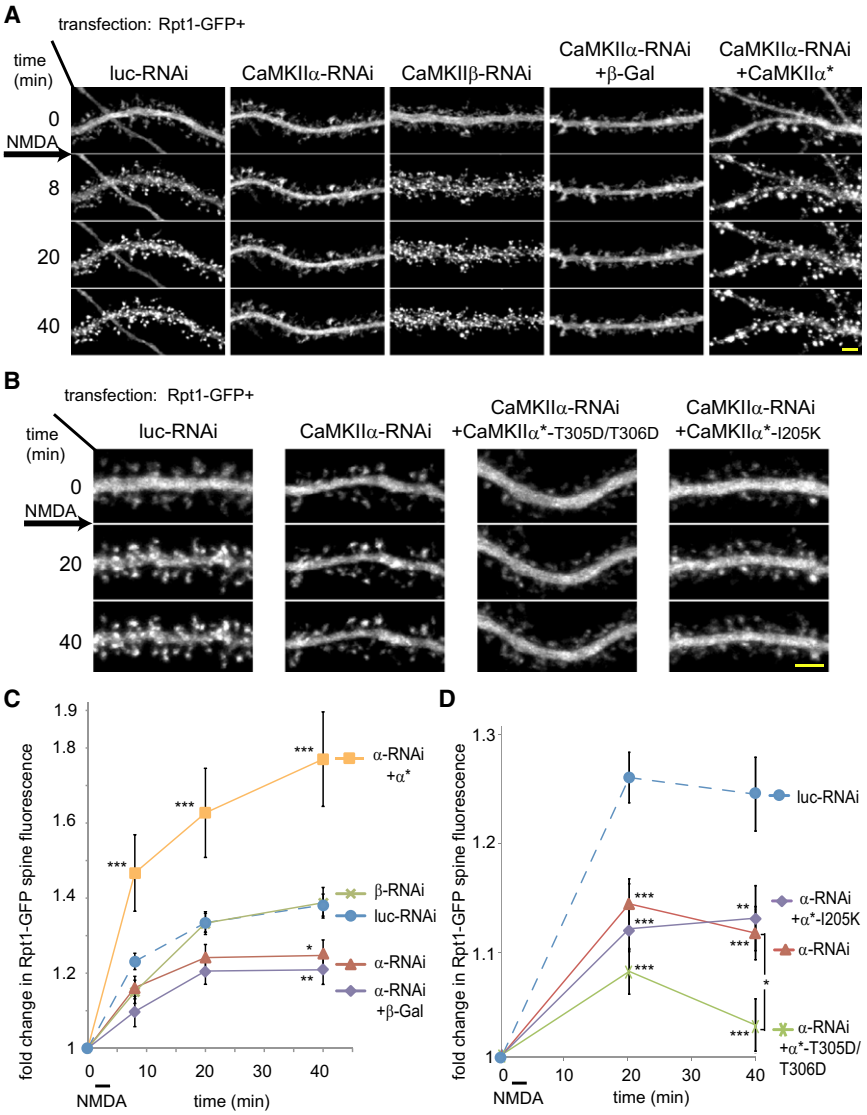

Figure 3. Activity-Dependent Redistribution of CaMKIIα Is Required for Proteasome Redistribution

(A and B) Time-lapse imaging of NMDA-induced proteasome (Rpt1-GFP) localization in live neurons transfected with Rpt1-GFP plus the indicated RNAi constructs, and RNAi-resistant CaMKIIα* wild-type or translocation-deficient mutants (-T305D/T306D or -I205K) or β-Gal (as control). The scale bar represents 3 μm.

(C and D) Time course of mean spine Rpt1-GFP fluorescence intensity (n = 20-54 dendrites; ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ compared to luciferase (luc)-RNAi control (dashed line); two-way ANOVA; Error bars denote SEM). See also Figures S2, S3, and S6.

CaMKII and other signaling pathways—we exploited the FK506 binding protein 12 (FKBP) and FKBP-rapamycin binding domain (FRB) dimerization system (Figure 4A) (Banaszynski et al., 2005). FKBP and FRB bind to each other in the presence of rapamycin, so by coexpressing FRB-tagged CaMKIIα and FKBP-tagged PSD-95 (the latter localizes to the PSD), rapamycin can be used to induce the binding of these two proteins in neurons. Validating this approach, in triply transfected heterologous cells, rapamycin induced the association of CaMKIIα-mCherry-FRB and CaMKIIβ-FLAG with PSD-95-FKBP, dependent on FRB being fused to CaMKIIα (Figure 4B).

We then cotransfected neurons with PSD-95-FKBP and CaMKIIα-mCherry-FRB and monitored the CaMKIIα-mCherry-FRB distribution by time-lapse imaging. Before rapamycin treatment, CaMKIIα-mCherry-FRB exhibited a diffuse distribution with modest concentration of signal in spines, similar to CaMKIIα-mCherry without the FRB domain (Figure 4C). Upon rapamycin addition, CaMKIIα-mCherry-FRB redistributed rapidly (within minutes) and dramatically to dendritic spines (Figure 4C) and precisely colocalized with PSD-95-FKPB (Figure 4D). CaMKIIα-mCherry, lacking the FRB domain, showed no change in distribution with rapamycin. Thus the FKBP-FRB-rapamycin system can be used to rapidly and specifically direct CaMKIIα localization to the PSD.

