Translocation of CaMKII to dendritic microtubules supports the plasticity of local synapses

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The processing of excitatory synaptic inputs involves compartmentalized dendritic Ca 2+ oscillations. The downstream signaling evoked by these local Ca 2+ transients and their impact on local synaptic development and remodeling are unknown. Ca 2+/calmodulin-dependent protein kinase II (CaMKII) is an important decoder of Ca 2+ signals and mediator of synaptic plasticity. In addition to its known accumulation at spines, we observed with live imaging the dynamic recruitment of CaMKII to dendritic subdomains adjacent to activated synapses in cultured hippocampal neurons. This localized and transient enrichment of CaMKII to dendritic sites coincided spatially and temporally with dendritic Ca 2+ transients. We show that it involved an interaction with microtubular elements, required activation of the kinase, and led to localized dendritic CaMKII autophosphorylation. This process was accompanied by the adjacent remodeling of spines and synaptic AMPA receptor insertion. Replacement of endogenous CaMKII with a mutant that cannot translocate within dendrites lessened this activity-dependent synaptic plasticity. Thus, CaMKII could decode compartmental synaptic plasticity to support remodeling of local synapses.

Introduction

Input-specific synaptic plasticity is thought to involve an activity-dependent and synapse-specific recruitment of proteins that affect synaptic strength. The “synaptic tag and capture” hypothesis proposed by Morris and colleagues (Frey and Morris, 1998; Redondo and Morris, 2011) postulates that modifications in the activated synapse lead to a tag that can then capture specific material among all of which is delivered throughout the dendritic arbors. However, to ensure appropriate delivery of material to activated synapses, a missing mechanism is one to guide such material from the dendrite into those synapses. How could this be achieved? One possibility would involve a signal, arising at the base of active spines within the dendritic shaft, which would impact on the local production and/or release of material for its subsequent translocation into active spines for capture.

Activity-dependent dendritic signals known to occur near active synapses are Ca 2+ spikes (Augustine et al., 2003), and an emerging concept in dendritic processing is the compartmentalization of the Ca 2+ events, possibly linked to clustered localized inputs (Chalifoux and Carter, 2011; Kleindienst et al., 2011; Takahashi et al., 2012). It has been proposed that the preferred site for long-term memory storage in neurons would reside in dendritic branches and subcompartments (Govindarajan et al., 2006, 2011), but the supporting signal processing inside dendrites is unknown. A major decoder of Ca 2+ spikes is CaMKII, a key enzyme in activity-dependent synaptic plasticity (Lisman et al., 2012). An interesting feature of CaMKII, which has been revealed by time-lapse imaging techniques, is that its Ca 2+/CaM activation can lead to its recruitment to sites where Ca 2+ rises, such as within activated synapses (Shen and Meyer, 1999; Colbran, 2004; Hudmon et al., 2005; Bayer et al., 2006). If CaMKII was also recruited to dendritic sites where Ca 2+ events occur, it might then engage specific signaling that is required to support the plasticity of neighboring synapses.

However, there is no clear evidence that CaMKII is timely located in relevant dendritic sites to be able to affect the plasticity of activated synapses. Using combinations of fluorescent proteins transfected in cultured hippocampal neurons for dual-color time-lapse optical imaging, we examined the spatial and temporal dynamics of CaMKII with those
of Ca$^{2+}$, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA receptors), and spines during protocols that induce synaptic activity and remodeling. Our experiments newly describe a dynamic process of localized dendritic translocation of CaMKII that requires local Ca$^{2+}$ activity and microtubular interactions, and which correlates with localized synaptic plasticity.

A role for CaMKII dendritic translocation in supporting synaptic plasticity is further corroborated by replacing CaMKII with a mutant that cannot translocate specifically to dendritic domains. Our experiments suggest that CaMKII has a role in dendritic processing, impacting on localized synaptic remodeling.

**Results**

**Synaptic stimulation leads to the local accumulation of CaMKII to dendritic domains**

Several studies have shown that CaMKII translocates to postsynaptic sites after the application of glutamate or NMDA in cultured neurons (Shen and Meyer, 1999; Bayer et al., 2001, 2006; Otmakhov et al., 2004; Hudmon et al., 2005). This translocation requires Ca$^{2+}$ influx and the Ca$^{2+}$/CaM activation of the enzyme which in turn binds to postsynaptic partners including the N-methyl-D-aspartate receptor (NMDAR). Meanwhile, the activation of a subset of excitatory synapses has been shown to increase Ca$^{2+}$ in dendritic subdomains (Augustine et al., 2003; Kleindienst et al., 2011; Takahashi et al., 2012). We thus wondered whether these dendritic Ca$^{2+}$ events might recruit CaMKII. We performed time-lapse imaging on cultured neurons that were transfected with mGFP-αCaMKII and stimulated with a protocol (0Mg$^{2+}$/Glycine) known to favor synaptic NMDAR activation and long-term potentiation in culture (cLTP; Lu et al., 2001). Whereas glutamate/glycine (Glu/Gly) stimulation led to the translocation of mGFP-αCaMKII to nearly all postsynaptic sites (Fig. S1 A; Hudmon et al., 2005; Bayer et al., 2006), cLTP stimulation led to its translocation to a subset of excitatory synapses (Fig. 1 A; Fig. S1 B), presumably those where sufficient presynaptic glutamate was released. However, we also observed an increase in mGFP-αCaMKII fluorescence immediately adjacent to several of those synapses, as dendritic hot spots (Fig. 1 A; Video 1).

The accumulation of mGFP-αCaMKII in dendritic domains generally appeared immediately at the base of activated excitatory synapses, whereas the surrounding domains (~5 µm away from the activated synapse) exhibited a decrease in fluorescence (Fig. 1 B; Fig. S1 B). In cases where neurons responded more strongly to the stimulation by exhibiting synaptic translocation of CaMKII to every spine, we did not observe localized dendritic hot spots (as with bath application of glutamate or NMDA; Fig. S1 A). Spontaneous neuronal activity in standard solution (Fig. S1 C; Video 2) or during increased activity in the presence of GABA$_A$ receptor blocker bicuculline (Fig. S1 D) also triggered dendritic translocation, but not in activity-blocking solution. We also observed, by immunostaining, the accumulation of endogenous αCaMKII into local dendritic hot spots (Fig. S2 A).

Dendritic hot spots of mGFP-αCaMKII ranged from ~1–10 µm in length and could be observed anywhere throughout the dendritic arbors. We counted from 0 to 20 dendritic translocation sites per neuron analyzed during a 5-min stimulation period (mean 7.4 ± 1.5; n = 16 neurons). Although a fraction of mGFP-αCaMKII that translocated to postsynaptic sites during stimulation was retained upon washout, as described previously (Bayer et al., 2006), the dendritic accumulation of CaMKII was fully reversible (Fig. 1 B; percentage of retention: synapses [32.2 ± 7.0], dendrites [1.4 ± 4.8], P = 0.0024 two-sided signed rank test; n = 12 pairs of synaptic/dendritic regions, 6 neurons). Repetitive 0Mg$^{2+}$/Gly stimulations could induce dendritic hot spots of mGFP-αCaMKII, either at the same location or occasionally at new dendritic sites (Video 3). Blocking NMDARs with 50 µM D-AP5, Na$^{+}$ channels with 1 µM TTX, or voltage-gated Ca$^{2+}$ channels with 10 µM Cd$^{2+}$ strongly reduced the accumulation of CaMKII to localized dendritic sites. Blocking Ca$^{2+}$ release from intracellular stores with 10 µM CPA partially reduced (~40%) the subdendritic translocation of the kinase (Fig. 1 C). The effects of these pharmacological blockers indicate that the translocation of CaMKII to subdendritic regions is dependent on the rise of intracellular free Ca$^{2+}$.

