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Autophosphorylated CaMKII\(\alpha\) 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 CaMKII\(\alpha\)—an abundant postsynaptic protein kinase—mediates the activity-dependent recruitment of proteasomes to dendritic spines in hippocampal neurons. CaMKII\(\alpha\) is biochemically associated with proteasomes in the brain. CaMKII\(\alpha\) translocation to synapses is required for activity-induced proteasome accumulation in spines, and is sufficient to redistribute proteasomes to postsynaptic sites. CaMKII\(\alpha\) autophosphorylation enhances its binding to proteasomes and promotes proteasome recruitment to spines. In addition to this structural role, CaMKII\(\alpha\) stimulates proteasome activity by phosphorylating proteasome subunit Rpt6 on Serine 120. However, CaMKII\(\alpha\) 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 CaMKII\(\alpha\) in activity-dependent proteasome redistribution, which is commensurate with the great abundance of CaMKII\(\alpha\) 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, CaMKII\(\alpha\) and -\(\beta\), form either CaMKII\(\alpha\) homomultimers or CaMKII\(\alpha\) and -\(\beta\) heteromultimers (Brocke et al., 1999). CaMKII\(\beta\) predominates in early brain development and it promotes neurite extension (Fink et al., 2003) whereas CaMKII\(\alpha\) is expressed postnatally during brain maturation and stabilizes dendritic arbor structure (Wu and Cline, 1998).

CaMKII\(\alpha\) and -\(\beta\) 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). CaMKII\(\alpha\) 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, CaMKII\(\beta\) 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 CaMKII\(\alpha\).

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
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.

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...
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 CaMKIIα 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 CaMKIIα with UBL. Two amino acid substitutions in GST 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 CaMKIIα 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. (Vemana 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 CaMKIIα 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 CaMKIIα 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 CaMKIIα enhances activity-dependent recruitment of proteasomes to spines.

To test if CaMKIIα 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-RNAi showed significantly 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
also is required, for activity-dependent proteasome accumulation in spines, with little contribution from CaMKIIβ.

How does CaMKII 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 (Egersma 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
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 CaMKII\(\alpha\) 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 a modest concentration of signal in spines, similar to CaMKII\(\alpha\)-mCherry without the FRB domain (Figure 4C). Upon rapamycin addition, CaMKII\(\alpha\)-mCherry-FRB redistributed rapidly (within minutes) and dramatically to dendritic spines (Figure 4C) and precisely colocalized with PSD-95-FKBP (Figure 4D). CaMKII\(\alpha\)-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 CaMKII\(\alpha\) localization to the PSD.

Is CaMKII\(\alpha\) 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 CaMKII\(\alpha\)-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 \(\beta\)-Gal accumulated in spines with CaMKII\(\alpha\)-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 CaMKII\(\alpha\)-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 CaMKII\(\alpha\) to the PSD is sufficient to enhance proteasome accumulation at postsynaptic sites, strongly supporting the idea that CaMKII\(\alpha\) recruits proteasomes via physical interaction.

**Autophosphorylated CaMKII\(\alpha\) Is a Postsynaptic Scaffold for the Proteasome**

To gain further insight into the mechanisms of CaMKII\(\alpha\) and proteasome interaction, we made use of known CaMKII mutations that affect autophosphorylation and translocation to synapses...
Experimental Procedures) increased by 2- to 3-fold the amount a was coimmunoprecipitated with CaMKII CaMKII immunoprecipitation of endogenous Rpt6 with the exogenous significantly with endogenous proteasomes, as assayed by coimmunoprecipitation of endogenous Rpt6 with the exogenous wild-type Flag-tagged CaMKII CaMKII mutants; nor did Rpt6 coprecipitate with PSD-95. Consistent with enhanced binding of proteasomes to autophosphorylated CaMKIIa, stimulating CaMKII phosphorylation on T286/T287 in P2 lysates (by Ca2+/calmodulin and ATP; see Experimental Procedures) increased by 2- to 3-fold the amount

(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 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...
neurons, wild-type CaMKIIx 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 CaMKIIx-T286D or the kinase-dead double mutant CaMKIIx-T286D/K42R was more effective than wild-type CaMKIIx in boosting the concentration of endogenous proteasomes in spines (Figures S5–S7E). CaMKIIx-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 CaMKIIx-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 CaMKIIx, but not its kinase activity toward heterologous substrates, is important for enhancing proteasome accumulation in spines.