Is CaMKIIα translocation to the PSD *sufficient* to recruit endogenous proteasomes to postsynaptic sites? We examined endogenous proteasome distribution by immunostaining for Rpt3. In neurons expressing PSD-95-FKBP and CaMKIIα-mCherry-FRB, rapamycin induced a ~60% increase in the amount of Rpt3 signal that colocalized with PSD-95 (Figures 4E and 4G; Rpt3 signal colocalizing with PSD-95 puncta is shown in "heat" color scale). This result was confirmed with immunostaining for another endogenous proteasome subunit, Rpt2 (Figures S4A and S4C). Unlike proteasomes, neither endogenous ERK nor exogenously expressed β-Gal accumulated in spines with CaMKIIα-mCherry-FRB (Figures 4F and 4G and Figures S4B and S4C), indicating some specificity of the effect. Moreover, in control neurons that expressed PSD-95-FKBP and CaMKIIα-mCherry (lacking FRB), rapamycin treatment had no effect on Rpt3 distribution (Figure S4D, E). Collectively, these results show that the artificial recruitment of non-activated CaMKIIα to the PSD is sufficient to enhance proteasome accumulation at postsynaptic sites, strongly supporting the idea that CaMKIIα recruits proteasomes via physical interaction.

Autophosphorylated CaMKIIα Is a Postsynaptic Scaffold for the Proteasome

To gain further insight into the mechanisms of CaMKIIα and proteasome interaction, we made use of known CaMKII mutations that affect autophosphorylation and translocation to synapses

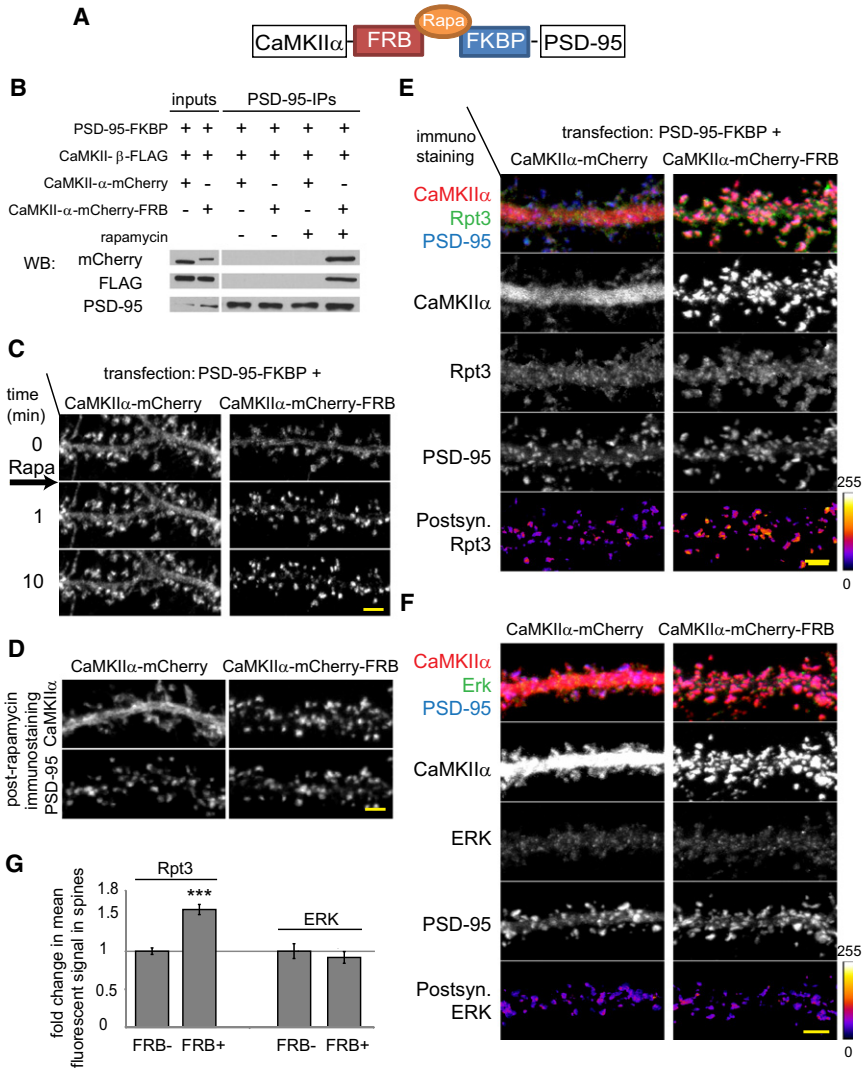

Figure 4. Driving CaMKIIα to Postsynaptic Sites is Sufficient to Recruit Proteasomes

(A) Schematic showing rapamycin (Rapa)-induced interaction between PSD-95 and CaMKIIα, using the FKBP-FRB hetero-dimerization system.

(B) Immunoblots showing rapamycin-induced coimmunoprecipitation of PSD-95-FKBP with CaMKIIα-mCherry-FRB and CaMKIIβ-FLAG in transfected HEK cells, only when FRB was fused to CaMKIIα.

(C and D) Time-lapse images showing rapamaycin (1nM - arrow) rapidly redistributes CaMKIIα-mCherry-FRB, but not CaMKIIα-mCherry, to postsynaptic sites in neurons expressing PSD-95-FKBP. Post hoc immunostaining with anti-PSD-95 antibody is shown in (D). The scale bar represents 3 μm.

(E and F) Immunostaining showing endogenous Rpt3 (E), but not ERK (F), accumulates in PSD-95-FKBP with translocated CaMKIIα-mCherry-FRB. Cultures were exposed to rapamycin (1 nM) and tetrodotoxin (1 μM) for 20 min before fixation. The intensity of Rpt3 and Erk pixels overlapping with PSD-95 are shown in "heat" color scale (bottom panels). The scale bar represents 3 μm.