**CaMKII translocates to dendritic sites of higher Ca$^{2+}$ concentration**

To test whether this localized dendritic accumulation of CaMKII spatially and temporally coincides with Ca$^{2+}$ elevation, we monitored in the same neurons the dynamics of mCherry-αCaMKII and GCaMP2, a genetically encoded Ca$^{2+}$ indicator (Talini et al., 2006). At the onset of the 0Mg$^{2+}$/Gly stimulation, Ca$^{2+}$ transiently increased throughout the neurons, but then hot spots of elevated free Ca$^{2+}$ oscillations appeared in dendritic segments (Fig. 2 Aii; Video 4). Interestingly, mCherry-αCaMKII gradually accumulated in those same dendritic regions, but not in regions where the elevation in Ca$^{2+}$ was less pronounced (Fig. 2 A). These results indicate that CaMKII translocates to regions where Ca$^{2+}$ reaches a significant level during synaptic transmission, which includes active spines and specific dendritic domains.

**Confined dendritic translocation of CaMKII evoked by local glutamate/glycine application**

To control more directly the localization of αCaMKII translocation inside dendrites, we caused a local elevation of Ca$^{2+}$ by stimulating a short dendritic segment with Glu/Gly application from a patch pipette placed within a few micrometers of a dendrite. We confirmed that the response was local by monitoring free Ca$^{2+}$ elevation with GCaMP2, while observing CaMKII translocation with mCherry-αCaMKII (Fig. 2 B). The application of Glu/Gly led to a rapid and localized increase in dendritic Ca$^{2+}$ near the pipette, which was maintained during a continuous train of stimulating puffs. Meanwhile, a gradual accumulation of mCherry-αCaMKII occurred in the same confined dendritic location (Fig. 2 B).

**Dynamic translocation of CaMKII to microtubular elements**

To test whether the local accumulation of CaMKII within dendritic segments arises from its binding to intracellular domains, we performed FRAP measurements (Fig. 3 A) during...
During the stimulation, CaMKII was less mobile at the sites where it was concentrated compared with the adjacent region, whereas there was no significant difference between those regions after the stimulation. This result suggests that...
**iii)** mCherry-αCaMKII GCaMP2 (Ca^{2+})

Before

0Mg^{2+}/Gly 5min

Wash 30min

Intensity scale bar

Max.Min

**ivi)** D+

50% ΔF/F₀

2min

**v)** Mean ΔF/F₀ at stim

![Graph showing mean ΔF/F₀ at stim for D+ and D- conditions.](image)

**vii)** mCherry-αCaMKII GCaMP2 (Ca^{2+})

Before

Puffs Glu/Gly 5min

Wash 10min

Intensity scale bar

Max.Min

50% ΔF/F₀

5min

**viii)** D+

![Graph showing mean ΔF/F₀ at stim for D+ and D- conditions.](image)
localized differences in CaMKII binding lead to its accumulation in dendritic domains.

Which dendritic domains could CaMKII bind to? Many years ago, Schulman (1984) identified a microtubule-associated protein (MAP2) as a major substrate of CaMKII. Other MAPs have since then been described as CaMKII substrates (Baudier and Cole, 1987; Baratier et al., 2006). Electron microscopy studies have also revealed CaMKII to be in close proximity with microtubules and neurofilaments (Vallano et al., 1986). Our optical measurements cannot easily resolve microtubules in thin dendrites. However, in larger proximal dendrites, a fiber-like pattern of fluorescence at the base of postsynaptic sites during the 0Mg²⁺/Gly stimulation became apparent (Fig. 3 B; Video 5).

If CaMKII indeed binds to microtubular elements upon Ca²⁺ elevation, then global stimulation of a neuron should trigger microtubular patterns of mGFP-αCaMKII fluorescence in the soma, where microtubules are easier to detect. Incubating the cells in a ringer solution that blocks much of the synaptic and electrical activities (high Mg²⁺/low Ca²⁺ solution) revealed a diffuse pattern of fluorescence of mGFP-αCaMKII throughout the neurons (Fig. 3 C, top). By contrast, a global stimulation (40 mM KCl or Glu/Gly) led to the rapid appearance of fiber patterns of fluorescence clearly visible in the soma and proximal dendrites (Fig. 3 C, middle; Video 6), accompanied by the postsynaptic translocation of the enzyme throughout the dendritic trees. To confirm that CaMKII indeed “decorates” microtubular elements after stimulation, we used immunostaining for endogenous αCaMKII and cytoskeletal markers. The enzyme colocalized strongly with tubulin (Fig. S2, B and D), but less with neurofilaments (NF-M; Fig. S2, C and D). To observe this microtubule decoration dynamically, we also cotransfected neurons with mCherry-αCaMKII and GFP-MAP2B, which revealed a clear increase in overlap during depolarization (Fig. 3 D). Interestingly, the overexpression of GFP-MAP2B by itself increased the level of mCherry-CaMKII localization to microtubular elements, even before stimulation (Fig. 3 F). To validate further the interpretation that CaMKII binds to microtubular elements during neuronal activity, we treated neurons with nocodazole for 90 min, to partially destabilize microtubules (Conde and Cáceres, 2009; Fig. S3). Fig. 3 E shows that indeed the partial destabilization of microtubules reduced CaMKII binding in dendrites during stimulation. The remaining fraction of activity-dependent binding occurs potentially on the stable microtubules that resisted nocodazole treatment (Fig. S3). These results argue that the binding to microtubular domains is responsible for the reduced mobility of CaMKII in active dendrites, and that upon neuronal stimulation CaMKII translocates to distinct sites: microtubular elements and postsynaptic sites.

βCaMKII is known to interact with F-actin filaments, but when neurons are stimulated, Ca²⁺/CaM activation of the enzyme disrupts the interaction (Shen and Meyer, 1999). We imaged mGFP-βCaMKII and observed that its fluorescence went from an F-actin–like punctuated pattern to a mixture of microtubular and postsynaptic patterns (Fig. S4 C). After synaptic stimulation (0Mg²⁺/Gly), we also observed the confined dendritic accumulation of βCaMKII near activated synapses (Fig. S4, A and B). Thus, the two main subunits of CaMKII in the brain translocate to microtubular domains and postsynaptic sites upon neuronal stimulation.

Mutagenesis analysis of CaMKII translocation to microtubular elements

To investigate the mechanisms of CaMKII binding to microtubular domains, we compared the activity of various CaMKII mutants to redistribute to somatodendritic fiber-like regions in response to KCl stimulation. To minimize the impact of possible mixing of endogenous CaMKII with the mutants, we co-transfected shRNAs for both the α and β isoforms, along with shRNA-resistant mGFP-CaMKII mutants. This protocol reduced the amount of endogenous α- and βCaMKII down to ~19 ± 2% and 26 ± 2%, respectively (Fig. S5, B and C). Those shRNA treatments did not impact on the peak Ca²⁺ response of the neuron during a KCl stimulation (Fig. 4 A), and cotransfected mGFP-αCaMKII wild-type (WT) resistant to shRNA normally exhibited microtubular translocation (Fig. S5 A; Fig. 4 B).