Is autophosphorylated CaMKIIx 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 CaMKIIx-T286A was able to support the NMDA-induced spine accumulation of proteasomes in neurons transfected with CaMKIIx-RNAi, but significantly less effectively than wild-type CaMKIIx (Figures 6A and 6B). Although lacking autophosphorylation, CaMKIIx-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 CaMKIIx-T286A with Rpt6 threefold (Figures 6C and 6D - note phospho-mimic CaMKIIx-T286D still bound more Rpt6 than calcium/calmodulin-treated CaMKIIx-T286A). Thus, CaMKIIx-T286A could partially support...
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 autophosphorylation 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.

**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.

**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 Ca2+/calmodulin and ATP, in a CaMKII activity dependent manner. 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 Ca2+/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...
analysis, we found that Ser-120 of 19S subunit Rpt6 (a predicted CaMKII site by ScanSite) was strongly phosphorylated by CaMKII. Addition of purified CaMKII 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 CaMKII stimulation of proteasome activity.

Functional Significance of CaMKIIz-Mediated Recruitment of Proteasomes
What is the functional significance of CaMKIIz-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 CaMKIIz-mediated recruitment of proteasomes.

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), supporting the authenticity of the K48-polyubiquitin staining. The spine K48 polyubiquitin signal detected with Apu2.07 fell ~50% within 20–30 min

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

Figure 7. CaMKIIz 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, CaMKIIz-RNAi plus RNAi-resistant wild-type or activation- and translocation-deficient T305D/T306D- or translocation-deficient I205K- or kinase-dead K42R-CaMKIIz*. 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).
contains so much CaMKII—an enzyme—to regulate synaptic data indicate that T286-autophosphorylated, and to a lesser thus defining a scaffold function of CaMKII some more effectively than CaMKII et al., 2006), has not been shown. CaMKII more abundant than CaMKII activation and activity of the proteasome. This study defines a novel role for CaMKII by binding and crosslinking actin filaments (Okamoto et al., 2007). However, a scaffolding role for CaMKII, which is 6 times more abundant than CaMKII in molar terms in the PSD (Cheng et al., 2006), has not been shown. CaMKII binds to proteasomes more effectively than CaMKII and promotes proteasome accumulation in spines even when its kinase activity is disrupted, thus defining a scaffold function of CaMKII. Specifically, our data indicate that T286-autophosphorylated, and to a lesser extent Ca2+/calmodulin-bound CaMKII, binds to proteasomes and that the translocation of these forms of CaMKII to synapses leads to the redistribution of proteasomes into spines. Signifying the functional importance of proteasome recruitment, CaMKII translocation—rather than CaMKII kinase activity—is critical for activity-induced postsynaptic turnover of endogenous proteasome substrates. Since Ca2+/CaM binding and subsequent autoprophosphorylation of CaMKII 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 Ca2+/CaM binding and T286 phosphorylation enhance CaMKII interaction with proteasomes? Upon Ca2+/CaM binding, CaMKII undergoes a conformational change that leads to T286 autoprophosphorylation, 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 CaMKII with its other synaptic partners (such as Densin-180 and NR2B subunit of NMDA receptors (Bayer et al., 2001; Wallkonis 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 CaMKII T286 autophosphorylation for normal proteasome redistribution. The modest but significant rescue afforded by T286A-CaMKII can be explained by its ability to translocate to synapses following synaptic stimulation (Shen and Meyer, 1999), its ability to bind Ca2+/CaM, and its enhanced proteasome association upon calcium/calmodulin binding.

The scaffold function of CaMKII for recruiting proteasomes is in addition to a catalytic role, in which CaMKII 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 CaMKII can function as a negative regulator of synaptic transmission (Hinds et al., 2003). Reminiscent of its postsynaptic redistribution, neuronal depolarization translocates CaMKII 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 CaMKII 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 CaMKII. However, it is important to note that both LTD and LTP require proteasome activity (Colledge et al., 2003; Fonseca et al., 2006), and both NMDA and chem-LTP stimulation protocols can induce CaMKII 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., 2001a). 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 CaMKII has a widely accepted role in synaptic potentiation and learning and memory (Lisman et al., 2002), proteasome recruitment and activation by CaMKII 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 CaMKII and decreases synaptic CaMKII (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 CaMKII is the primary recruiter of proteasomes, elevation of CaMKII 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, CaMKII\(\text{x}\) 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 SPS confocal microscope using a 63 x/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 HBSS-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 x/1.4 oil objective (1 μm = 105 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 (BIOLOG) 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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