(G) Quantification of the intensity of Rpt3 or ERK immunofluorescence signal colocalizing with PSD-95 puncta from (E) and (F). To control for size differences between PSD-95 puncta, total intensity of colocalized Rpt3 signal is normalized to the volume of PSD-95 puncta (n = 19-29 dendrites. ***$p < 0.001$; Mann-Whitney test. Error bars denote SEM). See also Figure S4.

(Shen and Meyer, 1999). When transfected into HEK293 cells, wild-type Flag-tagged CaMKIIα interacted weakly but significantly with endogenous proteasomes, as assayed by coimmunoprecipitation of endogenous Rpt6 with the exogenous CaMKIIα (Figures 5A and 5B). Notably, five times more Rpt6 was coimmunoprecipitated with CaMKIIα-T286D, a mutant that mimics autophosphorylation on Thr-286 and renders the enzyme calcium-calmodulin-independent and constitutively active (Figures 5A and 5B). A kinase-dead CaMKIIα double mutant (CaMKIIα-T286D/K42R) showed association with the proteasome similar to CaMKIIα-T286D. The autophosphorylation-disabled mutant CaMKIIα-T286A and kinase-dead mutant CaMKIIα-K42R bound proteasomes weakly—similar to wild-type CaMKIIα (Figures 5A and 5B). GAPDH, another abundant endogenous enzyme, did not coimmunoprecipitate with any of the CaMKIIα mutants; nor did Rpt6 coprecipitate with PSD-95. Consistent with enhanced binding of proteasomes to autophosphorylated CaMKIIα, stimulating CaMKII phosphorylation on T286/T287 in P2 lysates (by Ca^{2+}/calmodulin and ATP; see Experimental Procedures) increased by 2- to 3-fold the amount of endogenous CaMKIIα and β precipitated with UBL-beads, without changing the amount of proteasomes (Rpt6) pulled down (Figures 5C and 5D). Together with the mutant co-IP studies, these data indicate that proteasome interaction is enhanced by Thr-286 autophosphorylation of CaMKIIα, but does not otherwise depend on kinase activity of the enzyme.

The analogous CaMKIIβ mutants behaved similarly in this proteasome coimmunoprecipitation assay, however, the differences in the binding were smaller and less robust than seen with CaMKIIα mutants (Figures S5A and S5B). Even the CaMKIIβ mutant that most robustly associated with proteasomes (T287D/K43R) coprecipitated seven-fold less Rpt6 compared to the analogous CaMKIIα mutant T286D/K42R (Figures 5E and 5F). Finally, in the same proteasome coimmunoprecipitation assays, 4 times more proteasomes were associated with CaMKIIα homomers than with α/β heteromers (Figures 5G and 5H). Consistent with time-lapse imaging experiments, these data argue for a bigger role for CaMKIIα - mainly its homomers - in binding to proteasomes.

Given that autophosphorylation on T286 enhances CaMKIIα binding to proteasomes, and stabilizes CaMKIIα in the PSD (Shen and Meyer, 1999), we hypothesized that T286-phosphorylated CaMKIIα plays a crucial role in accumulating proteasomes at synapses. When overexpressed in cultured hippocampal

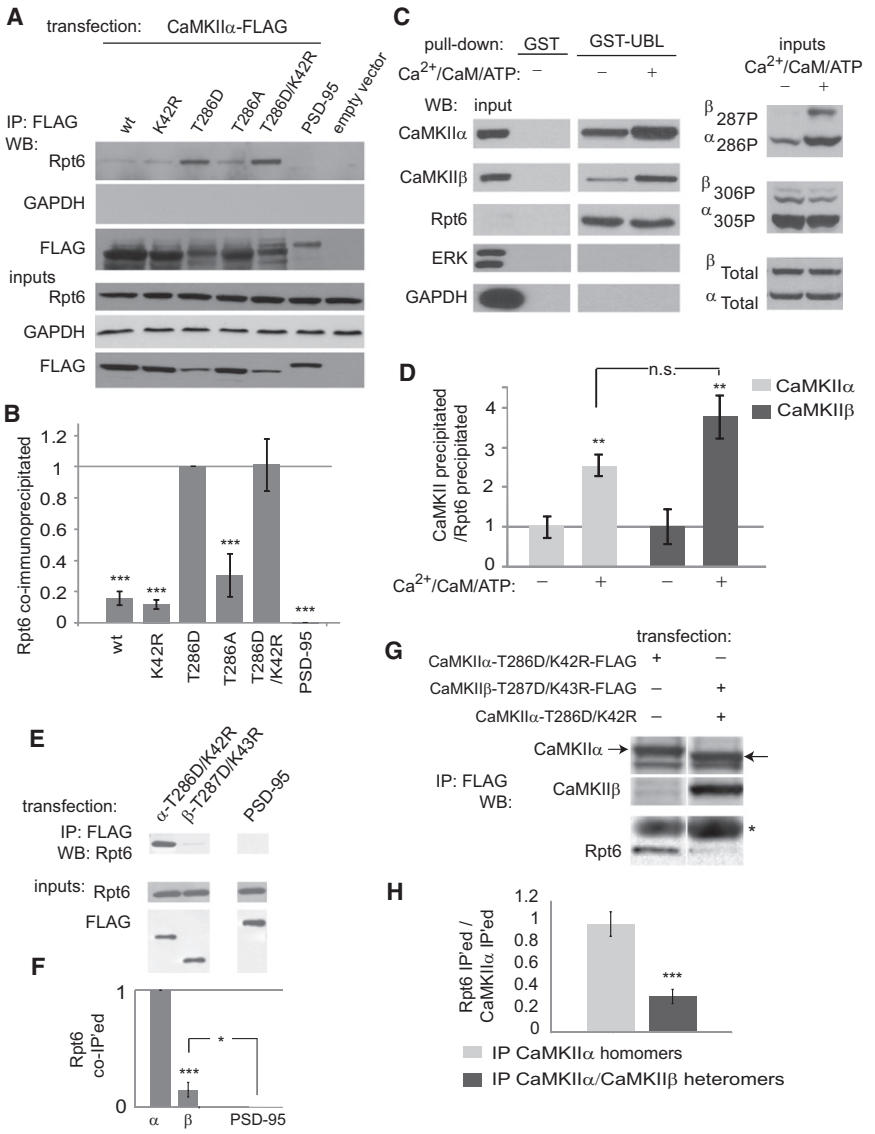

Figure 5. Autophosphorylation of CaMKIIα Enhances Its Association with the Proteasome