The tested CaMKII mutants were selected on the basis of previous work on synaptic translocation of CaMKII (Bayer et al., 2001, 2006; Hudmon et al., 2005; Marsden et al., 2010) and the mutagenesis analysis by Yang and Schulman (1999). In analyzing the KCl-evoked redistribution of the fluorescence for the different constructs, we can conclude that the activity-dependent translocation of CaMKII to microtubular elements shares a number of similarities with that to spines. First, it requires its Ca²⁺/CaM activation, as demonstrated by the lack of microtubular translocation of the mutant (T305-306D) that has impaired binding to Ca²⁺/CaM (Fig. 4 D; Rich and Schulman, 1998). Moreover, adding 10 µM KN93, a drug preventing CaMKII activation, also blocked its microtubular translocation (but not its inactive analogue KN92; Fig. 4 E). We found that preventing (T286A) or mimicking (T286D) autophosphorylation had little impact on microtubular translocation of the enzyme (Fig. 4, C and D). The complete multimeric structure of the enzyme is required for postsynaptic translocation (Bayer et al., 2006). Likewise, a CaMKII monomer (aa 1–326) or its association domains (aa 315–478) could not translocate to microtubular elements. The kinase domain T-site seems also involved in microtubular binding because the I205K mutation

Figure 2. αCaMKII translocates to dendritic sites of higher Ca²⁺ concentration. (A) Neurons expressing mCherry-αCaMKII and GCaMP2B stimulated for 5 min with 0Mg²⁺/Gly [Ai, iii] or [B] by local puffs (10-ms puffs at 2 Hz during 13 min) of Glu/Gly [100/10 µM; Bi, iii]. Brackets indicate CaMKII dendritic hot spots [D+, white] or adjacent dendritic region [D−, red]. (Ai and Bii) Kymograph of the change in intensity of GCaMP2 and mCherry-αCaMKII over time across the dendritic region shown above (white line). Stimulation period is indicated (0Mg²⁺/Gly or Puffs). [Ai and Bii] Time-lapse analysis of mCherry-αCaMKII (purple) and GCaMP2 (green) intensity in a region of dendritic CaMKII translocation [D+; top graph] or in an adjacent region [D−; bottom graph]. [Ai and Bii] Mean ±SEM ∆F/F0 (in paired regions as in ii–iii) of mCherry-αCaMKII (purple) and GCaMP2 (green) during the first 5 min of stimulation. [Ai] n = 12 neurons, 52 dendritic segments. [Bii] n = 5 neurons, 5 dendritic segments. Bars: [neurons] 10 µm; [dendrites] 5 µm. *, P < 0.01 paired t test. See also Video 4.
Figure 3. Activity-dependent translocation of αCaMKII to microtubules. (Ai) Time series of FRAP of mGFP-αCaMKII during a 0Mg²⁺/Gly stimulation in a dendrite, either where CaMKII has concentrated (D⁺, white brackets) or not (D⁻, red brackets). White circles indicate regions of photobleaching. Bar, 5 μm. (Aii) FRAP curves [bleaching at t = 3 s] and (Aiii) mobile fractions of CaMKII during and after a 0Mg²⁺/Gly stimulation in corresponding regions. (B) Dendrite from a neuron expressing mGFP-αCaMKII stimulated with 0Mg²⁺/Gly for 5 min. Bar, 5 μm. (C–Di) Time-lapse imaging of mGFP-αCaMKII (C) or mCherry-αCaMKII and GFP-MAP2B (D) during a 1-min stimulation with KCl. Bars: (neurons) 10 μm; (inset) 2 μm. (Ei) Time series of FRAP of mGFP-αCaMKII before (top) and during (bottom) a 1-min KCl stimulation. White circles indicate regions of photobleaching. Bar, 3 μm. (Eii) FRAP curves and (Eiii) mobile fractions before and during the stimulation on neurons treated or not with 5 μM nocodazole. Arrows in B, C, and D point to synaptic (red) or microtubule-like.
that blocked synaptic translocation (Bayer et al., 2001, 2006) also blocked the microtubular translocation (Fig. 4 D).

On the other hand, this analysis revealed differences between the mechanisms of microtubular and postsynaptic localization of CaMKII. The mutation E96K near the S-site kinase domain did not translocate to microtubules, but still translocated to synaptic sites. Surprisingly, two catalytically dead mutants of CaMKII, K42M and K42R, behaved differently. K42M was clearly already bound to microtubules at basal state, but not to postsynaptic sites, and stimulating the neurons did not lead to more microtubular localization (Fig. 4, C and D). Inversely, K42R did not bind microtubular domains, even after stimulation (Fig. 4, C and D), but did translocate to postsynaptic sites (Shen and Meyer, 1999). These results indicate that although the activity-dependent binding of CaMKII to microtubules bears significant similarities to that of postsynaptic sites, they nonetheless are different (Shen and Meyer, 1999; Marsden et al., 2010). The binding partners for CaMKII on microtubules versus postsynaptic sites are likely different, which may also explain the differences observed in the dissociation of the enzyme from both sites after stimulation (Fig. 1 Biv).

Activity of CaMKII in subdendritic hot spots

We next asked whether the CaMKII enriched in dendritic domains is engaged in signaling by measuring its autophosphorylation at state T286. Immunolabeling for phospho-T286 CaMKII revealed that the enzyme was indeed significantly more phosphorylated in dendritic segments where it was enriched compared with segments where it was not (Fig. 5 A and B). In larger dendrites of depolarized neurons, we observed autophosphorylated CaMKII decorating microtubular domains (Fig. 5 C). This observation indicates that CaMKII subdendritic accumulation coincides with increased local signaling of the enzyme.

Subdendritic translocation of αCaMKII correlates with synaptic GluA1-containing AMPARs

Because of the implication of CaMKII in dendritic exocytosis, cargo release and driving AMPARs to synapses (Maletic-Savatic et al., 1998; Hayashi et al., 2000; Karcher et al., 2001; Correia et al., 2008; Guillaud et al., 2008), as well as its renowned role in synaptic plasticity (Lisman et al., 2012), the dendritic translocation of the enzyme might allow for input-specific targeting of material that would be essential for such plasticity. Using the same culture preparation and cLTP protocol, we showed previously that CaMKII is involved in the activity-dependent synaptic recruitment of GluA1 tagged with pH-sensitive GFP (SEP-GluA1; Opazo et al., 2010). If the dendritic recruitment of CaMKII near active spines revealed here has an impact on this process, we would expect that it correlates with the recruitment of synaptic AMPARs. We therefore imaged SEP-GluA1 at synapses together with mCherry-αCaMKII to identify regions of synaptic and dendritic CaMKII translocation. We observed no significant AMPAR insertion at synapses that did not show synaptic CaMKII accumulation (S−) during the stimulation (Fig. 6). By contrast, synapses exhibiting CaMKII accumulation (S+) had increased levels of SEP-GluA1. However, within this category of CaMKII-positive synapses, we observed significantly more SEP-GluA1 in those associated with locally increased dendritic CaMKII (D+) compared with those without such increase (D−; Fig. 6). These results support a role for dendritic translocation of CaMKII in promoting further recruitment of AMPARs at synapses.

Subdendritic translocation of αCaMKII correlates with spine plasticity

In addition to its role in activity- and NMDAR-dependent accumulation of AMPARs to postsynaptic sites during LTP, CaMKII has been shown to promote spine volume increase (Matsuzaki et al., 2004; Xie et al., 2007; Yamagata et al., 2009; Pi et al., 2010) and the formation of new spines and filopodia (Toni et al., 1999; Jourdain et al., 2003). We thus also examined the possible correlation between dendritic CaMKII translocation and spine remodeling by cotransfected neurons with mCherry (as a structural marker) and mGFP-αCaMKII. Fig. 7 A shows an example of a spine growth in which CaMKII translocates to the spine before accumulating at the spine base during a 0Mg2+/Gly stimulation (Video 7). We quantified the changes in the number of spines and filopodia per dendritic segment (15 µm), containing (D+) or not (D−) dendritic hot spots of CaMKII, 30 min after stimulation. Although synaptic translocation occurred in both D+ and D− segments, we observed an increase in the number of protrusions in dendritic segments associated with increased CaMKII (Fig. 7 B), leading, 30 min later, to a greater increase in the number of dendritic protrusions compared with neighboring dendritic segments devoid of CaMKII accumulation (1.53 [D+] vs. 1.23 [D−]; Fig. 7 C). Interestingly, this differential increase in spine number in regions associated with dendritic CaMKII accumulation was partly due to a significant increase in the number of mushroom spines, which were 35% of all spines (Fig. 7 D). Thus, both spine formation and remodeling correlate with the local accumulation of CaMKII in adjacent dendritic regions. These results are consistent with the notion that CaMKII signaling on dendritic microtubules might regulate the local development and plasticity of synapses.