(A) Immunoblots showing coimmunoprecipitation of Rpt6 subunit of endogenous proteasomes (top row) with transfected FLAG-tagged wild-type and mutant CaMKIIα in HEK293 cells.

(B) Quantification of the coimmunoprecipitated Rpt6 from A, normalized to the amount of Rpt6 coimmunoprecipitated with CaMKIIα-T286D (n = 6 experiments, ***p < 0.001 compared to T286D; one-way ANOVA. Error bars denote SEM).

(C) Immunoblots showing that treatment of P2 brain lysates with calcium/calmodulin and ATP (to enhance CaMKII autophosphorylation on T286/T287 without affecting other major phosphorylation sites, right panels) increases binding of CaMKIIα and CaMKIIβ to GST-UBL beads, without affecting Rpt6 pull-down.

(D) Quantification of the amount of CaMKII precipitated with GST-UBL in (C), normalized to the amount of Rpt6 precipitated (n = 4 experiments. **p < 0.01, n.s.,non-significant (p = 0.41); Mann-Whitney test Error bars denote SEM).

(E) Immunoblots showing CaMKIIα-T286D/K42R associates with Rpt6 more than CaMKIIβ-T287D/K43R.

(F) Quantification of Rpt6 coimmunoprecipitated in E, normalized to CaMKIIα-T286D/K42R (n = 3 experiments, ***p < 0.001, and *p < 0.05; one-way ANOVA. Error bars denote SEM).

(G) Immunoblots showing CaMKIIα homomers associate with proteasomes more than CaMKIIα/β heteromers. CaMKIIα homomers or CaMKIIα/β heteromers were obtained by transfecting the indicated constructs into HEK293 cells followed by FLAG-immunoprecipitation. Arrows point to CaMKIIα. Asterisk indicates IgG heavy chain.

(H) Quantification of the coimmunoprecipitated Rpt6 in G, normalized to the amount of CaMKIIα precipitated (n = 3 experiments, ***p < 0.001; Mann-Whitney test. Error bars denote SEM). See also Figure S5.

neurons, wild-type CaMKIIα induced a small but significant increase in spine proteasome concentrations (~20%), as assayed by immunostaining for endogenous proteasome subunit Rpt3 and a spine volume marker, β-Gal (see Extended Experimental Procedures). Importantly, overexpression of either CaMKIIα-T286D or the kinase-dead double mutant CaMKIIα-T286D/K42R was more effective than wild-type CaMKIIα in boosting the concentration of endogenous proteasomes in spines (Figures S5C–S5E). CaMKIIα-T286A mutant did not change spine proteasome concentrations. The same constructs did not increase the spine concentration of α-actinin-2, another protein that interacts with CaMKII (Lisman et al., 2002) (Figure S5F). Because spine Rpt3 proteasome staining was normalized to the spine size and overall dendritic proteasome staining, differences in these quantities cannot explain the CaMKIIα-mediated enhancement of spine proteasomes. CaMKIIβ overexpression had little effect on steady-state proteasome levels in spines (Figure S5G). Thus, these results suggest that T286 autophosphorylation of CaMKIIα, but not its kinase activity toward heterologous substrates, is important for enhancing proteasome accumulation in spines.

Is autophosphorylated CaMKIIα also more effective in the *activity-induced* recruitment of proteasomes? In the molecular replacement experiment with Rpt1-GFP time-lapse imaging, we found that the autophosphorylation-deficient CaMKIIα*-T286A was able to support the NMDA-induced spine accumulation of proteasomes in neurons transfected with CaMKIIα-RNAi, but significantly less effectively than wild-type CaMKIIα* (Figures 6A and 6B). Although lacking autophosphorylation, CaMKIIα-T286A retains the ability to be activated by calcium/calmodulin (Shen and Meyer, 1999). In proteasome coimmunoprecipitation experiments, calcium/calmodulin added to the cell lysate stimulated the association of CaMKIIα-T286A with Rpt6 three fold (Figures 6C and 6D - note phospho-mimic CaMKIIα-T286D still bound more Rpt6 than calcium/calmodulin-treated CaMKIIα-T286A). Thus, CaMKIIα-T286A could partially support

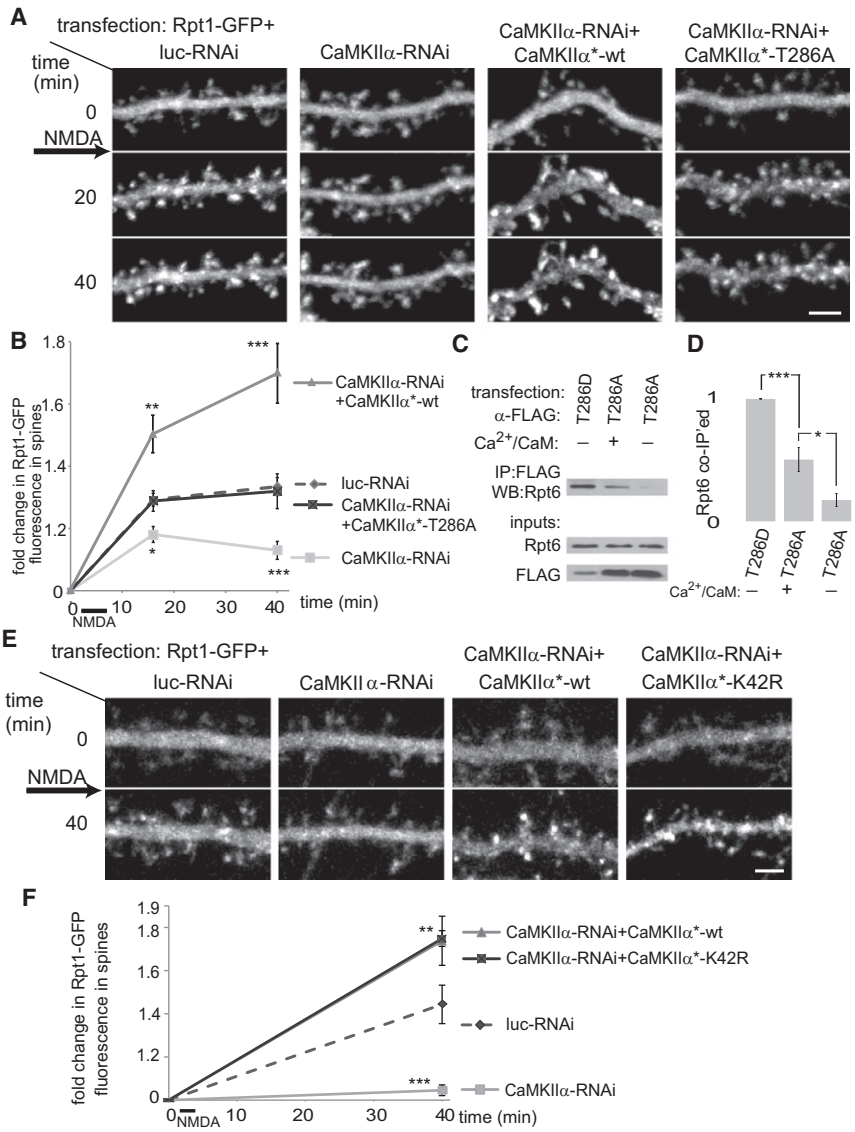

Figure 6. CaMKIIα T286 Autophosphorylation Promotes Activity-Dependent Proteasome Redistribution Independent of Kinase Activity

(A and E) Time-lapse imaging of NMDA-induced proteasome (Rpt1-GFP) localization in live neurons transfected with Rpt1-GFP and the indicated RNAi and RNAi-resistant wild-type or autophosphorylation-deficient T286A- or kinase-dead K42R-CaMKIIα* constructs. The scale bar represents 3 μm.

(B and F) Time course of mean spine Rpt1-GFP fluorescence intensity from A, E (n = 14-79 dendrites; *p < 0.05, **p < 0.01 and ***p < 0.001 compared to luc-RNAi (dashed); two-way ANOVA. Error bars denote SEM).

(C) Immunoblots showing addition of calcium/calmodulin to HEK293 lysates increases coimmunoprecipitation of Rpt6 subunit of endogenous proteasomes with transfected CaMKIIα-T286A-FLAG.

(D) Quantification of the amount of Rpt6 coimmunoprecipitated in C, normalized to CaMKIIα-T286D (***p < 0.001 and *p < 0.05; one-way ANOVA. Error bars denote SEM). See also Figure S3 and S6.

proteasome redistribution due to activation by calcium/calmodulin. Because the CaMKIIα-T286A mutant can move to synapses normally following stimulation (Figures S3E and S3F; [Shen and Meyer, 1999]), these data indicate an important role for T286 phosphorylation in activity-dependent proteasome redistribution.

In line with a kinase-independent mechanism for proteasome redistribution, molecular replacement with a kinase-dead CaMKIIα that can translocate to spines (CaMKIIα*-K42R) (Figure S3C, D and (Shen and Meyer, 1999)) rescued Rpt1-GFP redistribution as well as wild-type CaMKIIα* (Figures 6E and 6F). Moreover, inhibition of CaMKII activity with a drug inhibitor (KN-93) did not prevent proteasome redistribution compared to control neurons treated with the inactive analog KN-92 (data not shown).

Finally, to control for possible spine volume changes induced by stimulation and expression of CaMKIIα mutants, we coexpressed a soluble fluorescent protein, mCherry, and normalized Rpt1-GFP spine fluorescence to mCherry spine fluorescence over time (Figure S6). Similar results were obtained in these "volume-controlled" experiments, confirming that NMDA-induced Rpt1-GFP redistribution relies on CaMKIIα that can translocate to spines, but not the kinase activity of the enzyme. T286A mutant was less effective than wild-type or K42R mutant (Figure S6). Our findings suggest that T286 autophosphorylated CaMKIIα functions as a proteasome-binding scaffold to recruit proteasomes to spines without need for CaMKII phosphorylation of additional substrates.