αCaMKII-E96K impairs the activity-dependent increase in synaptic GluA1 and spine remodeling

To demonstrate causality between CaMKII dendritic translocation and synaptic plasticity, we needed to replace CaMKII in the...
Figure 4. Mutagenesis analysis of CaMKII translocation to microtubules. (A) Mean (±SEM) ΔF/F₀ of GCaMP2 and (B) mean (±SEM) fold increase in mGFP-α-CaMKII microtubular translocation (normalized to control without shRNA) with or without coexpression of shRNA against α- and β-CaMKII isoforms during a 1-min KCl stimulation. n = 17–18 neurons (A) and 5 neurons (B) per condition. *, P < 0.05 unpaired t test. (C) Neurons transfected with shRNA against α- and β-CaMKII and expressing different shRNA-resistant GFP-CaMKII mutants, before and during a 1-min stimulation with KCl. White arrows point to detectable fibers in CaMKII signal. Bar, 10 µm. (D) Fold increase in microtubular translocation during the KCl stimulation for various mutants. (n = 6–24 neurons per condition, 4 experiments; asterisk indicates different from GFP). (E) Fold increase in the microtubular translocation during a 1-min KCl stimulation with or without KN drugs. (Vehicle: 0.1% DMSO; n = 13–18 neurons per condition, 3 experiments; asterisk indicates different from vehicle). *, P < 0.05 Kruskal-Wallis followed by Tukey’s least-significant difference test. See also Fig. S4.
neurons with a mutant that cannot exhibit this translocation, but which retains its catalytic activity and postsynaptic translocation ability. The mutant E96K did not translocate to microtubular domains (Fig. 4, C and D) and did not exhibit subdendritic translocation during a 0Mg²⁺/Gly stimulation (only 1 hot spot observed out of 8 neurons compared with a mean of 7 events per neuron for WT CaMKII), but could translocate to synapses as much as the WT (Fig. 8A). This mutation is known to maintain kinase activity (Yang and Schulman, 1999), which we confirmed in HEK293 cells using the activity reporter CFP-cVim (Fig. 8 B). We thus tested the impact of replacing endogenous αCaMKII by shRNA with this E96K mutant (resistant to the shRNA) on the synaptic recruitment of SEP-GluA1 and spine growth. Fig. 8, C and D, show that after synaptic stimulation, neurons expressing mCherry-αCaMKII-E96K exhibited significantly less insertion of surface SEP-GluA1 at their synapses and extended less spines as compared with neurons expressing mCherry-αCaMKII WT. These results reinforce the proposal that localized dendritic CaMKII translocation plays a role in synaptic remodeling of nearby synapses.

**Discussion**

In Fig. 9, we present a model describing a possible mechanism by which CaMKII might transduce synaptic activity down to the adjacent dendrite, where local processes would take place to support input-specific spine plasticity and insertion of AMPARs. The recruitment of CaMKII to segments of dendrites may also serve to translate the dendritic Ca²⁺ spikes evoked by clustered inputs (Chalifoux and Carter, 2011; Kleindienst et al., 2011; Takahashi et al., 2012). We also observed local increases in dendritic Ca²⁺ during synaptic stimulation in our culture preparation. Dendritic CaMKII accumulation coincided spatially and temporally with these local Ca²⁺ elevations. The NMDAR-dependent increase in CaMKII to dendritic segments was partly due to intracellular store release of Ca²⁺ because blocking them with CPA reduced CaMKII subdendritic accumulation. This is consistent with the observation that CPA reduced synaptically evoked, NMDAR-dependent Ca²⁺ transients (Emptage et al., 1999). Blocking Na⁺ and Ca²⁺ channels reduced dendritic CaMKII accumulation, probably by blocking evoked release of glutamate as well as back-propagating action potentials. The necessity for a confined Ca²⁺ gradient to trigger the localized dendritic translocation of the kinase likely explains why we and other groups (Shen and Meyer, 1999; Otmakhov et al., 2004; Hudmon et al., 2005; Bayer et al., 2006) have not observed this process when imaging mGFP-CaMKII with bath application of glutamate or NMDA, which causes a global increase in intracellular Ca²⁺ and the translocation of the kinase to microtubular domains throughout the dendrites. Glutamate uncaging onto single spines has been shown to cause only low Ca²⁺ elevation in their dendritic base (Sobczyk et al., 2005; Higley and Sabatini, 2008) and has not been associated with CaMKII accumulation at the spine base (Lee et al., 2009). However, dendritic depolarization by retrograde action potentials coupled with synaptic stimulation should synergistically induce a higher level of dendritic Ca²⁺ (Higley and Sabatini, 2008) that may involve NMDA spikes (Schiller et al., 1998, 2000; Chalifoux and Carter, 2011), which would favor localized dendritic translocation of CaMKII.
Accumulation of endogenous CaMKII in dendritic sections, independently of protein synthesis, was in fact observed in hippocampal slices by activating specific lamina with trains of high-frequency stimuli during a few minutes (Steward and Halpain, 1999). Using trains of local application of Glu/Gly, we observed a localized dendritic accumulation of CaMKII, which coincided spatially and temporally with the confined Ca$^{2+}$ increase. This is in contrast with what Rose et al. (2009) observed with a single 10–25-ms puff evoking the delayed synaptic translation of the enzyme throughout the entire somatodendritic area of neurons. We never observed this phenomenon upon local puffs of Glu/Gly. The reasons for this difference are unclear.

Figure 6. Subdendritic translocation of αCaMKII correlates with an increase of surface GluA1 at neighboring postsynaptic sites. (A) Dendrite coexpressing mCherry-αCaMKII and SEP-GluA1 stimulated with 0Mg$^{2+}$/Gly for 5 min. Brackets indicate CaMKII dendritic hot spots (D+, white) or adjacent dendritic region (D−, red). Arrows point to a spine near (1) or adjacent (2) to an mCherry-αCaMKII dendritic hot spot. (n = 7 neurons, 24–43 synapses per group). Bar, 5 µm. (B) Representative examples of SEP-GluA1 intensity at postsynaptic sites for synapses without (blue, S−) or with (S+) synaptic CaMKII translocation, which are near (black, S+D+) or not (red, S+D−) a CaMKII dendritic hot spot. Synapses 1 and 2 are pointed in A. Bar, 0.5 µm. (C–E) Time-lapse analysis (C), histogram (D), and cumulative distribution (E) of the increase in SEP-GluA1 for synapses in the same category as described in B. *, P < 0.05 Anova followed by Tukey’s least-significant difference test.
In contrast to our observations, Rose et al. (2009) reported a global Ca²⁺ elevation in response to their local stimulation, which may have set in motion a different type of cell-wide response (Branco and Häusser, 2010).

Our results suggest that Ca²⁺ elevation causes CaMKII binding to microtubules and/or associated proteins. Any cellular organelle, cargo, or neurofilaments linked to microtubules could be targets for CaMKII binding. The list of known CaMKII substrates associated with microtubules, such as MAP2, Tau, MAP6, kinesins, and neurofilaments, to name a few (Schulman, 1984; Baudier and Cole, 1987; Hanson and Schulman, 1992; Baratier et al., 2006), is growing. It is likely that many of these support the rapid and reversible recruitment of the enzyme to microtubular domains upon Ca²⁺ elevation. In fact, overexpression of MAP2 increased the basal decoration of microtubular domains by CaMKII. Although this result might support MAP2 as a strong candidate, the overexpression of MAP2 could have also further stabilized microtubules and hence favored additional CaMKII-binding interactions.