Regulation of Proteasome Activity by CaMKII Phosphorylation

Although CaMKIIα kinase activity toward heterologous substrates is not required for proteasome recruitment to synapses, could it regulate the function of the proteasome via phosphorylation? We found that proteasome activity in P2 fractions of rat brain, as measured using a fluorogenic substrate, was stimulated by Ca^{2+}/calmodulin and ATP, in a CaMKII activity dependent manner. In in vitro kinase reactions, activity of purified 26S proteasomes, but not purified 20S proteasomes, was enhanced by addition of recombinant CaMKIIα in the presence of Ca^{2+}/calmodulin and ATP (data not shown), indicating direct phosphorylation of the subunits of the 19S complex by CaMKIIα (also see (Djakovic et al., 2009)). Using mass spectrometry

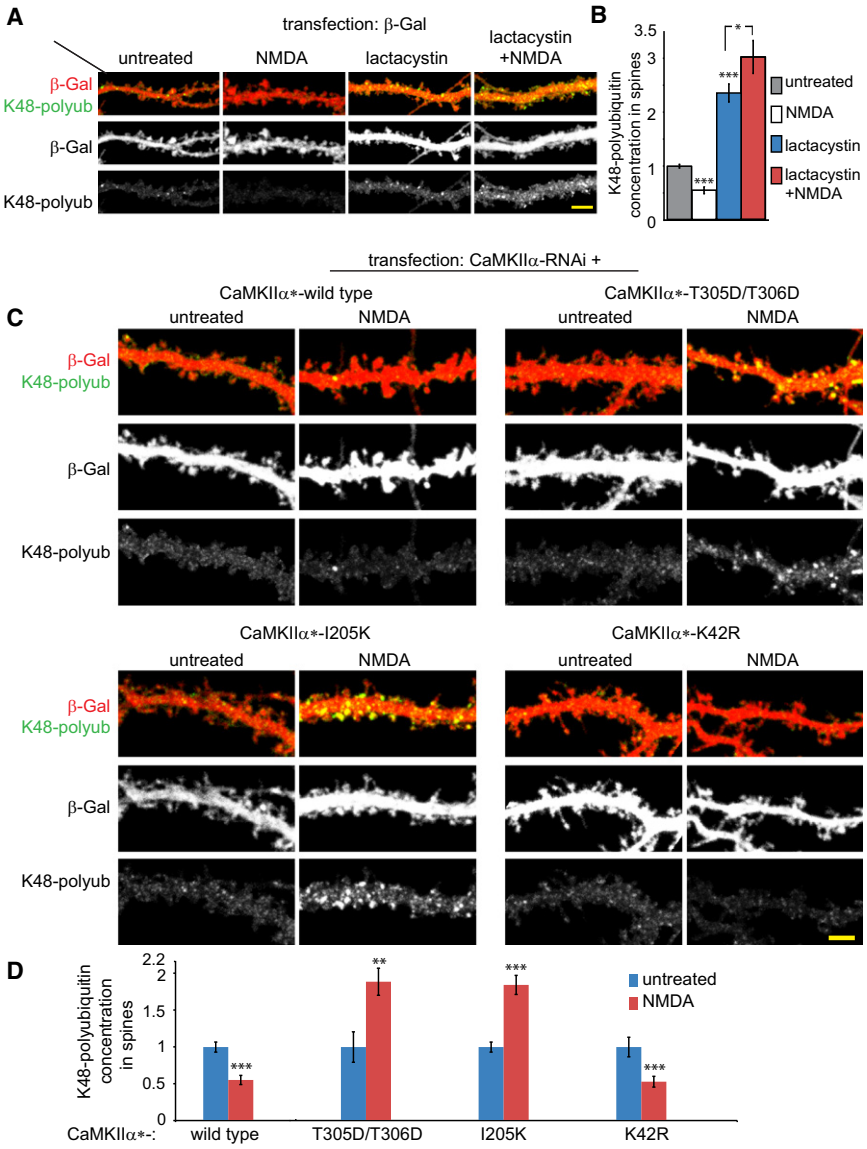

Figure 7. CaMKIIα Translocation is Required for Degradation of Endogenous Proteasome Substrates in Dendritic Spines

(A) Immunostaining of cultured hippocampal neurons stimulated with NMDA (30 μM, 3 min) in the presence or absence of lactacystin (10 μM). 30 min after NMDA stimulation, neurons were fixed and immunostained using anti-K48-polyubiquitin chain (to visualize endogenous proteasome substrates) and anti-β-Gal antibodies (to visualize transfected dendrite and spines). The scale bar represents 3 μm.

(B) Quantification of spine K48-polyubiquitin signal in A (n = 17-68 dendrites. *p < 0.05 and ***p < 0.001, using one-way ANOVA. Error bars denote SEM).

(C) Same as (A) except neurons were transfected with β-Gal, CaMKIIα-RNAi plus RNAi-resistant wild-type or activation- and translocation-deficient T305D/T306D- or translocation-deficient I205K- or kinase-dead K42R-CaMKIIα*. The scale bar represents 2 μm.

(D) Quantification of spine K48-polyubiquitin signal in C (n = 26-81 dendrites; **p < 0.01 and ***p < 0.001 compared to untreated; Mann-Whitney test. Error bars denote SEM).

analysis, we found that Ser-120 of 19S subunit Rpt6 (a predicted CaMKII site by ScanSite) was strongly phosphorylated by CaMKIIα in in vitro kinase reactions. Addition of purified CaMKIIα to lysates of HEK cells overexpressing wild-type Rpt6 enhanced proteasome activity ~1.8 fold, but had no effect in HEK cell lysates overexpressing the S120A mutant of Rpt6 (data not shown). Our findings imply that phosphorylation of Rpt6 on Ser-120 is important for CaMKIIα stimulation of proteasome activity.

Functional Significance of CaMKIIα–Mediated Recruitment of Proteasomes

What is the functional significance of CaMKIIα–mediated recruitment of proteasomes to postsynaptic sites? We hypothesized that NMDA receptor activation stimulates the degradation of polyubiquitinated substrates in spines and that this stimulation depends on CaMKIIα–mediated recruitment of proteasomes. Total level of endogenous proteasome substrates was quantified by immunostaining with an antibody ("Apu2.07") specific for K48-linked polyubiquitin chains (Newton et al., 2008), the ubiquitin modification that specifically marks proteins for proteasomal degradation. In control experiments, the K48-polyubiquitin signal in dendritic spines increased ~2.2-fold upon proteasome inhibition (10 μM lactacystin, 1 hr) (Figures 7A and 7B), supporting the authenticity of the K48-polyubiquitin staining. The spine K48 polyubiquitin signal detected with Apu2.07 fell ~50% within 20–30 min following treatment with NMDA (Figures 7A and 7B). Interestingly, when neurons were stimulated with NMDA in the presence of lactacystin, spine K48-polyubiquitin not only did not fall, but actually rose further (3-fold relative to untreated) (Figures 7A and 7B). The opposite effects of NMDA in the presence and absence of proteasome inhibitor suggest that NMDA receptor activation induces both ubiquitination and degradation of proteins. Consistently, NMDA treatment initially increased the spine K48-polyubiquitin signal at 5 min, and then decreased the staining below untreated control levels at 30 min time point (data not shown).

Next, we measured the NMDA-induced clearance of endogenous proteasome substrates in spines of neurons in which CaMKIIα was suppressed by RNAi and molecularly replaced with wild-type or mutant CaMKIIα. When neurons were transfected with CaMKIIα-RNAi plus wild-type CaMKIIα*, NMDA stimulation reduced the spine K48-polyubiquitin signal, as expected

(Figures 7C and 7D). However, when endogenous CaMKIIα was replaced with CaMKIIα*-T305D/T306D (a translocation- and activation-deficient mutant) or with CaMKIIα*-I205K (a mutant that can be enzymatically activated but cannot translocate to spines), NMDA failed to induce a loss of spine K48-polyubiquitin and instead caused a 2-fold increase (Figures 7C and 7D). The paradoxical increase resembled that seen in the presence of lactacystin and appeared to concentrate in dendritic spines (Figure 7C). Thus, without CaMKIIα translocation, postsynaptic ubiquitination was intact, but degradation was impaired. Finally, the kinase-dead but translocation-competent mutant, CaMKIIα*-K42R, completely restored NMDA-induced loss of spine K48-polyubiquitin signal (Figures 7C and 7D). In conclusion, CaMKIIα translocation, but not kinase activity, is required to support proteasome recruitment and activity-dependent turnover of ubiquitinated proteins in spines.

DISCUSSION

Despite a strong relationship between synaptic plasticity and proteasomal protein degradation, the molecular mechanisms by which synaptic activity regulates the UPS are not clear. This study defines a novel role for CaMKIIα in controlling the distribution and activity of the proteasome.

It has long been a puzzle why the postsynaptic compartment contains so much CaMKII–an enzyme – to regulate synaptic structure and function. CaMKIIβ may act as a structural protein by binding and crosslinking actin filaments (Okamoto et al., 2007). However, a scaffolding role for CaMKIIα, which is 6 times more abundant than CaMKIIβ in molar terms in the PSD (Cheng et al., 2006), has not been shown. CaMKIIα binds to proteasomes more effectively than CaMKIIβ and promotes proteasome accumulation in spines even when its kinase activity is disrupted, thus defining a scaffold function of CaMKIIα. Specifically, our data indicate that T286-autophosphorylated, and to a lesser extent Ca^{2+}/calmodulin-bound CaMKIIα, binds to proteasomes and that the translocation of these forms of CaMKIIα to synapses leads to the redistribution of proteasomes into spines. Signifying the functional importance of proteasome recruitment, CaMKIIα translocation–rather than CaMKIIα kinase activity–is critical for activity-induced postsynaptic turnover of endogenous proteasome substrates. Since Ca^{2+}/CaM binding and subsequent autophosphorylation of CaMKIIα occurs in response to calcium influx at stimulated synapses, this mechanism provides a means to recruit proteasomes specifically to activated synapses, where they can mediate local protein degradation.

How does Ca^{2+}/CaM binding and T286 phosphorylation enhance CaMKIIα interaction with proteasomes? Upon Ca^{2+}/CaM binding, CaMKIIα undergoes a conformational change that leads to T286 autophosphorylation, preventing the interaction between autoinhibitory and kinase domains thus rendering the enzyme constitutively active (Lisman et al., 2002). Similar to the enhanced interaction of T286-phosphorylated CaMKIIα with its other synaptic partners (such as Densin-180 and NR2B subunit of NMDA receptors (Bayer et al., 2001; Walikonis et al., 2001), the protein interaction surfaces exposed in the "open" conformation could be used to bind to proteasomes. RNAi molecular replacement experiments indicate the importance of CaMKIIα T286 autophosphorylation for normal proteasome redistribution. The modest but significant rescue afforded by T286A-CaMKIIα can be explained by its ability to translocate to synapses following synaptic stimulation (Shen and Meyer, 1999), its ability to bind Ca^{2+}/CaM, and its enhanced proteasome association upon calcium/calmodulin binding.

The scaffold function of CaMKIIα for recruiting proteasomes is in addition to a catalytic role, in which CaMKIIα phosphorylates and stimulates the proteolytic activity of the proteasome by phosphorylation of Rpt6 on Ser-120. The mechanisms by which Rpt6 Ser-120 phosphorylation enhances proteasome function and the regulation of Rpt6 phosphorylation in neurons remain to be elucidated.