On the kinase side, the mechanism of binding to microtubules bears similarities and differences in comparison to that of postsynaptic sites. Kinase activation, i.e., the displacement of the inhibitory domain but not its activity, per se, is required for both sites, as it is blocked by KN93 and by mutating T305/306 to D. Thus, displacement of the auto-inhibitory domain is likely to expose the required binding sites on the kinase.

The case of K42M is intriguing as it behaves differently than K42R, two mutants initially generated to prevent CaMKII catalytic activity by interfering with ATP binding (Hanson et al., 1994). It is possible that replacing the lysine with a methionine changes the enzyme conformation more drastically than the arginine, which retains the positive charge.

The unbinding kinetics observed between dendritic and postsynaptic translocation are distinct. We and Bayer et al. (2001, 2006) proposed earlier that the persistent binding of CaMKII to postsynaptic sites, after its activity-dependent translocation, is mediated through a tight interaction between the T-site of the kinase and the cytoplasmic tail of the NMDA receptor subunit GluN2B. By contrast, the microtubular binding did not show persistence after stimulation. The T-site nevertheless appears to be involved because the I205K mutant did not show microtubular translocation.

The multimeric arrangement of CaMKII is required for both sites of translocation, suggesting that binding of the enzyme is favored by multivalent interactions rather than a single binding interaction as in the monomeric mutant (1–326). Auto-phosphorylation at T286 seems to have little effect on dendritic translocation, whereas it can modulate its translocation in contrast to our observations, Rose et al. (2009) reported a global Ca²⁺ elevation in response to their local stimulation, which may have set in motion a different type of cell-wide response (Branco and Häusser, 2010).

Our results suggest that Ca²⁺ elevation causes CaMKII binding to microtubules and/or associated proteins. Any cellular organelle, cargo, or neurofilaments linked to microtubules could be targets for CaMKII binding. The list of known CaMKII substrates associated with microtubules, such as MAP2, Tau, MAP6, kinesins, and neurofilaments, to name a few (Schulman, 1984; Baudier and Cole, 1987; Hanson and Schulman, 1992; Baratier et al., 2006), is growing. It is likely that many of these support the rapid and reversible recruitment of the enzyme to microtubular domains upon Ca²⁺ elevation. In fact, overexpression of MAP2 increased the basal decoration of microtubular domains by CaMKII. Although this result might support MAP2 as a strong candidate, the overexpression of MAP2 could have also further stabilized microtubules and hence favored additional CaMKII-binding interactions.

On the kinase side, the mechanism of binding to microtubules bears similarities and differences in comparison to that of postsynaptic sites. Kinase activation, i.e., the displacement of the inhibitory domain but not its activity, per se, is required for both sites, as it is blocked by KN93 and by mutating T305/306 to D. Thus, displacement of the auto-inhibitory domain is likely to expose the required binding sites on the kinase.

Figure 7. Subdendritic translocation of αCaMKII correlates with spine remodeling. (A and B) Dendrite coexpressing mGFP-αCaMKII and mCherry stimulated with 0Mg²⁺/Gly for 5 min. Arrows point to spines (red), spine morphological change (pink), new protrusion (yellow), and spine elongation (green). White brackets indicate regions of dendritic CaMKII hot spot (D+). (C) Cumulative distribution and associated histogram of the fold increase in the number of spines on 15-µm dendritic sections, associated with regions revealing dendritic CaMKII accumulation (D+) or not (D–). (D) Histogram of the mean ± SEM increase in spine number classified by spine morphology and associated with regions revealing dendritic CaMKII accumulation (D+) or not (D–). (n = 19 neurons, 154 dendritic sections). * P < 0.05 two-sided rank sum test. Bars: (A) 2 µm; (B) 5 µm. See also Video 7.
some substrates. In fact, transient binding to microtubular substrates may be what supports the dendritic relocation that we have observed. Further investigation is needed to dissect the detailed mechanism of CaMKII microtubular translocation.

What are, then, the functions of CaMKII binding to microtubular domains? They likely depend on the localization of this binding, such as the soma, dendrites, the axon, or even the growth cone, and on the activity pattern that leads to this interaction.

Figure 8. E96K, an active mutant of αCaMKII that translocates to synapses, but not to microtubules, impairs activity-dependent increase in surface GluA1 synaptic insertion and spine remodeling. (Ai) Neurons transfected with mGFP-αCaMKII WT or E96K and Homer1c-DsRed and stimulated with KCl for 1 min. (ii) Fold increase in CaMKII postsynaptic translocation, cotransfected or not with Homer1c-DsRed during the KCl stimulation. Because CaMKII postsynaptic translocation was not different with or without Homer1c coexpression, all neurons were pooled together (n = 13–22 neurons per condition, 6 experiments). (Bii) HEK cells cotransfected with mGFP-αCaMKII WT, E96K or K42M, and CFP-cVim. The cells were stimulated (HBSS + 10 µM ionomycin + 5 mM Ca²⁺) or not (HBSS) for 5 min, fixed, and immunolabeled for phospho-Vim. (Bii) Quantification of cVim phosphorylation by αCaMKII WT, E96K, and K42M. The intensity of pVim immunolabeled was divided by the intensity of both total cVim and CaMKII. n = 351–536 cells per condition, 2 experiments. Bars, 5 µm. (A and B) *, P < 0.05 Kruskal-Wallis followed by Tukey’s least-significant difference test. (C) Histogram and cumulative distribution of the fold increase in SEP-GluA1 for synapses of neurons with synaptic translocation (S+) of mCherry-αCaMKII WT or E96K rescue, in the presence of shRNA against αCaMKII, during a stimulation with 0Mg²⁺/Gly for 5 min. (n = 8 neurons, 272–300 synapses per group). (D) Histogram and cumulative distribution of the fold increase in the number of spines on 15-µm dendritic sections in neurons expressing mGFP-αCaMKII WT or E96K rescue as well as mCherry and shRNA against αCaMKII. (n = 6–8 neurons, 63–82 dendritic sections). (C and D) *, P < 0.05 two-sided rank sum test.
Figure 9. **Model of CaMKII subdendritic translocation and its impact on synaptic plasticity.** (A) During basal activity, intracellular free Ca$^{2+}$ is low and a large fraction of CaMKII freely diffuses throughout the neuron. (B) During elevated synaptic activity, Ca$^{2+}$ increases in spines and in nearby dendritic compartments. CaMKII translocates to activated synapses and subdendritic regions by binding to synaptic and microtubular partners, respectively. This local accumulation where Ca$^{2+}$ activity is high leads to a depletion of the enzyme concentration on adjacent segments where Ca$^{2+}$ activity is lower. The localized dendritic activation of CaMKII leads to a confined phosphorylation of substrates in this region. Hypothetically, CaMKII target may be proteins involved in receptor trafficking. Cargos containing AMPARs could then be released by CaMKII phosphorylation, thereby facilitating their delivery to activated synapses. (C) After activation, CaMKII largely unbinds from microtubules but only partially from strongly activated synapses, which remain larger and retain more AMPARs.
In the case of global, high frequency firing of the neuron, leading to global free Ca$^{2+}$ elevation, we would expect the enzyme to bind throughout the microtubular network, and thereby transmit information about the activity state of the cell down to its core cytoskeleton. The large number of substrates for CaMKII on microtubules offers multiple downstream functions such as dendritic remodeling, active transport and delivery, and exocytosis. In the case of local synaptic activity into a specific dendritic branch or segment, the binding of CaMKII to microtubular elements is restricted to that subcellular region, leading to local signaling, which might impact on local synaptic remodeling.