In addition to postsynaptic functions, proteasomes on the presynaptic side regulate transmission by limiting neurotransmitter release (Yao et al., 2007). It has been reported that presynaptic CaMKIIα can function as a negative regulator of synaptic transmission (Hinds et al., 2003). Reminiscent of its postsynaptic redistribution, neuronal depolarization translocates CaMKIIα from the periphery of the presynaptic bouton to the core of the vesicle pool region (Tao-Cheng et al., 2006). Thus it is possible that CaMKIIα might orchestrate proteasome translocation and activity in the presynaptic terminal as well.

Although inducing opposite functional changes in synaptic strength, both NMDA application (a standard way to induce chemical LTD) and chem-LTP stimulation induce proteasome redistribution to spines, dependent on CaMKIIα. However, it is important to note that both LTP and LTD require proteasome activity (Colledge et al., 2003; Fonseca et al., 2006), and both NMDA and chem-LTP stimulation protocols can induce CaMKIIα translocation to synapses in cultured neurons (Otmakhov et al., 2004; Shen and Meyer, 1999).

LTP, LTD and long-term memory consolidation relies on proteasomes to degrade proteins (Colledge et al., 2003; Fonseca et al., 2006; Lopez-Salon et al., 2001). During reconsolidation and extinction of previously formed memory, protein degradation appears to be required to disassemble the preexisting memory and/or to incorporate the new updated memory (Lee et al., 2008). Protein degradation near synapses is elevated around 15-30 min post-LTP-induction (Karpova et al., 2006) and after learning (Lee et al., 2008), consistent with the time course of proteasome redistribution in culture. Because CaMKIIα has a widely accepted role in synaptic potentiation and learning and memory (Lisman et al., 2002), proteasome recruitment and activation by CaMKIIα could drive the elimination of "negative" regulators of plasticity and aid the physical reorganization of synapses for new learning.

During homeostatic synaptic plasticity, chronic elevation of excitatory activity (24 hr) enhances synaptic CaMKIIα and decreases synaptic CaMKIIβ (Thiagarajan et al., 2002). We note that chronic hyperactivity increases proteasome-dependent protein turnover at synapses (Ehlers, 2003), which likely contributes to homeostatic synaptic plasticity. Because CaMKIIα is the primary recruiter of proteasomes, elevation of CaMKIIα levels could promote postsynaptic accumulation and activity of proteasomes and contribute to enhanced turnover of synaptic proteins during homeostatic plasticity. In conclusion, beyond phosphorylating synaptic proteins involved in synaptic

potentiation, CaMKIIα can facilitate synaptic plasticity by directly modulating protein degradation at the level of proteasome distribution and proteasome activity.

EXPERIMENTAL PROCEDURES

GST-UBL Purification and Analysis of Proteasomes and the Associated Proteins

Rat forebrain P2 fractions were prepared as described (Bingol and Schuman, 2006) and lysed in Buffer A (20 mM HEPES, 10% glycerol, 1% Triton-X, and protease and phosphatase inhibitors (Roche)) containing 0.15 M NaCl. Following clearing at 25,000 g for 30 min, supernatants were passed through GST or GST-UBL columns (Schauber et al., 1998), and bound proteins were washed with Buffer A containing 0.2 M NaCl. Following elution of 5–10 fractions (half column volume) in Buffer A containing 2 M NaCl and 2% Triton-X, each fraction is analyzed by silver staining and tandem mass spectrometry as described (Choe et al., 2007).

Hippocampal Culture, Transfection, and Immunostaining and Time-Lapse Imaging

Dissociated hippocampal neuron cultures were prepared as described (Seeburg et al., 2008). Transfections were done using Lipofectamine 2000 at DIV 18-21. Constructs were expressed for 2–3 days posttransfection. Immunostaining was performed as described (Bingol and Schuman, 2006). All immunostaining images were acquired with a Zeiss LSM 510 or Leica SP5 confocal microscope using a 63×/1.4 oil objective (1 μm = 21 pixels) with a Z-step size of 0.2–0.25 μm. For details of image analysis, see Extended Experimental Procedures.

For time-lapse imaging, neurons were continuously perfused with an HBS-based imaging buffer containing 1.5 mM $MgCl_2$ and 2 mM $CaCl_2$ (Lu et al., 2001). After acquiring baseline images, cells were stimulated with 30 μM NMDA for 3 min in the imaging buffer without magnesium. All confocal images for live imaging were acquired using a 63×/1.4 oil objective (1 μm = 10.5 pixels) with a Z-step size of 0.4 μm. Quantification of the Rpt1-GFP localization and FRAP experiments were described previously (Bingol and Schuman, 2006) and detailed in Extended Experimental Procedures.

HEK293 Cell Transfection and Immunoprecipitation:

HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen). After 24 hr, cell lysates were prepared in RIPA lysis buffer and mixed with anti-FLAG M2 beads. For immunoprecipitation of Rpt6 from the brain, mouse anti-Rpt6 antibody (BIOMOL) was incubated with P2 lysates and precipitated with Protein A/G-conjugated beads (Pierce). Inputs and precipitates were resolved by SDS-PAGE and analyzed by Western blotting.

Activation of CaMKII in the P2 fraction

Rat brain P2 fraction lysates were stimulated with 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM ATP, 1 mM DTT and 4 μM calmodulin for 5 min at 4°C. Reactions were stopped with the addition of 2 mM EDTA. These lysates were used for either proteasome pull-down assays or mixed with Suc-LLVY-AMC to measure proteasome activity as described (Ehlers, 2003).

Statistical Analysis

Error bars indicate standard error of the mean. To compute p values, non-paired Student's t test, Mann-Whitney test and one- or two-way ANOVA with Bonferroni post-test were used, as indicated in figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.01.024.

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