We examined two specific features of synaptic remodeling: the recruitment of AMPARs and spine growth. Our results show an enhanced accumulation of GluA1 at synapses where CaMKII accumulated and particularly more in those located near a hot spot of dendritic CaMKII as well. Beyond this correlation, the replacement of endogenous CaMKII by the E96K mutant argues for causality between the localized dendritic translocation and the local plasticity. This enrichment of receptors might be due to their increased exocytosis in the CaMKII-rich dendritic region. Indeed, CaMKII regulates dendritic exocytosis (Maletic-Savatic et al., 1998), the exocytosis of AMPARs was observed in the portion of the dendrite near stimulated spines (Makino and Malinow, 2009; Patterson et al., 2010), and the insertion of new GluA1 in synapses correlates with a dendritic Ca$^{2+}$ transient (Korkotian and Segal, 2007). Recently, however, the activity-dependent exocytosis of AMPARs, evoked by glutamate uncaging, was shown to require the Ras-ERK pathway, independently of CaMKII (Patterson et al., 2010). Conceivably, the presence of TTX, used in this uncaging protocol, reduced Ca$^{2+}$ elevation and CaMKII activity in the dendrite, thereby masking a possible contribution of the enzyme in additional GluA1 exocytosis.

Another key function proposed for CaMKII has been the regulation of transport proteins involved in AMPAR trafficking. The kinase might release GluA1, contained in Rab11 endosomes located notably at the base of spines (Park et al., 2006), possibly by phosphorylating myosin V (Karcher et al., 2001). The activity- and myosin V–dependent translocation of recycling endosomes into spines was shown to support both spine enlargement and AMPAR delivery (Wang et al., 2008). Thus, the enrichment of CaMKII at the base of active spines via binding to microtubules provides a possible mechanism to specifically release AMPARs at the appropriate synapse. Clustering of AMPARs into the synaptic cleft can then be facilitated also by CaMKII via the phosphorylation of stargazin C terminus (Opazo et al., 2010). This phosphorylation could potentially occur on cargo vesicles or at the plasma membrane. In addition to CaMKII’s role in regulating morphology of existing spines, the enzyme plays a role in the formation and elongation of new protrusions during LTP (Toni et al., 1999; Jourdain et al., 2003). Synaptogenesis is accompanied by confined dendritic Ca$^{2+}$ (Lohmann and Bonhoeffer, 2008), which might support the developmental process through CaMKII. βCaMKII has been linked to filopodial extension and synapse formation (Fink et al., 2003). Because this subunit also exhibits subdendritic translocation, it may have contributed to the effects observed on GluA1 recruitment or spine remodeling in combination with αCaMKII, which would likely accumulate at the same sites. The enzyme might also participate in the activity-dependent change in microtubules that affects molecular transport (Maas et al., 2009). Localized CaMKII could also support local translation of mRNA—including its own αCaMKII mRNA, required for plasticity (Sutton and Schuman, 2006).

Our results describe a potential mechanism supporting input-specific synaptic plasticity (Fig. 9). By causing elevation in Ca$^{2+}$ at their base, active excitatory synapses could then act on the dendritic delivery of material that supports and/or enhances their own plasticity. This mechanism would complement the synaptic tagging and capture hypothesis, for which CaMKII has also been proposed to constitute a key element (Frey and Morris, 1998; Redondo and Morris, 2011). The role as a synaptic tag may be supported by its ability to reside in the PSD for a prolonged time after its translocation (Bayer et al., 2006), coupled to its locked active state when bound to GluN2B (Bayer et al., 2001, 2006), and its multivalent structure favoring multiple interactions with other synaptic partners and scaffolding with other CaMKII holoenzymes (Hudmon et al., 2005; Robison et al., 2005). It should be noted that LTP of an individual spine can be evoked by causing Ca$^{2+}$ elevation only inside the spine and not in the dendritic shaft, as shown with uncaging approaches (Lee et al., 2009). Thus, dendritic CaMKII is not necessary for certain forms of LTP, although in some cases it might enhance it. In fact, localized synaptic depolarization and/or dendritic spikes have been reported to induce stronger LTP than that induced by back-propagating action potentials (Hardie and Spruston, 2009). These observations suggest that spatial proximity of inputs could favor stronger LTP. Proximity to potentiated synapses helps weaker inputs to generate LTP, but only shortly after (Harvey and Svoboda, 2007); localized and transient dendritic modifications could support such a process, and CaMKII could thus be involved. The proposal that the dendritic branch or segment is the critical site of integration in long-term storage (Govindarajan et al., 2006, 2011) is appealing and the localized dendritic recruitment of CaMKII could be part of the process. CaMKII is a multifunctional enzyme that regulates many aspects of neuronal function and synaptic plasticity. Further studies are needed to better understand the functional consequences of CaMKII recruitment to microtubular domains near activated synapses, but numerous potential implications can be drawn.

Materials and methods

Rat hippocampal cultures, plasmids, and transfection
Dissociated hippocampal neurons were prepared as described previously (Hudmon et al., 2005; Nault and De Koninck, 2010). In brief, hippocampi dissected out of P1-P3 rats were dissociated enzymatically (papain, 12 U/ml; Worthington) and mechanically (trituration through Pasteur pipette). After dissociation, the cells were washed, centrifuged, and plated on poly-L-lysine–coated Aclar (12 mm) or glass (18 mm) coverslips at a density of ~350–450 cells/mm$^2$. Growth media consisted of Neurobasal and B27 (50:1), supplemented with penicillin/streptomycin (50 U/ml; 50 µg/ml) and 0.5 mM L-glutamax (Invitrogen). Fetal bovine serum (2%; Hyclone) was added at time of plating. After 5 d, half of the media was changed without serum and with Ara-C (5 µM; Sigma-Aldrich) to limit proliferation of non-neuronal cells. Twice a week thereon, half of the growth medium was replaced with serum- and Ara-C–free medium.
Neurons were transfected at 11–15 DIV using Lipofectamine 2000 (Invitrogen) as described previously (Hudsom et al., 2005). When shRNA were used, neurons were transfected at 8 DIV and imaged at 11 DIV. mGFP–CaMKII and mGFP–βCaMKII plasmids contained a mutation (A207K) in the eGFP (Takara Bio Inc.) to eliminate GFP-dependent aggregate (Zacharias et al., 2002; Hudsom et al., 2005). mCherry and mCherry–CaMKII plasmids were generated by PCR amplification of the cDNA fragment encoding mCherry from plenti-CaMKII-I-Sec82-mCherry-VPRE (a gift from Karl Deisseroth, Stanford University, Stanford, CA) with primers 5′-GCCGTACCCGTCGCCACCATGGTAGAAGGCGGAGGAGAATAAC3′ and 5′-CTTGAACACGTCGTCGCCGC3′. The PCR product was then digested with Agel–BsrGI and inserted into the Agel–BsrGI sites of mGFP–CF1 or mGFP–CaMKII. α- and β CaMKII shRNAs were obtained from Yasinari Hayashi (Riken Brain Science Institute, Saitama, Japan) and Sang Lee (Medical College of Wisconsin, Milwaukee, WI). mGFP–CaMKII WT-rescue was described previously (Opazo et al., 2010). mGFP–βCaMKII WT-rescue was generated by inserting silent mutations on mGFP–βCaMKII WT plasmid: 5′-GAGTACGGCAGAAAATAA3′ compared with the βCaMKII shRNA target sequence: 5′-GAGTATACGCTAAA–GATCA-3′. mGFP–CaMKII K42M and K42R-rescue were obtained by digestion of mGFP–CaMKII WT-rescue with PfiI and insertion into the PfiI sites of mGFP–CaMKII K42M or K42R (generated by mutagenesis). Rescue of αCaMKII E96K, I205K, and T305–306D were generated by mutagenesis to insert the silent mutations and verified by sequence analysis. αCaMKII T286A and T286D rescue were generated by digestion of αCaMKII WT-rescue with BamH1 and inserted into the BamH1–BbvCI sites in mGFP–CaMKII T286A and T286D, respectively (Hudsom et al., 2005). Monomeric αCaMKII (aa 1–236) and the association domain (aa 315–478) were subcloned into the mGFP–CaMKII backbone using a PflmI restriction insert (Hudsom et al., 2005). mCherry–CaMKII WT-rescue and E96K-rescue were obtained by replacing mGFP with mCherry using Agel–BsrGI. CFP–C-Vim–NES was constructed using FCT1–2×Vim–CFP–NES (provided by J. Tsai and H. Schuman, Stanford University, Stanford, CA) digested with BamH1 and Mefl and introduced into an enhanced GFP vector (Takara Bio Inc.). SEP–GluA1 plasmid was generated by PCR amplification of the cDNA encoding Superc elliptic pHLuorin (obtained from G. Miesenbock, University of Oxford, Oxford, UK) with primers 5′-GATCA-3′ and 5′-GATCCAGCTAGTGAAGAGAGAAACT3′ and 5′-GATGAACGGAATTCCTGTAACACGCTG3′ and 5′-GATCA-3′. The PCR product was then digested with NheI–BsiWI and inserted into the NheI–BsiWI sites of mGFP–GluA1 (obtained from G. Miesenbock, University of Oxford, Oxford, UK) digested with BamH1 and MfeI and introduced into an enhanced GFP vector (Takara Bio Inc.). SEP–GluA1 plasmid was then digested with NheI–BsiWI and inserted into the NheI–BsiWI sites of YFP–GluA1 (obtained from G. Miesenbock, Brandeis University, Waltham, MA). GCaMP2, subcloned into pcPEF-N1 vector (Takara Bio Inc.) with BgIII and NotI (Tallini et al., 2006), was obtained from J. Nakai (RIKEN Brain Science Institute, Saitama, Japan) and Sang Lee (Medical College of Wisconsin, Milwaukee, WI). To help the visual detection of CaMKII subcellular translocation, we substracted each image of a maximum projection of the images before the stimulation. Possible regions of CaMKII dendritic accumulation were determined in those treated images as a series of contiguous pixels with higher intensity during the stimulation. The region was subsequently verified by examining the raw images to avoid false positive. A line was traced along the dendritic region for each detected CaMKII hot spot and the sum of dendritic length of all those regions for a neuron was calculated and normalized to the total dendritic length in the field of view. To evaluate if the signal from synapses pruning toward the zaxis could be responsible for the subdendritic translocation observed, we acquired z-stacks of thin optical slices by confocal microscopy. We have not found evidence that superimposed spines could account for the dendritic signal.

**Dual Ca**2+ (GCaMP2) and mCherry–CaMKII analysis. Dendritic regions exhibiting CaMKII subcellular translocation were detected by analyzing an image at the end of the stimulation. Circular regions of φ = 1–3.5 μm were traced, one at the center of each detected site and another one at the end of the stimulation. Average intensity in those regions was measured over time (f) and corrected by the mean average intensity before the stimulation (F0) to obtain ΔF/Fo for both GCaMP2 and mCherry-CaMKII. Over the course of the imaging, we noticed that GCaMP2 signal slightly decreased, possibly due to bleaching and photo-isomerization (Bengtson et al., 2010). Analysis of mGFP–CaMKII translocation to microtubules. CaMKII translocation to microtubules was automatically analyzed in MetaMorph in a region that includes the soma and proximal dendrites. Each image of a z-stack was first deconvolved and a maximum projection was obtained for each time point. A sliding threshold ranging from the image minimum to maximum values in increments of 2 was applied. To detect above-threshold groups of contiguous pixels that corresponded to fiber-like structures, we used four criteria: pixel length corresponding to 2–100 μm, fiber breadth (1 μm and 5 μm, respectively) area from 0.2–5 μm2, and elliptical form factor (length/breadth) > 3.5. We summed the intensity of all regions detected for each image and normalized it to the total intensity of the cell. We averaged the ratio obtained for the images before the stimulation (before) and at the end of the stimulation (stim: last 30 s) and calculated a fold increase stim/before.

mGFP–CaMKII translocation to postsynaptic site. CaMKII translocation was automatically analyzed in MetaMorph by measurements of the contrast between synapses and neighboring non-synaptic areas. Each image was acquired separately. A sliding threshold in increments of 10 ranging from the image minimum to maximum values was applied. To detect above-threshold groups of contiguous pixels that corresponded to synapses, we used two criteria: pixel area corresponding to 0.15–2 μm2 and shape factor (4πA/P2; P, perimeter; A, area) > 0.6. We obtained a maximum projection for each time point of the background-subtracted cell and of the detected clusters. The "synaptic
contrast" is [mean pixel intensity of each object] – [threshold value at which it was detected]) / [mean value of all nonzero pixels of the image], averaged over all objects. When mGFP-CaMKII was cotransfected with Homer1c-DsRed (Fig. 8, Aii), Homer synaptic clusters were detected and tracked over time. For each cluster, a size-adapted square ROI was determined based on morphological analysis and was drawn on each image, according to the tracked position, to measure the associated average intensity of mGFP-CaMKII. For both types of analyses, we averaged the values obtained for the images before the stimulation [before] and during the stimulation and calculated a fold increase stim/before.

Synaptic insertion of GluA1-containing AMPARs and correlation with dendritic CaMKII translocation. Analysis for SEP-GluA1 was done in Matlab using custom software. Deconvolved images from a z-stack were summed for each time point. Those sum projections were then xy aligned before data analysis. Clusters of SEP-GluA1 were detected using local threshold and morphological analysis. Clusters with an intensity at least 3 standard deviations higher than the intensity of a 10–15-pixel box surrounding the cluster, with an area >0.5 µm² and an eccentricity <0.95, were selected. The position of these GluA1 clusters, considered as synaptic, was then tracked. For each cluster, the size of a region of interest was determined based on the morphological analysis and square ROI was drawn on each projection image, according to the tracked position, to measure the average intensity in both fluorescence channels. Each synapse trajectory was verified visually to avoid errors in detection. Synaptic CaMKII translocation was determined by comparing the intensity of mGFP-CaMKII in those synapses before and during the stimulation. CaMKII synaptic accumulation ([S+]: stim/before > 1), CaMKII synaptic accumulation ([S−]: stim/before ≤ 1), and CaMKII events of CaMKII dendritic translocation were manually identified and synapses either inside (D+) or outside (D−) of this dendritic region were selected for intensity analysis.

**Spine number and relationship with dendritic CaMKII translocation.** To count spine number, 15-µm sections of dendrites were analyzed separately, blind of the condition. Also blind of the condition, the presence of dendritic CaMKII translocation was determined visually. Using Neuron-Studio (Rodriguez et al., 2008), dendrites were drawn semi-automatically and spines were detected. We measured the number of spines for 3 images before and 3 images 30 min after the stimulation to calculate a fold change (after/before). We also classified the spine morphology using the measurements done by NeuronStudio and measured the difference in spine number by category, after versus before the stimulation. Thin spines: head diameter < 0.5 µm and length/head diameter > 2. Mushroom spines: head diameter > 0.5 µm, head/neck > 1.1 and length > 0.5 µm. Stubby spines: others. Occasionally, complex protrusions were detected as more than one spine; because such structure might represent more than one synaptic site, we counted each of them to reflect spine remodeling.

**FRAP measurements.** Neurons transfected with mGFP-CaMKII, preincubated (or not) with 5 µM nocodazole [Sigma-Aldrich] for 1 h in the culture medium, were stimulated as described above with OSt+/−/Gly or KCl. Photobleaching was performed either before, during, or after the stimulation. Images of dendrites were acquired on a confocal system (LSM 510 Meta-Axioskop FS2 Plus; Carl Zeiss) with a 63x 0.95 NA, Achroplan water-immersion objective. Scanning illumination was provided by the 488-nm line of a 30-mW argon ion laser set to 25% of maximum output power. Transmission was set to 4% for prebleach and recovery imaging. The optical slice was 7 µm to collect information from inside (D+) and outside (D−) of this dendritic region were selected for intensity analysis.  

The corrected intensity is calculated by the function:  

$$ \text{corrected intensity} = \frac{\text{mean intensity of each object}}{\text{threshold value at which it was detected}} \times \frac{\text{mean value of all nonzero pixels of the image}}{\text{mean value of all nonzero pixels of the image}}$$

To correct for photobleaching and focus changes during acquisition, we used a correction factor obtained with reference region intensity (I_ref) using the equation:

$$ \text{correct factor} = \frac{I_{\text{pre-bleach}} - \text{I_bleach}}{I_{\text{pre-bleach}} - \text{I_bleach}}$$

To collect information from the normalized intensity, we next fitted the function:

$$ f(t) = A(1 - \sum \text{exp}^{-\text{bt}^n})$$

in Matlab, using the least-squared fit method. The value of (n) is 2. "A" represents the mobile fraction of proteins, "B" the time constant and "b" the magnitude of the exponential. For the OSt+/−/Gly experiment (Fig. 3 A), we fitted only the first 15 s after the photobleaching to minimize the impact of new binding of mGFP-CaMKII (i.e., continued accumulation) post-bleaching.

**HEK293 cell transfection, stimulation, and analysis.** Human embryonic kidney 293 (HEK293) cells were grown to 30–40% on poly-lysine–coated Aclar coverslips (12 mm in diameter) in DME growth media supplemented with 10% fetal bovine serum and Glutamax-1 (Invitrogen). Cells were transfected using Lipofectamine 2000 as described by the manufacturer and treated 16–24 h later. Cells were stimulated or not in a solution containing HBSS supplemented with 5 mM CaCl₂, and 10 µM ionomycin (1:1,000 dilution of a methanol stock) for 5 min or a solution containing only HBSS, respectively. ROIs were drawn in the cells’ cytosolic area (outside of nucleus) to measure average intensity in each channel (CYP, Cypheos, and mGFP-CaMKII).

**Immunocytochemistry and colocalization analysis.** Neurons were stimulated for 5 min with OSt+/−/Gly solution, for 90 s with depolarizing solution, or for 60 s with Glu/Gly solution. Immediately after the stimulation, they were fixed for 10 min in 4% PFA solution (0.1 M phosphate buffer, 4% sucrose, and 2 mM EGTA, pH 7.4). To assess microtubule stability, using antibodies against tyrosinated and acetylated tubulin, cells were simultaneously permeabilized and fixed in PHEM buffer (60 mM Pipes, 25 mM Hepes, 5 mM EGTA, and 1 mM MgCl₂) containing also 0.25% glutaraldehyde, 3.7% PFA, 3.7% sucrose, and 0.1% Triton X-100 (based on Witte et al., 2008). Neurons were then washed once with PBS and twice with 0.1 M PBS/glycine and were permeabilized for 30 min in blocking solution (PBS, 2% normal goat serum, and 0.1% Triton X-100). The following primary antibodies were diluted in blocking solution and incubated for 2 h at room temperature: mouse anti-CaMKII (clone CB2b; 1:500; Invitrogen), rabbit anti-active CaMKII phosphoT286 (1:500; Promega), rabbit anti-tubulin (1:200; Sigma-Aldrich), rabbit anti-MAP2 (1:500; EMD Millipore), rabbit anti-Neurofilament M (1:500; EMD Millipore), guinea pig anti-VGlut1 (1:500; EMD Millipore), mouse anti-phospho-Vimentin (Ser82; clone MONO2, 1:5,000; MBL International), mouse anti-PSD95 (clone 6G6-1C9; 1:500; Cedarlane), mouse anti-β-CaMKII (clone CB61; 1:500; Invitrogen), mouse anti-acetylated tubulin (1:500; Sigma-Aldrich), and rat anti-tyrosinated tubulin (1:100; Abcam). After washes in PBS, secondary antibodies (goat anti-mouse Alexa Fluor 488, anti–rabbit 546, anti–rabbit 594, anti–guinea pig 633 [1:1,000], or anti–rat 546 1:500; Invitrogen) were diluted in blocking solution and incubated for 45 min at room temperature. Coverslips were mounted in Prolong Gold Antifade mounting media (Invitrogen). Images were acquired on a confocal system (LSM510 META Axiophot F52 Plus; Carl Zeiss) using a 63x 1.4 NA oil immersion objective and an optical slice of 1–1.5 µm. Alexa Fluor 488 was excited with an argon laser at 488 nm and detected through a band-pass filter (505–530 nm). Alexa Fluor 546 and 594 were excited with a HeNe laser at 543 nm and detected through a band-pass filter (565–615 nm) and a long-pass filter (560 nm), respectively. Alexa Fluor 633 was excited with a HeNe laser at 633 nm and detected through a band-pass filter (650–710 nm). To analyze the colocalization between CaMKII and microtubules or neurofilaments, images from 40 neurons per condition were treated in MetaMorph to remove background noise and extract regions of interest. Colocalization was assessed by a correlation analysis using a Matlab script derived from Jaksolksi et al. (2005), which was originally based.
on Li et al. (2004). The $I_{\text{max}}$ value per neuron was obtained by averaging the $I_{\text{max}}$ from two ROIs selected in proximal dendrites near the soma. The correlation images in Fig. S2 show the normalized mean deviation product (nMDP) pixel map of paired signals. The nMDP index can vary from 0 to 1. The $I_{\text{max}}$ index is the ratio of correlated pixels (nMDP > 0) over total pixels. The index varies between 0 and 1, with 0.5 representing a non-colocalized, random signal.

To analyze the decrease in endogenous CaMKII levels caused by shRNA treatments, we fixed the neurons after live imaging and immunolabeled half of the coverslips for aCaMKII and half for pCaMKII because both antibodies are from mouse. We imaged transfected neurons and measured the average intensity of CaMKII immunolabeling in their soma and divided it by the somatic average intensity from other nontransfected neurons in the same field of view.

Online supplemental material

Fig. S1 shows the patterns of mGFP-αCaMKII dendritic translocation after either Glu/Gly, OMeG/ Gly, or bicuculline stimulation, or during spontaneous activity of the neurons. Fig. S2 describes the colocalization of endogenous αCaMKII with microtubules after OMeG/Gly stimulation or global depolarization with 40 mM KCl. Fig. S3 shows the effect of nocodazole on microtubule destabilization assessed by measuring changes in ratio of acetylated/tyrosinated tubulin. Fig. S4 shows that pCaMKII also translocates to local microtubular domains after OMeG/Gly stimulation or to somatodendritic microtubules upon global stimulation with Glu/Gly. Fig. S5 illustrates the morphometric analysis used to quantify microtubular translocation of mGFP-CaMKII during KCl stimulation as well as the effectiveness of shRNA knockdown of α- and βCaMKII in cultured neurons. Video 1 shows a time-lapse sequence of the localized and reversible dendritic translocation of mGFP-αCaMKII in cultured hippocampal neurons stimulated with OMeG/Gly. Video 2 shows a time-lapse sequence of mGFP-αCaMKII dendritic translocation during spontaneous neuronal activity. Video 3 shows a time-lapse sequence demonstrating the repetitive accumulation of mGFP-αCaMKII in subdendritic regions during multiple OMeG/Gly stimulations. Video 4 shows time-lapse sequence of mCherry-αCaMKII translocation to dendritic regions exhibiting elevated Ca2+ levels, revealed by coincident GCaMP2 imaging. Video 5 shows a time-lapse sequence of mGFP-αCaMKII translocation to a synapse, followed by the dendritic accumulation of mGFP-αCaMKII onto microtubular-like domains adjacent to that synapse. Video 6 shows a time-lapse sequence of mGFP-αCaMKII decorating somatodendritic microtubules upon neuronal depolarization with KCl. Video 7 shows a time-lapse sequence of mGFP-αCaMKII translocating to a dendritic spine, followed by dendritic accumulation below that spine, and long-lasting change in spine volume, as revealed by simultaneous mCherry time-lapse sequence. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201202058/DC1.

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